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Production, characterization, and antitumor efficiency of L-glutaminase from halophilic bacteria

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Abstract

Background: Halophiles are an excellent source of enzymes that are not only salt stable, but also can withstand and carry out reaction efficiently under extreme conditions. L-glutaminase has attracted much attention with respect to proposed applications in several fields such as pharmaceuticals and food industries. The aim of the present study was to investigate the anticancer activity of L-glutaminase produced by halophilic bacteria. Various halophilic bacterial strains were screened for extracellular L-glutaminase production. An attempt was made to study the optimization, purification, and characterization of L-glutaminase from *Bacillus* sp. DV2-37. The antitumor activity of the produced enzyme was also investigated.

Results: The potentiality of 15 halophilic bacterial strains isolated from the marine environment that produced extracellular L-glutaminase was investigated. *Bacillus* sp. DV2-37 was selected as the most potent strain and optimized for enzyme production. The optimization of fermentation process revealed that the highest enzyme activity (47.12 U/ml) was observed in a medium supplemented with 1% (w/v) glucose as a carbon source, 1% (w/v) peptone as a nitrogen source, 5% (w/v) NaCl, the initial pH was 7.0, at 37 °C, using 20% (v/v) inoculum size after 96 h of incubation. The produced crude enzyme was partially purified by ammonium sulfate precipitation and dialysis. Of the various parameters tested, pH 7, 40 °C, and 5% NaCl were found to be the best for L-glutaminase activity. The enzyme also exhibited high salt and temperature stability. The antitumor effect against human breast (MCF-7), hepatocellular (HepG-2), and colon (HCT-116) carcinoma cell lines revealed that L-glutaminase produced by *Bacillus* sp. DV2-37 showed potent cytotoxic activity of all the tested cell lines in a dose-dependent manner with an IC_{50} value of 3.5, 3.4, and 3.8 µg/ml, respectively.

Conclusions: The present study proved that L-glutaminase produced by marine bacteria holds proper features and it has a high potential to be useful for many therapeutic applications.

Keywords: L-glutaminase, Halophilic bacteria, Optimization, Characterization, Antitumor agent

Background

L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid and ammonia (Nandakumar et al. 2003). L-glutaminase is an amide enzyme that has a significant contributory role in cellular nitrogen metabolism in all living cells (Kiruthika and Saraswathy 2013). In food industry, L-glutaminase is used as a flavor and aroma enhancing agent (Rajeev and Chanrdasekaran 2003). L-glutaminase is generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauce (Renu et al. 2003). L-glutaminase has been considered to play a significant role in enzyme therapy for cancer treatment, especially in acute lymphocytic leukemia. It was also found to be effective against human immunodeficiency virus (HIV) (Zhao et al. 2004).

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Another important application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need for separate measurement of glutamic acid (Padma and Singhal 2007).

L-glutaminase enzyme is ubiquitous in nature and reported in animals, plants, bacteria, actinomycetes, yeast, and fungi (Ardawi and Newsholme 1983; Binod et al. 2017). Attempts are being made to replace enzymes, which traditionally have been isolated from animal tissues and plants to enzymes from microorganisms because microbial enzymes are cheaper to produce, more predictable, controlled, and reliable (Teja et al. 2014; Aishwariyaa et al. 2020). Many bacteria synthesize extracellular and intracellular glutaminases such as *Bacillus sp*., *Pseudomonas*, *Actinobacterium sp*, and *E. coli.* (Amobonye et al. 2019). The focal sources of fungal glutaminases are *Aspergillus sp*. and *Trichoderma sp*. (Singh and Banik 2013).

Reports showed that the majority of microbes producing L-glutaminase have been isolated from soil and aquatic (marine) environment (Iyer and Singhal 2010; Yulianti et al. 2012). L-glutaminases produced by terrestrial microorganisms have been reported to have some disadvantages such as unstable in extreme conditions, incompatible with human blood, and may induce a lot of side effects to patients. Thus, there is a great urgency to investigate other enzymatic sources (Sabu 2003).

Halophilic microorganisms are a potential source of extremozymes called halozymes which are capable of functioning under high concentrations of salt, a wide range of pH values, and temperatures (Balagurunathan et al. 2010). Furthermore, over the past few decades, Several efforts have been made to discover the potential abilities of these extremophiles and using them efficiently for therapeutic purposes. The important features of these enzymes that distinguish them from all other types of drugs are high affinity, specificity, and catalytic efficiency (Aishwariyaa et al. 2020). Hence, there is an increasing interest in the identification of marine microbial strains and developing the practical bio-processing technique to improve their productivity for therapeutic purpose.

Methods

Enrichment and screening of L-glutaminase isolates

Halophilic bacterial strains were isolated from water samples collected from the mangrove region on the western coast of the Red Sea, Egypt. About 1.0 ml of seawater was serially diluted in sterilized distilled water up to 10^6 dilutions. Hundred microliters of aliquot from 10^3 to 10^6 dilutions were spread on minimal glutamine agar (MGA) medium using the sterile L-rod. The constituents of MGA (g/l) include 1.0 NaCl, 0.5 KCl; 0.5 $MgSO_4$; 1.0 KH_2PO_4; 0.1 $FeSO_4$; 0.1 $ZnSO_4$; 10 glutamine, 0.12 Phenol red and cycloheximide (20 µg/ml) (Hymavathi et al. 2009). L-glutamine acts as the sole carbon and nitrogen source. Phenol red acts as a pH indicator. Cycloheximide retards the fungal growth. The plating was done in triplicate and all the plates were incubated at 37 °C for 48 h. Only the bacteria which synthesize L-glutaminase can grow in MGA medium and the extracellular production of L-glutaminse was detected. The formation of a pink zone around colonies indicated a positive response due to accumulation of ammonia, which has resulted in a change in pH indicator color from yellow to pink due to the increase in pH value which is caused by L-glutamine use.

The secondary screening for the highly producer isolates was done by culturing the positive strains for enzyme production in liquid minimal glutamine medium. All the flasks were incubated at 37 °C for 72 h in a rotary shaker at $150 \times g$. After centrifugation of the cultures by using a cooling centrifuge at $10{,}000 \times g$ for 30 min at 4 °C, the optical density (OD) of each supernatant was measured at 540 nm using a visible spectrophotometer (Abd-Alla et al. 2013). A culture, giving the highest L-glutaminase production was used further for L-glutaminase production. Regular subculturing of the selected isolate was performed at an interval of every 4 weeks and preserved at refrigerated conditions as slant culture.

Identification of bacterial stain

Bacterial isolate showing the highest L-glutaminase production was tested for species identity using the 16S rRNA sequencing method (Rochelle et al. 1995). The gene sequencing was done at Macrogen (South Korea). DNA sequences were aligned using Gene Mapper v4.1 & Data Collection v 3.1 Communication Patch1. To extract the genomic DNA, bacterial colonies are picked with a sterilized toothpick and suspended in 0.5 ml of sterilized saline, then centrifuged at $10{,}000 \times g$ for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA), incubated at 56 °C for 30 min, and then heated to 100 °C for 10 min. After heating, the supernatant can be used for the PCR reaction. Bacterial 16S rRNAs were amplified using the following universal bacterial 16S rRNA primers: forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1792 R (5′-TACGGYTACCTTGTTACG ACTT-3′). The polymerase chain reaction was performed using kits with Ampli Taq DNA polymerase (FSenzyme; Applied Biosystems). One microlitre of template DNA was added to 20 µl of PCR reaction solution. Amplification was performed using 35 cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. The PCR amplicon was purified using a Montage PCR clean-up kit (Millipore). The purified PCR products of approximately 1400 bp

were sequenced using 2 primers 518 F (5′-CCA GCA GCC GCG GTA ATA Cg-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′). Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA). Sequence analysis was performed with sequences in the National Center for Biotechnology Information (NCBI), USA database using Basic Local Alignment Search Tool for Nucleotides (BLASTN) (Altschul et al. 1997).

Inoculum preparation

The inoculum of the tested bacterial strain was prepared in 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth liquid medium (pH 7). Prepared medium was autoclaved and then inoculated with a loopful of culture from 24 h old nutrient agar slant. The inoculated flasks were kept on a shaker at 150 × *g* for 24 h and used as the inoculum.

Production and extraction of L-glutaminase

Five ml of the prepared inoculum was transferred aseptically to 45 ml of minimal glutamine (MG) production medium. All the flasks were incubated at 37 °C in a rotary shaker at 150 × *g* for 72 h. After every 24 h, 10 ml of sample was removed aseptically and centrifuged using cooling centrifuge at 10,000 × *g* for 30 min at 4 °C. The clear supernatant was used for enzyme estimation (Chanakya et al. 2010).

L-glutaminase assay

Assay of L-glutaminase was carried out as described by Chanakya et al. (2010) utilizing L-glutamine as a substrate and the released ammonia was measured using Nessler reagent. Briefly, 0.5 ml of 0.04 M glutamine was taken in a test tube, to which 0.5 ml of 0.05 M buffer (Tris HCl, pH 7.2), 0.5 ml of enzyme, and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml, the reaction mixture was incubated at 37 °C for 30 min. After incubation, the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloro-acetic acid). The precipitated proteins were removed by centrifugation (1000 × *g* for 20 min). The blank was prepared similarly without adding enzyme preparation. 0.1 ml was taken from the above-mentioned reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml of Nessler's reagent was added, incubated for 10 min, and the absorbance (OD) was measured at 450 nm using a UV–visible spectrophotometer. Then a standard curve was plotted using ammonium sulfate as the standard for estimation of ammonia liberated. One unit of L-glutaminase is the amount of enzyme which liberates 1 µmol of ammonia per minute per ml (µmole/ml/min) under optimal assay conditions. Assays were done in triplicate, and the mean enzyme activity was expressed as unit per ml (U/ml).

Protein estimation

Protein content in the crude enzyme source was estimated by Lowry's method (Lowry et al. 1951) using bovine serum albumin as the standard, and the values were expressed as mg/ml.

Optimization of L-glutaminase production

Various process parameters that enhance the yield of L-glutaminase by *Bacillus* sp. DV2-37 was investigated. The effect of incorporation of additional carbon sources (glucose, fructose, sucrose, dextrose, maltose, galactose, lactose, mannitol and soluble starch at 1% w/v) and nitrogen sources (peptone, yeast extract, beef extract, malt extract, urea, ammonium chloride, ammonium sulfate, ammonium nitrate, and sodium nitrate and amino acids (L-glutaminase, L-asparagine, L-glutamic acid, L-arginine, L-methionine, L-proline, L-lysine at 1% w/v) were investigated. Moreover, the effect of different concentrations of sodium chloride (1–9%), initial pH values (5–9), incubation temperatures (25–40 °C), inoculum concentrations (5–25%), and incubation periods (24–120 h) was studied. During this optimization process, once a particular parameter was optimized, the same optimum condition of that specific parameter was employed in the subsequent studies wherein another parameter is to be optimized. All the extracts obtained from above parameters were studied for L-glutaminase activity. The experiments were conducted in triplicate, and the mean values were reported.

Partial purification of L-glutaminase

The purification was carried out using 500 ml of crude enzyme extract. Finely powdered ammonium sulphate was slowly added into cell-free supernatant (crude enzyme) so as to reach 40% saturation. The whole content was stirred at 4 °C using a magnetic stirrer. The precipitated crude enzyme was removed by centrifugation at 10,000 × *g* at 4 °C for 20 min. Fresh ammonium sulfate was added to the supernatant to increase the saturation to 50%. The obtained precipitate was re-suspended in a minimal volume of 0.01 M phosphate buffer (pH 8). Precipitated protein was removed by centrifugation as described earlier. Once again the fresh ammonium sulfate was added to the cell free supernatant to increase the concentration to 80%. The obtained enzyme precipitate was re-suspended in a minimal volume of 0.01 M phosphate buffer (pH 8) and precipitated protein was recovered by centrifugation (Aly et al. 2017). The enzyme precipitate obtained after ammonium sulphate

precipitation was dialyzed against 0.01 M phosphate buffer (pH 8) for 24 h at 4 °C with continuous stirring and occasional changes of the buffers. The dialyzed fractions were collected and freeze-dried, and the lyophilized enzyme was used for the following assays.

Enzyme characteristics

The effect of pH on enzyme activity and stability

The effect of pH on L-glutaminase activity was determined by assaying the enzyme activity at different pH values ranging from 4.0 to 10.0 as described above. The used buffers were as follows: 50 mM of citrate–phosphate buffer (pH 4.0–6.0), phosphate buffer (pH 7.0 –8.0), and glycine–NaOH buffer (pH 9.0–10.0). The pH stability of the enzyme was investigated in the same pH range and incubated for 1 h. Afterward, aliquots of the mixtures were taken to measure the residual L-glutaminase activity (%) with respect to the control, under standard assay conditions (Jeong et al. 2010).

The effect of temperature on enzyme activity and stability

The effect of temperature on L-glutaminase activity was determined by incubating the reaction mixture at optimum pH value under different temperature ranging from (30–80 °C). In order to determine the thermostability of the enzyme, experiments were conducted by measuring the residual activity after incubation in the same temperatures range for 1 h under standard assay conditions (Jeong et al. 2010).

The effect of different NaCl concentrations

The effect of different NaCl concentrations on L-glutaminase activity was examined by incubating 100 µl of partial purified enzyme with 100 µl of each concentration (1, 3, 5, 7, 9 and 12%) and the activity was then measured by a standard enzyme assay. In order to determine the stability of the enzyme, experiments were conducted by measuring the residual activity after incubation in the same concentrations range for 1 h under standard assay conditions (Jeong et al. 2010).

Anticancer activity

The anticancer activity of the produced L-glutaminase was studied against human breast (MCF-7), hepatocellular (HepG-2), and colon (HCT-116) carcinoma cell lines besides one normal cell line, namely, human epithelial retina cells (RPE- 1), which were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were propagated in 24-well plate (BD Biosciences, San Jose, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50 µg/ml gentamycin. The cell suspension (10^5cells/ml) was seeded in every well and incubated at 37 °C for 48 h in 5% CO_2 for the formation of a confluent monolayer. The cell viability was measured using MTT assay (Renugadevi and Venus 2012). The MTT assay is based on the reduction of yellow 2,5-diphenyl tetrazolium bromide to purple formazan by actively growing cells. The monolayer of cells in 24-well plates was incubated alone or with an enzyme at different concentration (50, 25, 12.5, 6.25, 3.125, and 1.56 µg/ml) for 48 h. After incubation, 20 µl MTT stock solution (5 mg/ml in phosphate-buffered saline or PBS, pH 7.5, filtered through 0.22-µm cellulose acetate filter; Sigma, St. Louis) was added to each well, incubated for 4 h at 37 °C then the solution was decanted. To stop succinate-tetrazolium reductase activity and solubilize formazan crystals, 100 µl of propanol was then added to each well. The viable cells yield was determined by a colorimetric method. The percentage of viable cells was calculated as follows: Cell proliferation (%) = (OD experimental group/OD control group) × 100. The 50% inhibitory concentration (IC_{50}), enzyme concentration causing 50% inhibition of intact cells was calculated from the graph plotting percentage of cell viability against enzyme concentration. The lower the IC_{50} value indicates high antitumor capacity.

Statistical analysis

Data were expressed as means ± SD. The mean values were calculated based on the data taken from at least three independent experiments ($n = 3$). Statistical analysis was performed by using the Student's t-test. Differences were considered significant at $P < 0.05$.

Results

In the present study, totally, 25 morphologically different bacterial isolates were selected from the sediment samples collected from marine ecosystems. Of these, 15 bacterial isolates produced measurable pink color zone around the colony on minimal glutamine agar (MGA) medium ranged from 7.5 to 18 mm that was proportional to their ability to produce L-glutaminase. The quantitative screening for L-glutaminase production was evaluated using submerged fermentation revealed that these isolates produced L-glutaminase with a range activity of 0.9–5.16 U/ml. The isolate showing the highest production of L-glutaminase (5.16 U/ml) was selected for further study. To confirm the identification of the selected isolate, 16SrRNA gene sequence analysis was performed. The sequence alignment using BLASTN software for the comparison of up to 1500 bp indicated that the 16S rRNA gene sequence of the selected strain exhibits a high homology (98%) with that of *Bacillus* sp. DV2-37.

Optimization of L-glutaminase production

The effect of carbon sources

The results cited in Table 1 showed that incorporation of additional carbon sources enhanced the enzyme yield from 5.16 to 18.13 U/ml by the tested marine bacterium. Among the various sugars tested for their effects on the L-glutaminase production, glucose was found to be the best carbon source, yielding maximum L-glutaminase production (18.13 U/ml) followed by maltose (17.09 U/ml).

The effect of nitrogen sources

The results of the effect of the addition of different inorganic and organic nitrogen sources on enzyme production level revealed that peptone enhanced the enzyme yield from 18.85 to 36.08 U/ml. Among the different amino acids tested, L-glutamine was observed to enhance L-glutaminase synthesis (36.12 U/ml) (Table 1).

The effect of sodium chloride concentrations

The results cited in Table 1 showed that the yield of L-glutaminase was increased with increasing NaCl concentration up to 5% as maximum as 40.06 U/ml and it was low in 1% and 3% of NaCl concentrations. Yield was decreased, when the concentration was increased above 5%. Hence, 5% of NaCl concentration was the optimum for the production of L-glutaminase from *Bacillus* sp. DV2-37.

The effect of initial pH

In the present study, the optimum L-glutaminase production reached 40.32 U/ ml was recorded at pH 7.0 and any further alteration either increase or decrease of the

Table 1 Effect of various nutritional and environmental factors on L-glutaminase production by *Bacillus* sp. DV2-37. Data represent the mean of 3 different readings ± standard deviation

Factors	Enzyme activity (U/ml)	Factors	Enzyme activity (U/ml)
Carbon source (1% w/v)		NaCl (%)	
Control	5.16 ± 0.2	1	36.55 ± 1.5
glucose	18.13 ± 1.5	3	38.00 ± 2.4
Fructose	7.11 ± 0.65	5	40.06 ± 5.6
Sucrose	15.10 ± 0.66	7	36.70 ± 2.06
Dextrose	16.9 ± 0.85	9	29.12 ± 1.56
Maltose	17.09 ± 0.95	pH	
Galactose	10.08 ± 0.30	5	23.58 ± 5.4
Lactose	13.92 ± 1.0	6	30.92 ± 4.2
Mannitol	8.20 ± 0.98	7	40.32 ± 1.2
Starch	6.07 ± 0.20	8	36.47 ± 1.5
Nitrogen source (1% w/v)		9	34.10 ± 2.3
Control	18.85 ± 2.30	Temperature	
Peptone	36.08 ± 1.45	25	24.48 ± 1.90
Yeast extract	32.90 ± 1.63	30	32.33 ± 1.04
Beef extract	27.25 ± 2.10	35	38.56 ± 2.20
Malt extract	29.00 ± 1.25	37	40.80 ± 1.4
Urea	18.95 ± 1.12	40	29.00 ± 1.0
NH4Cl	25.30 ± 1.65	Inoculum size (%)	
NH4SO4	23.40 ± 1.44	5	33.00 ± 1.25
NH4NO3	20.80 ± 3.12	10	40.84 ± 1.12
NaNO3	19.11 ± 2.54	15	42.55 ± 5.62
Amino acids (1% w/v)		20	45.05 ± 2.58
L-glutamine	36.12 ± 1.10	25	35.25 ± 4.20
L-asparagine	28.86 ± 0.12	Incubation time (h)	
L-glutamic acid	12.60 ± 2.14	24	20.08 ± 2.0
L-arginine	18.01 ± 0.02	48	29.00 ± 1.5
L-methionine	14.52 ± 0.4	72	45.14 ± 7.34
L-proline	11.35 ± 0.31	96	47.12 ± 6.66
L-lysine	12.20 ± 1.1	120	39.68 ± 5.64

medium pH negatively influenced the enzyme production. Critical analyses of the L-glutaminase productivity values do suggest that alkaline conditions are more supportive compared to the acidic environment during fermentation by this isolate (Table 1).

The effect of temperature

Growth temperature is another critical parameter that needs to be controlled. Maximum L-glutaminase production of 40.80 U/ml was noticed at a temperature of 37 °C. Variation of the temperature on either side of this resulted in decrease in L-glutaminase production. The loss of activity is more at the higher temperature when compared to the lower temperatures (Table 1).

The effect of inoculum size

The results cited in Table 1 revealed that the production of L-glutaminase by *Bacillus* sp. DV2-37 increased as the inoculum size increased until it reached its maximum productivity (45.05 U/ml) at 20%, after that it decreased as the inoculum size increased.

The effect of incubation period

The maximum enzyme productivity (47.12 U/ml) by *Bacillus* sp. DV2-37 was obtained at 96 h of cultivation period (Table 1). After that, the enzyme production decreased, suggesting its association with growth parameters. It is noteworthy to state that by optimizing the above-mentioned components and culture conditions, the production of L-glutaminase has been increased about ninefold from 5.16 U/ml of the initial medium composition to 47.12 U/ml under the final conditions.

Properties of the partially purified enzyme

The L-glutaminase activity and protein content in each fraction obtained were determined and further used for the estimation of specific activity, yield, and recovery. The specific activity and the purity fold of the enzyme increased gradually with each progression of purification (ammonium sulfate precipitation and dialysis), although the total protein, total activity, and yield reduced relatively (Table 2). Using ammonium sulfate precipitation, the crude enzyme was purified about 1.75 times more and the yield rate was enhanced up to 75.19% with a specific activity of 53.07 U/mg. The enzyme purity was increased with the dialysis process; the yield and the final specific activity reached 51.15% and 176.2 U/mg, respectively.

The description and characterization of the enzymes produced by microorganisms are relevant for their possible application in many industries. In the present study, the conditions for enzyme activity reaction were optimized by studying different parameters like pH of the reaction mixture, temperature, and the effect of NaCl concentrations. Testing the pH dependence of enzyme activity revealed that the maximum activity of L-glutaminase was observed at pH 7 (47.45 U/ml). The pH stability of the enzyme also showed a similar trend, the enzyme being most stable at pH 7.0 (Fig. 1).

Temperature is a critical factor for the maximum enzyme activity. Upon incubation in different temperatures, the reaction mixture showed an increased rate of enzymatic reaction, from 30 to 40 °C. The enzyme was optimally active (50.50 U/ml) at a temperature of 40 °C. Incubation above 40 °C promoted a remarkable inactivation of L-glutaminase from *Bacillus* sp. DV2-37. Furthermore, L-glutaminase showed thermal stability over a temperature range of 30–50 oC and retained 90% of its activity at 60 °C (Fig. 2).

Results presented in Fig. 3 showed that, as NaCl concentration increased, the activity of the enzyme increased

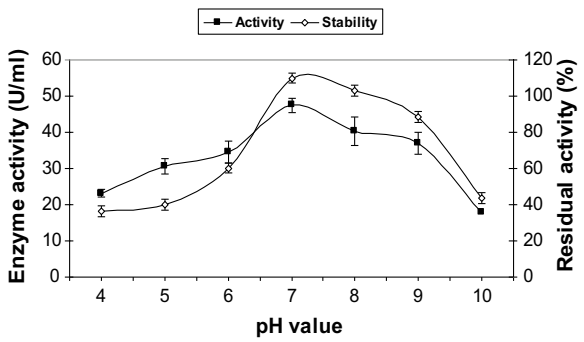

Fig. 1 Activity and stability of L-glutaminase produced by *Bacillus* sp. DV2-37 as a function of pH values. Results are means of three independent determinations. Data represent the mean of 3 different readings ± standard deviation

Table 2 Purification steps of L-glutaminase produced by *Bacillus* sp. DV2-37

Purification Procedure	Enzyme Activity (U)	Protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Cell free extract	1336	565	30.23	1.0	100
Ammonium sulphate	9871	225	53.07	1.75	75.19
Dialysis	7053	58	176.2	5.8	51.15

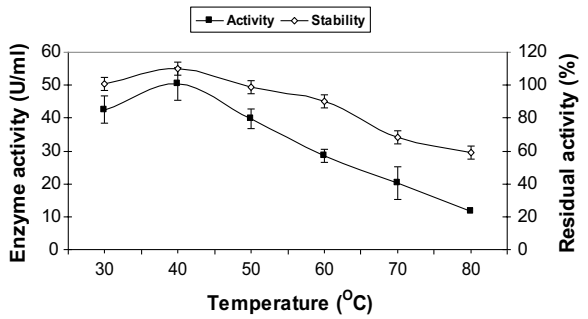

Fig. 2 Activity and stability of L-glutaminase produced by *Bacillus* sp. DV2-37 as a function of temperature. Results are means of three independent determinations. Data represent the mean of 3 different readings ± standard deviation

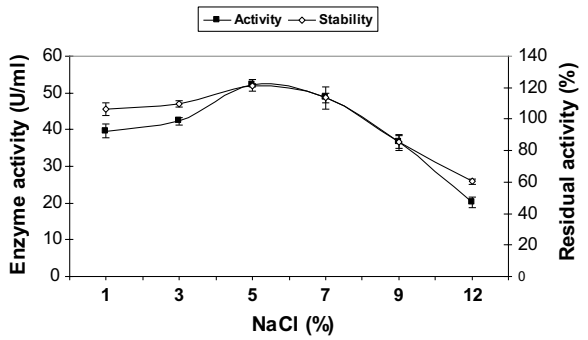

Fig. 3 Activity and stability of L-glutaminase produced by *Bacillus* sp. DV2-37 as a function of NaCl concentrations. Results are means of three independent determinations. Data represent the mean of 3 different readings ± standard deviation

until it reached its maximum activity (52.11 U/ml) at 5% (w/v) NaCl, after that any increase in NaCl concentration leads to decrease in enzyme activity. Such results indicated the high salt tolerance of the enzyme. Data presented in Fig. 3 indicated the salt tolerance nature of L-glutaminase from the marine *Bacillus* sp. DV2-37. It exhibited more than 100% activity at 1–7% NaCl and retained 85.5% of activity at 9% NaCl concentration, whereas glutaminase from non-marine microbes was found to be inactivated at high salt concentrations, limiting their industrial potential.

Anticancer activity

In the present study, In vitro anticancer activity of L-glutaminase produced by *Bacillus* sp. DV2-37 was studied against the human breast (MCF-7), hepatocellular (HepG-2), and colon (HCT-116) carcinoma cell lines at different concentration (50, 25, 12.5, 6.25, 3.125, and 1.56 µg/ml) by the MTT assay and compared with the standard Doxrubcin. L-glutaminase produced by *Bacillus* sp. DV2-37 showed a potent cytotoxic activity of all cell lines in a dose-dependent manner as shown in Table 3. The results showed that MCF-7, HepG-2 and HCT-116 cell proliferation were significantly inhibited by L-glutaminase with IC_{50} values of 3.5, 3.4, and 3.8 µg/ml, respectively (Table 4). The produced L-glutaminase exhibited weak toxicity to the normal epithelium retina cell line (RPE-1) with IC_{50} of 50 µg/ml. A blank experiment was conducted containing the same extract concentrations, but heat-inactivated to confirm that this antitumor activity is related to the produced L-glutaminase not to other bacterial proteins. Thus, L-glutaminase produced by *Bacillus* sp. DV2-37 was found to be a potent cytotoxic agent against MCF-7, HepG-2, and HCT-116 cell lines.

Table 3 Cytotoxicity activity of L-glutaminase produced by *Bacillus* sp. DV2-37 against human breast (MCF-7), hepatocellular (HepG2), and colon (HCT-116) carcinoma cells

L-glutaminase concentration (µg/ml)	Viability (%)		
	MCF-7	Hep G-2	HCT-116
50	8.97	6.89	9.83
25	15.91	11.93	14.47
12.5	24.59	20.47	23.65
6.25	39.42	35.29	35.54
3.125	51.47	51.43	53.92
1.56	74.92	82.94	68.17
0	100	100	100

Table 4 Inhibition concentrations (IC_{50}) for cytotoxicity activity of L-glutaminase produced by *Bacillus* sp. DV2-37

Human carcinoma cells	L-glutaminase concentration (µg/ml)	Doxrubcin (Standard)
MCF-7	3.5	0.426
HepG-2	3.4	1.2
HCT-116	3.8	0.469

Discussion

In recent years, marine bacteria play a major role in the fields of health, medicine, and industry. Marine environment harbors a huge pool of novel enzymes that have very unique properties. In this respect, L-glutaminases extracted from marine microorganisms are expected to be characterized by special properties i.e. salt-tolerant and thermo-stable as needed by many industries (Patel et al. 2020).

In the present study, upon screening of the bacterial strains isolated from the marine environment, the strain showed the highest production of L-glutaminase was selected and identified as *Bacillus* sp. DV2-37. In the

same line, various halophilic bacterial strains producing L-glutaminase were isolated from marine habitats and identified based on 16S rRNAs such as *Bacillus subtilis*, *Bacillus cereus* MTCC 1305, *Aeromonas veronii*, *Providencia* sp., *Acinetobacter calcoaceticus* PJB1, and *Halomonas* (Zolfaghar et al. 2019; Hussein et al. 2020).

The production of pink color zone around the bacterial colony grown on minimal glutamine agar (MGA) medium is an indication of L-glutaminase production (Hymavathi et al. 2009). Earlier method involves the isolation of microorganisms from certain environments by routine isolation procedures and then screened for enzymatic activity. However, the use of selective media and the presence of antibiotics, NaCl, and pH indicators make MGA medium suitable for direct and selective isolation of L-glutaminase producing marine isolates (Balagurunathan et al. 2010).

Carbon source represents the energy source required for the growth of microorganisms. Carbohydrates and related compounds are considered as the favorable carbon sources for many genera of microbes. The enhanced production of L-glutaminase by incorporation of carbon sources may be attributed to the positive influence of additional carbon sources along with glutamine for enhancing the enzyme biosynthesis (Chitanand and Shete 2012). In the present study, glucose was used as an additional carbon source for yielding the maximum L-glutaminase by *Bacillus* sp. DV2-37. Similarly, glucose was the best carbon source for glutaminase production by *Pseudomonas aurignosa* (Al-Zahrani et al. 2020). On the other hand, rhamnose caused the highest L-glutaminase production of the marine bacterial isolate *Bacillus subtilis* OHEM11 (Orabi et al. 2020).

Nitrogen source has got a profound influence on enzyme production as it is the ultimate precursor for protein biosynthesis. Besides, the nitrogen source can also affect the pH of the medium, which in turn may influence the activity and stability of the enzyme. The results of the present study revealed that peptone enhanced the enzyme yield. On the contrary, Kiruthika and Nachimuthu (2014) reported that glutaminase production by marine *Bacillus subtilis* JK-79 was enhanced by using yeast extract. Furthermore, amino acids were reported to be a common growth factor required for the synthesis of enzyme as the major nitrogen source (Cruz Soto et al. 1994); hence, the yield of L-glutaminase produced was varied when the amino acid was changed. In the present study, L-glutamine was found to enhance L-glutaminase synthesis by *Bacillus* sp. DV2-37. This observation suggests that L-glutamine acts as an inducer for the production of extracellular L-glutaminase enzyme. Similar results were reported by Prakash et al. (2010) who indicated that L-glutamine and L-asparagine resulted in a high yield of L-glutaminase. Moreover, Al-Zahrani et al. (2020) showed that the maximum glutaminase activity by *Pseudomonas aurignosa* was achieved with glutamine out of various nitrogen sources.

It is well-known that the pH of the culture medium affects the availability of certain metabolic ions and permeability of bacterial cell membranes, which in turn supports cell growth and enzyme production (Kapoor et al. 2008; Krishna-kumar et al. 2011). In general, the pH range 6.0 to 8.0 was reported to be the most favorable range for L-glutaminase production by the majority of microbial organisms (Sathish and Prakasham 2010). In the present study, it was noticed that pH 7.0 is optimum for the L-glutaminase production by *Bacillus* sp. DV2-37. In the same line, Lakshmi and Jaya (2012) stated that the optimum pH for L-glutaminase production by *Aspergillus oryzae* NCIM 1212 was at pH7. In addition, the best L-glutaminase production was observed at pH 7 for the forest soil isolated bacterial strain of *Bacillus* sp. (Nagaraju and Raghu Ram 2018). However, marine *Vibrio azureus* JK-79 bacterial strain exhibited the maximum glutaminase production at pH 8 (Kiruthika 2013).

Furthermore, growth temperature influenced the microbial metabolism both with respect to the rates of cellular processes run and the enzymatic reactions occur. Sivakumar et al. (2006) reported that any temperature beyond the optimum range is found to have some adverse effects on the metabolic activities of the microorganisms. In the present study, the highest L-glutaminase production by *Bacillus* sp. DV2-37 was noticed at a temperature of 37 °C. In the same line, Kiruthika (2013) confirmed that the maximum glutaminase activity by *Vibrio azureus* JK-79 isolated from marine environment was at 37 °C. On the other side, Al-Zahrani et al. (2020) stated that a temperature of 35 °C is the best one for glutaminase production by *Pseuedomonas* NS16.

Quantum of initial biomass controls the kinetics of growth and several biological metabolic functions that leading to the overall biomass and extracellular enzymatic production (Wakayama et al. 2005). The highest inoculum concentration caused the reduction of the lag phase, that yielding maximum enzyme productivity (Tobin et al. 2001). In addition, it was reported that maximum enzyme production could be obtained only after a certain incubation time, which allows the culture to grow, after which it decreases. It might be due to the depletion of nutrients in the medium which stressed the bacterial physiology. This reduction of the nutrients resulted in the inactivation of the secretary machinery of the enzymes (Alexandra et al., 2003). In the present study, the maximum enzyme productivity by *Bacillus* sp. DV2-37 was obtained at 96 h of incubation period. In the same manner, Krishna-kumar et al. (2011) mentioned that the

highest production of L-glutaminase by the marine alkalophilic *Streptomyces* sp. SBU1 was at 96 h of incubation period. However, maximum L-glutaminase production was achieved at 18 h of incubation time by marine isolated bacterial strain *Bacillus subtilis* (Zhang et al., 2019) and at 72 h of incubation period for *Pseudomonas VJ-6* (Jyothi and Shivaeerakumar 2011).

The results of the present study showed that upon optimizing the above- mentioned medium components and culture conditions, L-glutaminase production by *Bacillus* sp. DV2-37 has been increased from 5.16 U/ml using the initial medium composition to 47.12 U/ml (ninefold) under optimum conditions. In the same line, statistical optimization has been reported to enhance the production of L-glutaminase from marine *Bacillus subtilis* JK-79 up to 3.48 fold when compared with the basal medium (Kiruthika and Murugesan 2020) and up to 2.88 fold under solid state fermentation as compared to the basal wheat bran medium (Kiruthika et al. 2018).

In the present study on the L-glutaminase production of *Bacillus* sp. DV2-37, the maximum activity was reported at pH 7. As the pH value diverged from the optimum level, the efficient functioning of the enzyme affected, and this could be ascribed to the decreased saturation of the enzyme with substrate due to a decreased affinity and/or due to the effect of pH on the stability of the enzyme.

Cancer is one of the most dangerous diseases. It is the second bigger disease of human beings (Rashmi et al. 2012). Although several kinds of treatments are available, enzyme therapy is reported to be the more effective one. Cancer therapy using enzymes relies on the low molecular weight protein, specific in their action and has less or no toxic effects. In addition, the enzymatic approach was reported to be more promising in cancer therapy (Pandian et al. 2014).

Cancer cells, especially, cannot synthesize L-glutamine as they lack the proper functioning glutamine biosynthetic machinery (L-glutamine synthetase) and therefore require large amount of L-glutamine for their rapid growth. These cells depend on the exogenous supply of L-glutamine for their survival and rapid cell division, as it is a primary tool for donation of its nitrogen, which aid in protein, nucleic acid, lipid formation and participate in oxidative metabolism. Furthermore, glutaminase is already present in mitochondria, but it must be at the level that allows sequential and fast degradation of glutamine (Lukey et al. 2013). Hence, the use of L-glutaminase deprives the tumor cells of L-glutamine and causes selective death of L-glutamine dependant tumor cells (Nathiya et al. 2011; Elshafei et al. 2014). The glutamine-deprivation therapy with L-glutaminase that hydrolyzes L-glutamine to L-glutamic acid and ammonia, not only selectively inhibits tumor growth by the blocking of the de novo protein synthesis, but also increase in the superoxide level of oxidative stress that promotes the death of the cancer cells (Mustafa et al. 2020). Thus, it can act as a possible candidate for enzyme therapy. For example, L-glutaminase has been receiving more attention as an antileukemic agent for treatment of acute lymphoblastic leukemia (ALL) and other types of cancer (Orabi et al. 2019).

L-glutaminase enzyme produced by halotolerant isolates can be used for increasing the level of glutamine catabolism and stopping cancer development. In addition, the chemical nature of seawater could provide microbial sources producing enzymes that could have fewer side effects when used in therapeutic applications (Kiruthika and Swathi 2019). Thus, marine bacteria have recently attracted attention for the L-glutaminase production (Zolfaghar et al. 2019). *Halomonas meridiana* was first reported as an L-glutaminase producer that is used as an anti-colon cancer agent by Mostafa et al. (2021).

In the present study, the produced L-glutaminase by the isolated marine bacterial strain *Bacillus* sp. DV2-37 showed potential anticancer activity against all the tested cell lines with IC_{50} values of 3.5, 3.4 and 3.8 µg/ml for MCF-7, HepG-2, and HCT-116, respectively. Furthermore, it is worth mentioning that the produced L-glutaminase exhibited weak toxicity to the normal epithelium retina cell line (RPE-1) that confirmed that this enzyme possesses great selectivity to cancer cells. In the same trend, many studies investigated the cytotoxicity of L-glutaminase from various microorganisms. For instance, Pandian et al. (2014) reported that purified L-glutaminase by *Alcaligenes faecalis* KLU102 inhibited the growth of Hela cells with an IC_{50} value of 12.5 µg/ml. Moreover, Nathiya et al. (2012) mentioned that L-glutaminase purified from a bacterium was able to stop a breast carcinoma with IC_{50} of 256 µg/ml. Also, Fifi (2015) indicated that L-glutaminase has a noteworthy efficiency contrary to Hep-G2 cell (IC_{50}, 6.8 µg/ml) and a reasonable cytotoxic result against HCT-116 cell (IC_{50}, 64.7 µg/ml). In addition, L-glutaminase produced by *Aspergillus oryzae* showed significant cytotoxic activity against MCF-7 with IC_{50} of 283.288 ug/ml (Sunil et al. 2014). Furthermore, the cytotoxicity effect of L-glutaminase produced by *Bacillus subtilis* OHEM11 indicated significant safety on Vero cells with high anticancer activity against NFS-60, HepG-2, and MCF-7 cancer cell lines (Orabi et al. 2020). Overall, all these data confirmed that L-glutaminase possesses great selectivity to cancer cell and can display potential application in cancer chemoprevention and chemotherapy.

Conclusions

Glutaminase enzyme is an important enzyme in the medical, industrial, and economic aspects. The potential of the isolated *Bacillus* sp. DV2-37 for L-glutaminase production was analyzed with different process parameters and medium constituents. Maximum production was noticed in a medium supplemented with 1% (w/v) glucose as carbon source, 1% (w/v) peptone as nitrogen source, 5% (w/v) NaCl, the initial pH of 7.0, at 37 °C, using 20% (v/v) inoculum size after 96 h of incubation. Under optimal conditions, the glutaminase production improved to 47.12 U/ml. The produced L-glutaminase holds proper features in comparison with others formerly described in the literature. It is active and steady over a widespread range of pH and temperatures. The produced L-glutaminase showed a pronounced antitumor activity against human breast (MCF-7), hepatocellular (HepG-2), and colon (HCT-116) carcinoma cell lines. Thus, it can play an important role in cancer chemoprevention and chemotherapy.

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Authors' contributions
The author contributed to the design and implementation of the research, the analysis of the results, and the writing of the manuscript. All authors read and approved the final manuscript.

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