

Full Length Research Paper

Purification and characterisation of a novel broad spectrum anti-tumor L-glutaminase enzyme from marine *Bacillus subtilis* strain JK-79

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L-glutaminase is a therapeutic enzyme used in the treatment of acute lymphoblastic leukemia (ALL). In this study, the extracellular L-glutaminase produced by *Bacillus subtilis* strain JK-79 was purified to homogeneity. The purified L-glutaminase has specific activity of 937 U mg⁻¹ with K_M and V_{Max} value of 8 × 10⁻² mM and 200 μM/ml min, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) confirmed it to have a molecular mass of 64 kDa. Further biochemical characterization revealed that the purified enzyme attained maximal activity at pH 7 and temperature 37°C. The enzyme was stable at 37°C for 60 min at physiologic-ph. The enzyme showed high specificity towards L-glutamine. The effect of metal ions on enzyme activity showed that NaCl, KCl and CaCl₂ enhanced the activity while AgCl₃ and CuCl₂ strongly inhibited the activity of the enzyme. The *in-vitro* antioxidant activity of the enzyme was investigated and the IC₅₀ value for DPPH and ABTS assay were 400 and 600 μg/ml, respectively. The purified enzyme showed cytotoxic activity against various Leukemic cell lines tested with IC₅₀ value of 231, 480, and 500 μg/ml for K562, U932 and Jurkat cell lines, respectively. Interestingly, L-glutaminase also revealed cytotoxic effect on other cancer cell lines such as MCF-7, OV1063 and HCA 7 with IC₅₀ value of 500, 526 and 750 μg/ml, respectively. Hence this enzyme from marine bacteria would possibly be an attractive candidate for further pharmaceutical use as a broad spectrum anti-tumor drug.

Key words: L-glutaminase, purification, characterization, anti-oxidant activity, anti-cancer activity.

INTRODUCTION

L-glutaminase (L-glutamine *amide hydrolase* EC.3.5.1.2) has identified applications in many fields. This enzyme catalyze the deamidation of L-glutamine to L-glutamic acid and ammonia. L-glutaminase plays an important role

in cellular metabolism of plants, animals and microorganisms such as bacteria, fungi and yeast (Brosnan et al., 1995; Riberg et al., 1995; Gurung et al. 2013; El-Ghonemy, 2014). It is widely used in food industry as

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flavor enhancer (Nakadai and Nasuno, 1989 and Sabu et al., 2000a), and in pharmaceutical sector as an effective therapeutic agent in the treatment of HIV (Zhao et al., 2004; Roberts et al., 2001) and acute lymphocytic leukemia (Schmid and Roberts, 1974). In recent years, L-glutaminase in combination with or as an alternative to L-asparaginase, could be used in enzyme therapy for cancer particularly leukemia (Nandakumar et al., 2003; Sabu, 2003). Sea water which is saline in nature and chemically closer to human blood plasma could provide biomolecules especially enzymes that could have no or less side effects when used in therapeutic applications (Iyer and Singhal, 2009). The L-glutaminase causes selective death of glutamine dependent tumor cells by blocking glutamine and thus helps in the treatment of malignancies, and also used as an analytical reagent in the determination of glutamate and glutamine (Mulchandani and Bassi, 1996; Botre et al., 1993; Noura et al., 2014). In food industry, L-glutaminase has replaced the use of monosodium glutamate as flavor enhancer in Chinese foods (Tachiki et al., 1998), and also used in the manufacture of threonine by gamma glutamyl transfer reactions (Sabu et al., 2000b).

There is need for further discovery of new anti-tumor compounds, as the available anti-cancer drugs in the treatment of cancer is often unsatisfactory to the normal cells. Cancers of the lung, female breast and colorectal are the top three cancer types in terms of incidence, and are ranked within the top five in terms of mortality (first, fifth and second, respectively) (World Health Organization, 2018). From 2010 to 2014, leukemia was the sixth most common cause of cancer deaths in both men and women. Leukemia, lymphoma and myeloma are expected to cause the deaths of an estimated 58,100 people in the US in 2018. These diseases are expected to account for 9.5% of the deaths from cancer in 2018, based on the estimated total of 609,640 cancer deaths (Facts and Statistics, Leukemia and Lymphoma society, 2018).

In recent years, biomedical sciences accentuate the involvement of glutaminase and other amino acid-depleting enzymes as agents for treating tumors (Holcenberg, 1982). L-asparaginase which is currently used in the treatment of acute lymphoblastic leukemia, exhibits lot of side effects in patients due to allergic responses (Narta et al., 2007; Yogendra et al., 2013), and show little or no cytotoxic activity on other types of neoplasms (Roberts et al., 2001). Hence, there is a need to find a novel source of L-glutaminase that is therapeutically suitable and also reveals a broad spectrum of anti-tumor activity.

This study focused on purification and characterisation of L-glutaminase from potent marine microbe *Bacillus subtilis* strain JK-79. Also, L-glutaminase was analyzed for its anti-oxidant activity (DPPH and ABTS assay) and *in vitro* anti-cancer activity against four different human cancer cell lines *viz* MCF-7, JURKAT, U937, K562 and HCA-7 using MTT assay (Mosmann, 1983).



Figure 1. Growth of *Bacillus subtilis* strain JK-79 (KC492745) on MGA (Minimal Glutamine Agar) media.

METHODOLOGY

Production media

The production of L-glutaminase was performed on an optimized sea water based media containing D-fructose-2.0, L-glutamine-2.5 and yeast extract 1.3%. The salinity of sea water was 25 ppt. The *B. subtilis* strain JK-79 (KC492745) used in this study was isolated from marine sediment collected from Parangipettai coastal area (Lat. 11° 29'N; Long. 79° 46'E) (Kiruthika and Saraswathy, 2014). The culture was maintained in Zobell's marine agar slant (Himedia, India) at 4°C and was periodically sub-cultured (Figure 1). The inoculum was prepared by adding a loop full 24-h old culture into a sterile medium in a 500 ml capacity Erlenmeyer flask. The culture flask was incubated at 37°C, 120 rpm on an orbital shaking incubator for 18 h until the mid-logarithmic phase was reached. Batch submerged fermentation was carried out with the production media inoculated with 2% (v/v) culture and incubated at 37°C, 120 rpm for 18 h. The culture was centrifuged after fermentation at 10000 g for 10 min at 4°C to harvest the cells, and the supernatant was used as an enzyme source.

Glutaminase assay

The enzyme activity was checked by the glutaminase assay as described by Imada et al. (1973). 0.5 ml of enzyme supernatant, 0.5 ml of 0.04 M L-glutamine, 0.5 ml of 0.01 M phosphate buffer and 0.5 ml of distilled water were added and incubated at 37°C for 30 min. After incubation, 0.5 ml of 1.5 M TCA (Tri Chloroacetic acid) was added and mixed thoroughly. 50 µL of the above mixture was added to 3.7 ml of distilled water and 0.2 ml of Nessler's reagent and the absorbance was measured at 450 nm. Ammonium sulfate (Himedia) was used as a standard to detect the presence of ammonia in crude enzyme. One unit (U) of enzyme activity is defined as the enzyme required to liberate 1 µmole of ammonia per ml per min at standard assay conditions.

Protein assay

Protein content in the sample was estimated by the Lowry's method (Lowry et al., 1951) using BSA as the standard and the values were expressed in mg/ml.

Purification of L-glutaminase

All enzyme purification steps were carried out at 4°C. Solid ammonium sulphate was slowly added to the crude enzyme filtrate with gentle stirring to bring 40 to 80% saturation in a sequential manner. The enzyme precipitate obtained from saturation was dissolved in a minimal volume of 0.1 M phosphate buffer (pH 7) and dialyzed against the same buffer for 24 h at 4°C with continuous stirring and occasional change of buffer. The concentrated protein solution was loaded on a diethylaminoethyl (DEAE)-cellulose column which was pre-equilibrated with 20 mM Tris HCl. Bound proteins were then eluted with a concentration gradient of sodium chloride (0.5, 0.25, and 0.1 M) in same buffer. The fractions were collected until A_{280} becomes zero and each fraction was assayed for L-glutaminase. Active protein fraction with L-glutaminase activity were pooled and reloaded on to pre-equilibrated Sephadex G-100 column (Himedia) and then eluted with the same buffer. Fractions collected were assayed for glutaminase activity and protein concentration. L-glutaminase active fractions were pooled and lyophilized (Christ, Alpha 1-4, LSC, Germany) for further characterization. Sodium dodecyl sulfate (SDS)-PAGE was carried out using Laemmli (1970) method with a 12.5% separating acrylamide gel (pH 8.8) and a 5% stacking gel (pH 6.8) containing 0.1% SDS. The proteins in the gel were stained with Coomassie brilliant blue R-250. The molecular weight of L-glutaminase was determined using standard molecular weight markers.

Matrix Assisted Laser Desorption/Ionization – time of flight (MALDI-TOF)

The molecular mass was confirmed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) using Bruker Ultraflex MALDI-TOF mass spectrometer equipped with a 337 nm nitrogen laser. Matrix solution A and B were prepared by mixing a saturated solution of Sinapic acid in ethanol and in TA30 solvent (30:70 [v/v] acetonitrile and 0.1% trifluoroacetic acid in deionized water), respectively. The matrix solution A, B and the purified enzyme were mixed in the ratio (1:1) and 2 μ l of the sample was spotted onto a well sample plate, dried at room temperature and analyzed.

High Performance Liquid Chromatography (HPLC)

HPLC analysis of purified L-glutaminase was performed on an Agilent series 1100 HPLC system fitted with a reversed phase high performance liquid chromatography (RP-HPLC) C 18 column. The solvent system was prepared with acetonitrile and water in the ratio of 50:40 at flow rate of 0.5 ml/min. A highly sensitive photodiode array (PDA) detector was set to read the absorbance at 280 nm.

Characterization of the L-glutaminase

Effect of substrate concentration on L-glutaminase activity

Kinetic parameters such as Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of the purified enzyme L-glutaminase were determined using L-glutaminase substrate in the range between 0.01 and 0.1 M. The Lineweaver–Burk plot (Lineweaver–Burk, 1932) for L-glutaminase catalyzed reaction was plotted.

Effect of pH on L-glutaminase activity

Effect of different pH (4–10) on the activity of purified L-glutaminase was studied using 100 mM sodium acetate buffer for pH 4 and 5;

100 mM Tris–maleate buffer for pH 6; 100 mM Tris–HCl buffer for pH 7 and 8 and 100 mM glycine–NaOH buffer for pH 9 and 10. The stability of the purified L-glutaminase enzyme was analyzed by determining the residual enzyme activity for the different pH at the time interval of 30 min.

Effect of temperature on L-glutaminase activity

The optimum temperature of L-glutaminase was assessed by incubating the enzymes at different temperatures of 30 to 60°C with an interval of 5°C. The residual enzyme activity was measured for the different temperatures at an interval of 30 min.

Effect of incubation time on L-glutaminase activity

To observe the effect of incubation time, the L-glutaminase assay was carried out at various time intervals from 10 to 60 min at an interval of 10 min.

Substrate specificity

Substrate specificity for L-glutaminase was determined in the presence of different substrates (0.04 mM) such as L-glutamine, D-glutamine, L-glutamic acid, L- asparagine, L-aspartic acid and L-glu+L-asp.

Effect of metal ions on L-glutaminase activity

Different metal ions such as $BaCl_2$, $CuCl_2$, $FeCl_3$, Fe_2SO_4 , $MgSO_4$, $MnCl_2$, $NaCl$, $AgNO_3$, $CaCl_2$ and KCl were added to the standard assay mixture at three concentrations viz. 0.1, 0.5 and 1 mM, and the residual enzyme activity thus obtained was compared to the control.

Determination of anti-oxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Free radical scavenging activity of L-glutaminase was determined by using rapid, simple and inexpensive method involving the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Peng et al., 2000). The enzyme stock solution (1.0 mg/ml) was diluted to final concentrations of 50, 100, 200, 400, 600, 800 and 1000 μ g/ml, in ethanol. One (1) mL of a 0.3 mM DPPH ethanolic solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min, the degree of reduction of absorbance was recorded in ultraviolet visible (UV-Vis) spectrophotometer at 518 nm.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

The ABTS+ assay was performed based on the procedure described by Re et al. (1999). The stock solutions included 7 mM ABTS+ solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the above mentioned two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS+ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS+ solution was prepared for each assay. 950 μ l of ABTS radical solution, 50 μ l of enzyme solutions

Table 1. Purification of L-glutaminase from marine *Bacillus subtilis* JK-79

Fractions	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification (X)
Crude	950	654,427	9550	68.52	100	1
60% Ammonium sulphate	850	423,084	5650	74.8	64.64	1.09
DEAE-Cellulose	18	23,270	72	323.20	3.55	4.71
Sephadex G-100	10	14994	16	937.125	2.29	13.6

were added and the reaction mixture was vortexed for 10 s. After 6 min the absorbance was recorded at 734 nm and compared with the control ABTS solution.

Determination of cell viability by MTT (dimethyl thiazolyl diphenyl tetrazolium bromide) assay

The anti-proliferative effect of purified L-glutaminase was evaluated on different human tumor cell lines viz Jurkat, K562, U937, OV1063 (ovarian cancer cell lines), MCF-7 (breast cancer cell lines) and HCA 7 (colon cancer cell lines) procured from NCCS, Pune, India. The leukemic cell lines (Jurkat, U937) and OV1063 were grown in RPMI-1640 medium supplemented with 10% heat FCS, 10 mM Hepes, 100 µg/mL penicillin and 100 µg/mL streptomycin. K562 was grown in Iscove's modified DULBECCOS medium with 4 mM L-Glutamine adjusted to contain 1.5 g/l sodium bicarbonate, MCF 7 cell line in EAGLES minimum essential medium with 0.01 mg/ml human insulin and HCA7 in DMEM culture medium. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cell viability was measured using MTT assay according to the method of Mosmann (1983). Cancer cells (5 x 10⁴) were seeded in each well of 96 well plate containing 0.1 ml of medium. L-glutaminase of different concentration (100-1000 µg/mL) was added and after 24 h of incubation, the cell viability was checked by adding 10 µl/well of MTT dye (5 mg/ml stock). The formazan blue crystal, formed by viable cells was dissolved in 100 µl of DMSO and the rate of color production was measured at 570 nm in an ELISA reader. The percentage of viability of cells at various concentrations of L-glutaminase was plotted and the effective dose required to inhibit 50% of the cancer cells (IC₅₀) were determined for all the cancer cell lines.

RESULTS AND DISCUSSION

Enzyme purification

L-glutaminase produced extracellularly by marine *B. subtilis* strain JK 79 was purified to apparent homogeneity using a combination of ammonium sulphate precipitation and chromatographic procedures. Homogeneity was achieved by using 60% ammonium sulphate precipitation followed by DEAE cellulose and gel filtration chromatography using Sephadex G-100. The homogeneity of the purified enzyme was also confirmed by HPLC analysis. The elution profile for the purified enzyme L-glutaminase revealed a single clear peak with a retention time of 4 min which confirms homogeneity. It was also compared with elution profile of the standard

(G8880, glutaminase from *Escherichia coli*) which also showed a single peak with same retention time thus confirming that the purified enzyme is only glutaminase (Figure 4 and 5). Analysis of enzyme activity suggested that this enzyme was purified for about 13.6 fold with specific activity of 937.12 U/mg and 1.75% yield Table 1. The purified enzyme showed high recovery when compared to enzyme purification reported for *Bacillus* sp. LKG-01, *Bacillus licheniformis* GlsA and *B. cereus*. The molecular weight of the enzyme was determined by SDS-PAGE and MOLTI-TOF MS (matrix assisted laser ionization/desorption mass spectroscopy) which was estimated to be 64 kDa (Figures 2 and 3). The monomeric nature of L-glutaminase have been reported in *B. subtilis* RSP-GLU (Sathish and Prakasham, 2010), *Bacillus* sp. LKG-01 (Lokendra et al., 2012), *B. licheniformis* GlsA (Sinsuwan et al., 2012), *Bacillus cereus* (Singh and Banik, 2013), *Stenotrophomonas maltophilia* NYW-81 (Wakayama et al., 2005) and *Cryptococcus albidus* (Iwasa et al., 1987). The dimeric forms of glutaminase is also reported in *Micrococcus luteus* K-3 (Moriguchi et al., 1994), *Bacillus pasteurii* (Klien et al., 2002), *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010) and *A. oryzae* (Levintow, 1954).

Effect of pH and Temperature on L-glutaminase activity and stability

The pH dependence on enzyme activity was determined within the range of 4 to 10 and optimum pH of 7 (Figure 6). The purified enzyme retained 100% activity at the optimum level and more than 50% of its activity at pH 5, for 60 min (Figure 7). Iwasa et al. (1987) reported that pH 6.0 is optimum for L-glutaminase obtained from *C. albidus*. Optimum pH is species specific and is reported as 7.5 for *B. cereus* (Singh and Banik, 2013) and *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010), 8.0 for *B. subtilis* RSP-GLU (Sathish and Prakasham, 2010) 9.0 for *B. pasturii* (Klien et al., 2002) and 11.0 for *Bacillus* sp. LKG-01 (Lokendra et al., 2012). Glutaminases with optimum pH at and above neutrality are suitable for clinical purposes (Singh and Banik, 2013). Furthermore, the source of the enzyme used in this research (*B. subtilis* JK-79) is of marine origin, hence, it would be less

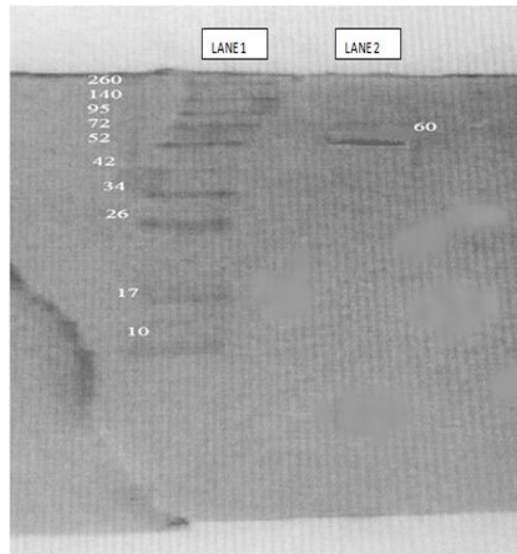


Figure 2. SDS-PAGE analysis of the purified L-glutaminase from marine *B. subtilis* JK-79. Lane 1: Protein marker, Lane 2: Sephadex G-100 fraction

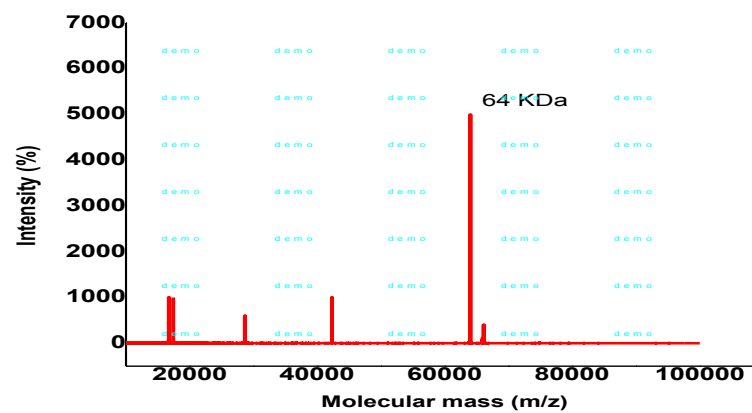


Figure 3. MALDI-TOF MS of L-glutaminase from marine *B. subtilis* JK-79.

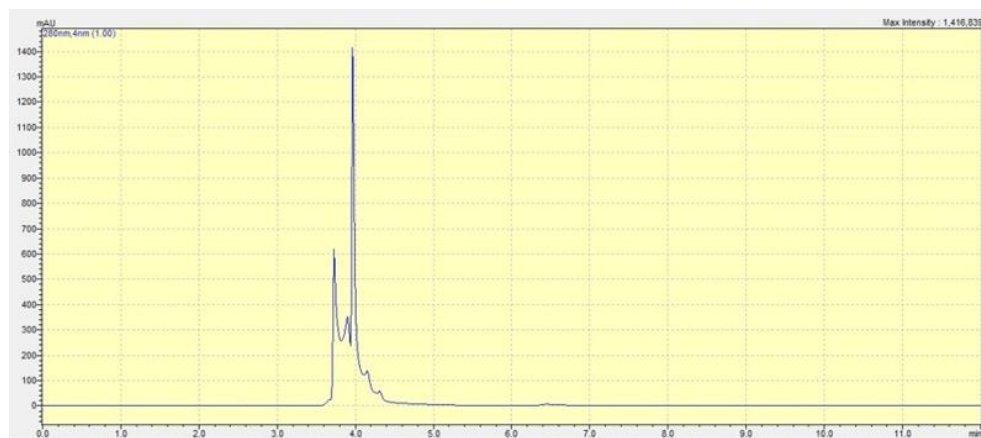


Figure 4. HPLC analysis for the standard Glutaminase(G8880, Sigma Aldrich)

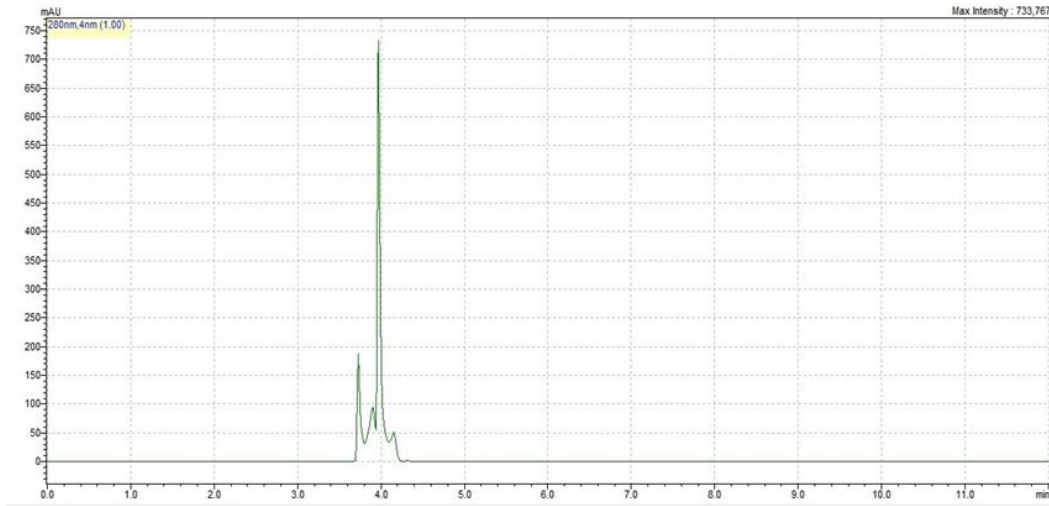


Figure 5. HPLC analysis for the purified enzyme Glutaminase from *B.subtilis* JK-79.

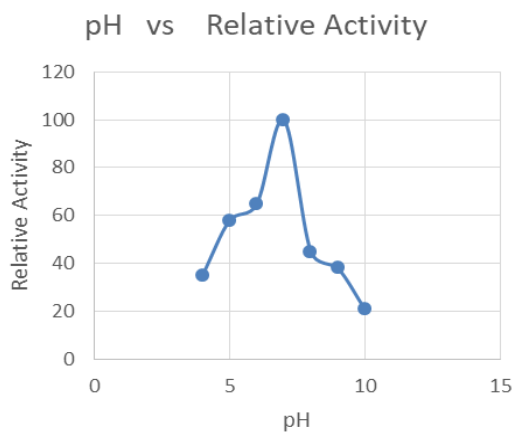


Figure 6. Effect of pH on the activity of L-glutaminase

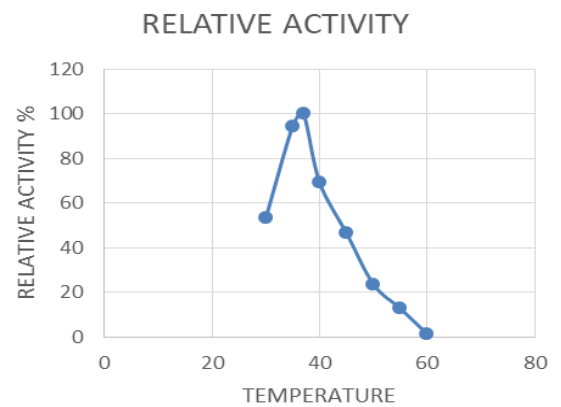


Figure 8. Effect of temperature on L-glutaminase activity.

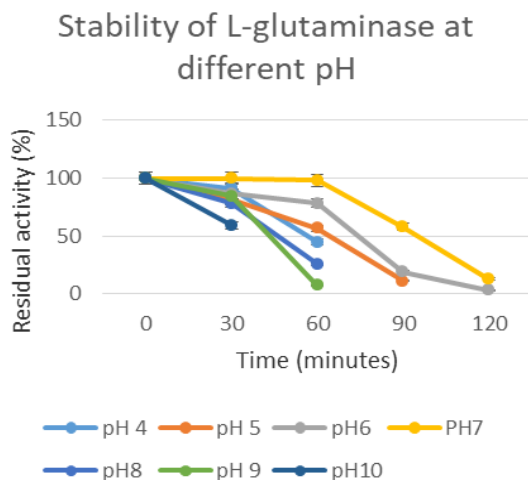


Figure 7. Effect of pH on the stability of L-glutaminase

immunogenic when used as therapeutic agent as the sea water is saline in nature and chemically closer to human blood plasma (Sabu, 2003).

The optimum temperature of the purified L-glutaminase was 37°C and retained 100% activity up to 60 min at this temperature (Figures 8 and 9). The thermostability analysis revealed that the enzyme retained more than 50% of its activity at 40°C for 60 min. The optimum temperature of glutaminase is 37°C for *B. pasturii* (Klien et al., 2002), 37°C for *B. cereus* (Singh and Banik, 2013), 50°C for *B. subtilis* RSP-GLU (Sathish and Prakasham, 2010), 70°C for *C. albidus* (Iwasa et al., 1987) and 50°C for *Aspergillus oryzae* (Yano et al., 1988).

Effect of substrate on L-glutaminase activity

In order to determine the substrate kinetics of L-glutaminase, the enzyme activity was analyzed by

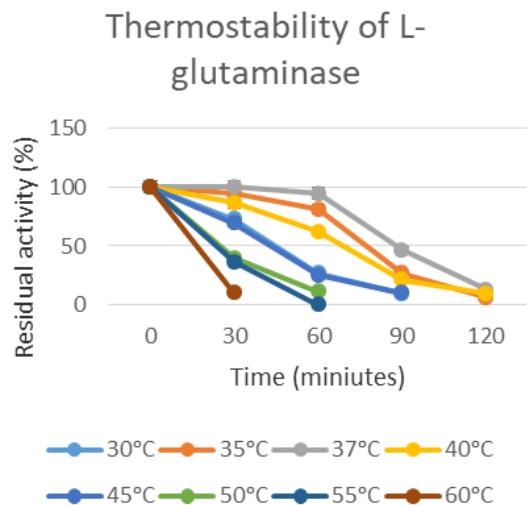


Figure 9. Thermo stability of L-glutaminase.

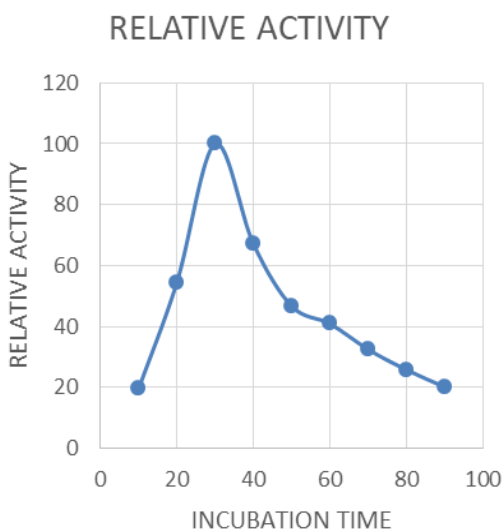


Figure 10. Effect of incubation time.

supplementing with varied L-glutamine concentration ranging from 0.01 M to 0.1 M in the reaction mixture; it was observed that highest activity of the enzyme was noticed with 0.04 M concentration of L-glutaminase incubated for 30 min. Further, the optimum incubation time for the purified enzyme was 30 min as depicted in Figure 10. L-glutaminase showed highest specificity (100% relative activity) for L-glutamine compared to D-glutamine (39%). These results confer that the enzyme L-glutaminase from marine *B. subtilis* JK-79 is highly specific for L-glutamine (Figure 11). Glutaminase from *B. cereus* showed higher specificity for L-glutamine (100% relative activity) compared to D-glutamine with only 48.40% relative activity. Similarly L-glutaminase from

Effect of substrate on L-glutaminase activity

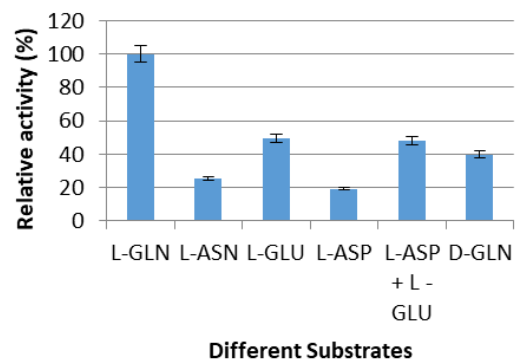


Figure 11. Substrate specificity of L-glutaminase

Bacillus sp. LKG-01 (Lokendra et al., 2012) had good specificity for L-glutamine with 100% relative activity while it showed no activity for glutamic and aspartic acid. The kinetic parameters K_M and V_{Max} of the enzyme were determined by Lineweaver-Burk double reciprocal plot and were estimated to be 0.08 mM and 200 $\mu\text{M}/\text{ml min}$, respectively as shown in Figure 12. This K_M value was higher than the substrate specificity reported by *B. cereus* (Singh and Banik, 2013), *Bacillus sp.* LKG-01 (Lokendra et al., 2012) and *C. albidus* (Lokendra et al., 2012). Glutaminase enzymes are therapeutically suitable if they display high enzyme activity at physiologic pH, that is, between about pH 6.5 and 8.5 and have a low K_M , that is, between 10^{-6} and 10^{-4} M (Roberts et al., 2001). Thus, the L-glutaminase enzyme from marine *B. subtilis* strain JK-79 with low K_M value and optimum pH as 7 could possibly be a potential candidate for anti-cancer drug therapy.

Effect of metal ions on L-glutaminase activity

There were effects of varied concentrations of metal ions on L-glutaminase activity of the purified enzyme. It was evident from Figure 13, that Na^+ , K^+ and Ca^{2+} have increased the enzyme activity, whereas Ba^{2+} , Mg^{2+} , Mn^{2+} and Fe^{2+} showed moderate inhibition on L-glutaminase activity. However, Ag^{3+} and Cu^{2+} strongly inhibited the purified enzyme activity.

Determination of anti-oxidant activity

DPPH and ABTS assay

The damage caused by free radicals plays a significant pathological role in human diseases. Anti-oxidant

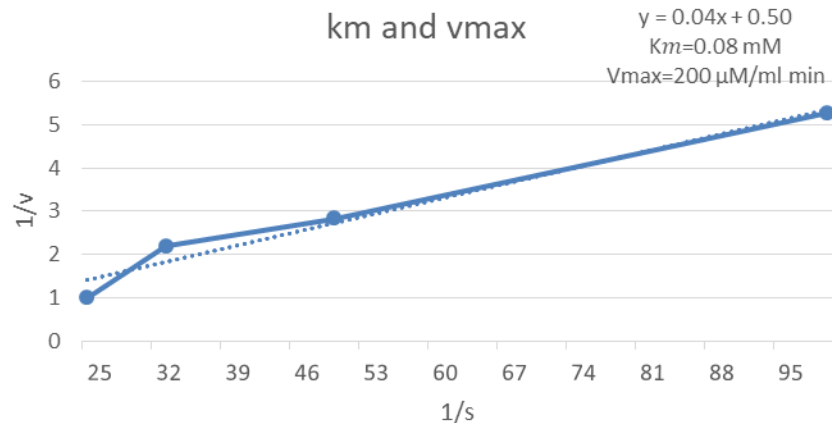


Figure 12. The Lineweaver-Burk plot to determine the K_m and V_{max} for L-glutaminase.

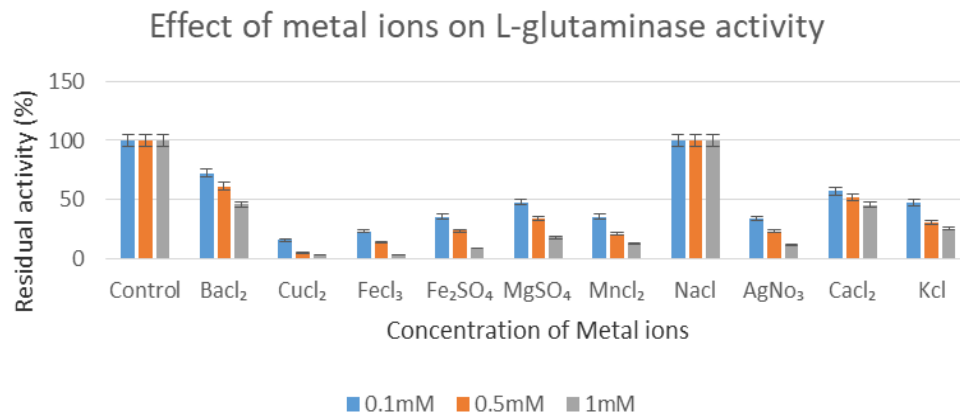


Figure 13. Effect of different metal ions on L-glutaminase activity.

properties, especially radical scavenging activities are very important in controlling the free radicals. At present, a lot of anti-oxidants are available; however, chemically synthesized anti-oxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) are restricted by legislation as its usage might cause deleterious effects in humans. So there is an increased interest in finding new anti-oxidants from natural sources. DPPH and ABTS are the two most commonly used methods to determine the anti-oxidant capacity of pure compounds. Hence, the anti-oxidant capacity of the purified L-glutaminase was analysed. L-glutaminase can scavenge the free radicals generated in vitro by DPPH and ABTS assays by donating their hydrogen atoms.

Figures 14 and 15 illustrate the DPPH and ABTS radical scavenging ability of L-glutaminase and the standard (L-ascorbic acid). L-glutaminase showed a significant DPPH and ABTS radical scavenging activity that was enhanced with increasing concentration. The

IC_{50} value for DPPH and ABTS radicals were found to be 400 and 600 $\mu\text{g/ml}$, respectively.

Determination of anti-tumor activity of L-glutaminase

The two major characteristics that make glutaminase enzymes therapeutically suitable are high enzyme activity at physiologic pH, that is, between about pH 6.5 and 8.5 and low K_M value, that is, between 10^{-6} and 10^{-4} M. Additionally desirable properties of glutaminase enzymes for therapeutic use include: high stability at physiologic pH, not strongly inhibited by the products of the reaction it catalyzes, it does not require co-factors or prosthetic groups, narrow substrate specificity, effective irreversibility of the enzymatic reaction under physiologic conditions, available from an organism that contains low levels of endotoxin and low immunogenicity (Roberts et al., 2001).

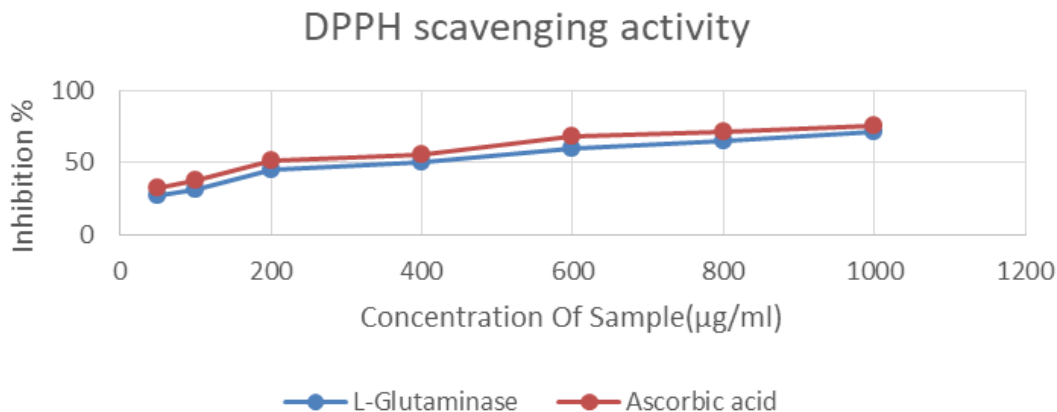


Figure 14. DPPH scavenging activity of L-Glutaminase from marine *B. subtilis* strain JK-79.

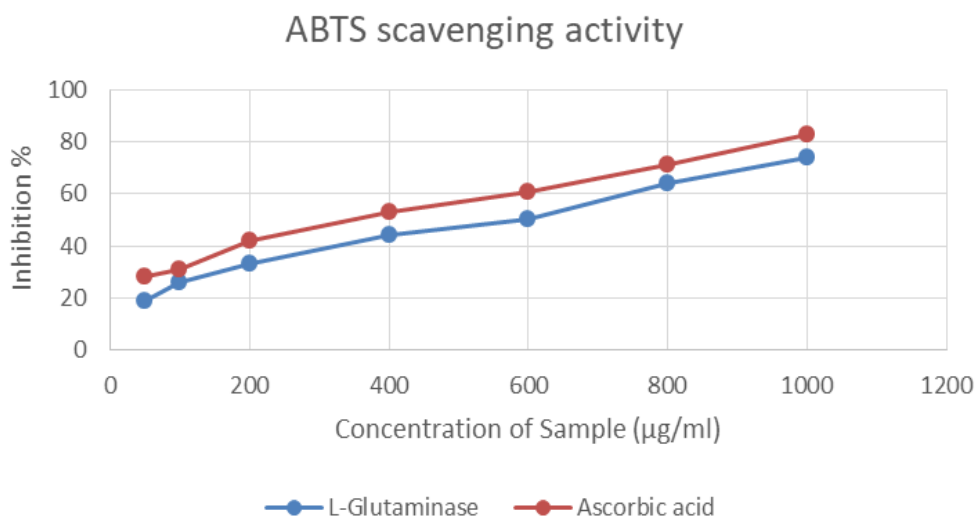


Figure 15. ABTS scavenging activity of L-glutaminase from marine *B. subtilis* strain JK-79.

The properties of the enzyme L-glutaminase from marine *Bacillus subtilis* strain JK-79 with low K_M value, high specificity for L-glutamine, optimum pH 7.0, optimum temperature 37°C and anti-oxidant activity indicate the suitability of this enzyme as a potent anti-cancer agent. Therefore the cytotoxic effect of this enzyme was determined by various human tumor cell lines such as K562, U937, Jurkat, MCF-7, OV1063 and HCA 7 using MTT assay, a non-radioactive, fast and economical assay widely used to quantify cell viability and proliferation. The incubation of the above leukemic cell lines (K562, U937, Jurkat) with increasing concentration of L-glutaminase enzyme leads to a gradual inhibition of cell growth with IC_{50} values of 231 µg/ml for K562 cell lines, 480 µg/ml for U932 and 500 µg/ml for Jurkat cell lines (Figures 16,17 and 18). The cytotoxic activity was compared with Topotecan and PD-184352. Topotecan (tradename

Hycamtin) is a chemotherapeutic agent that is a topoisomerase inhibitor. It is a water-soluble derivative of camptothecin. It is used in form of the hydrochloride to treat ovarian cancer and lung cancer, as well as other cancer types. PD-184352 is a MEK inhibitor. Singh and Banik (2013) studied the cytotoxic effect of L-glutaminase from *B. cereus* MTCC 1305 on the viability of hepatocellular carcinoma cells (Hep-G2) and reported the IC_{50} value as 82.27 µg/ml.

The cytotoxic activity of L-glutaminase purified from marine *B. subtilis* JK-79 was demonstrated on other cell lines such as MCF-7 a breast cancer cell line, OV1063 (ovarian cancer cell line) and HCA 7 (colon cancer cell line). Surprisingly, the enzyme also showed good activity against the three cell lines tested. The IC_{50} value obtained for MCF-7, OV1063 and HCA -7 were 500 µg/ml, 526 µg/ml and 750 µg/ml respectively (Figures 19, 20 and 21).

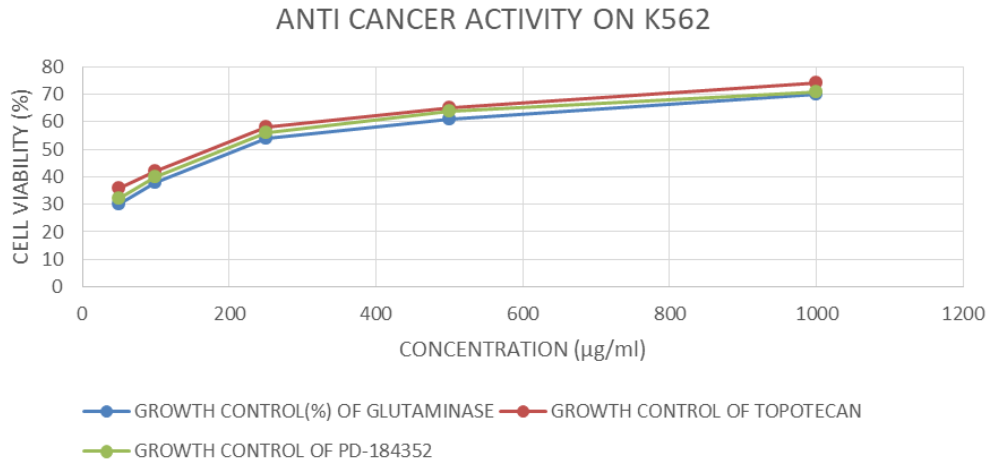


Figure 16. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of K562 cell lines.

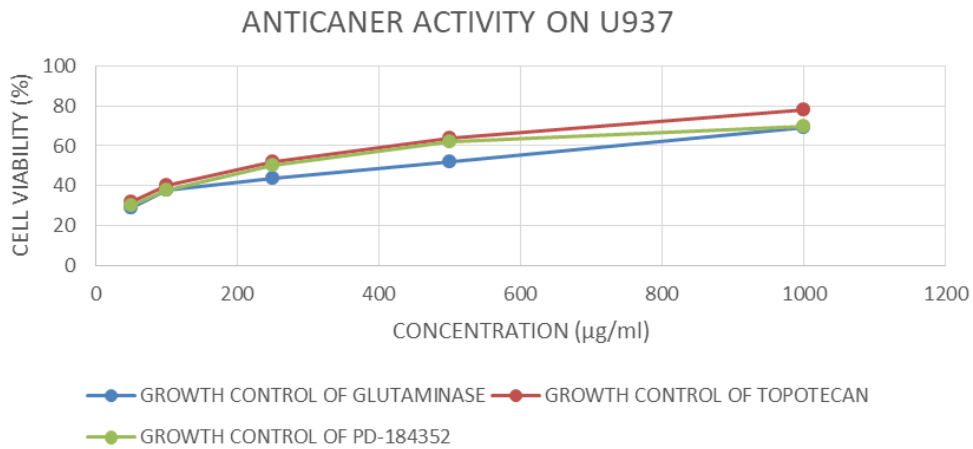


Figure 17. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of U937 cell lines.

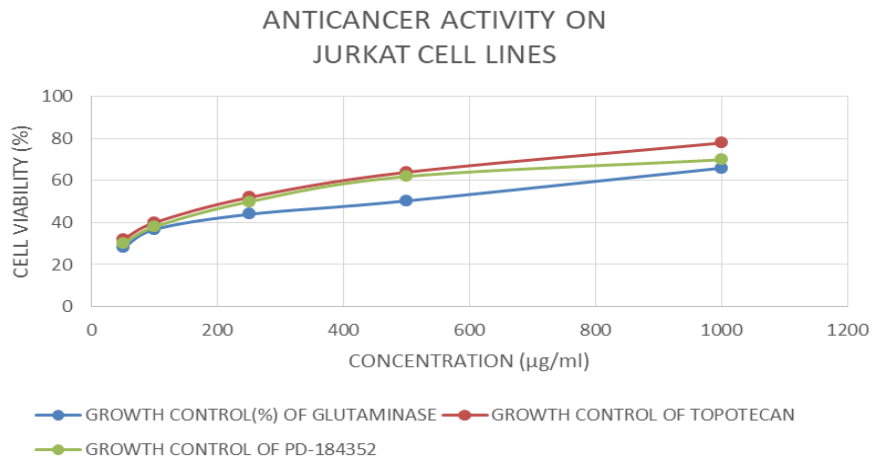


Figure 18. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of Jurkat cell line

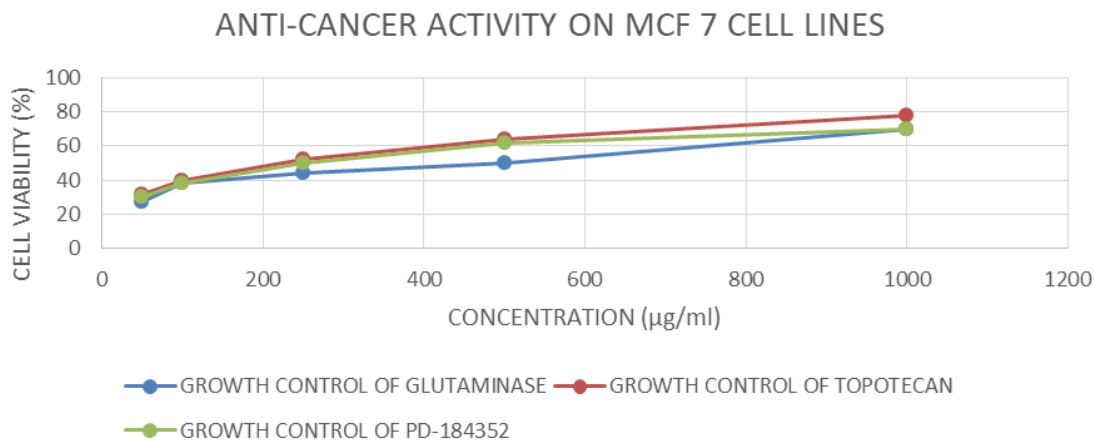


Figure 19. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of MCF-7 cell lines.

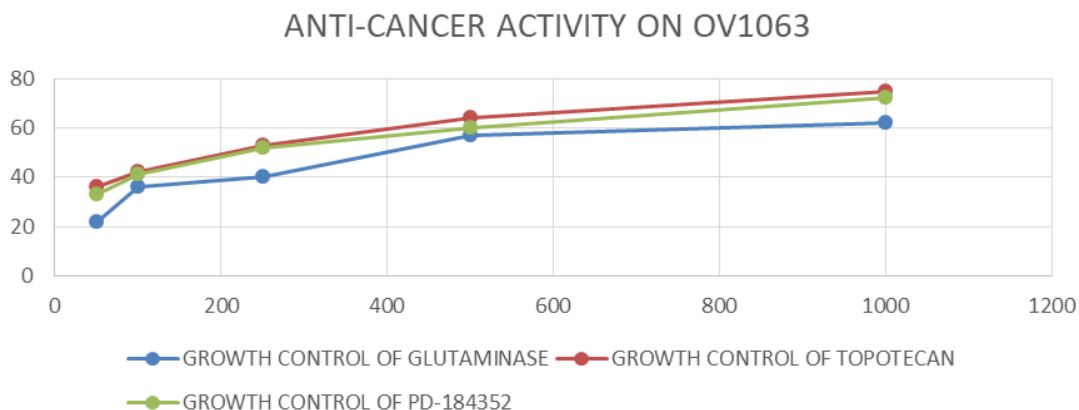


Figure 20. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of OV1063 cell lines.

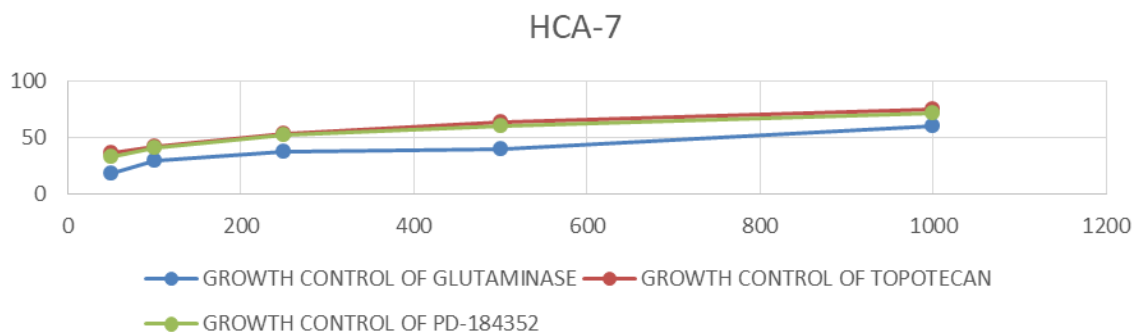


Figure 21. Effect of different concentrations of L-glutaminase from marine *B. subtilis* JK-79 and standard Topotecan, PD-184352 on the viability of HCA-7 cell lines.

Conclusion

Despite the promise of glutaminase as a therapeutic agent, there are only scanty reports on the application of marine

glutaminase enzyme as an anti-cancer agent. To the best of our knowledge, there are no reports available in the literature on marine L-glutaminase enzyme having a broad spectrum of anti-tumor activity. This study demonstrates

the purification of L-glutaminase enzyme from marine *B. subtilis* strain JK-79. Characterisation of the enzyme revealed that it exhibited maximum enzyme activity at physiological pH 7.0 at 37°C and has low Km value with high substrate specificity. This shows that the enzyme is best suited for clinical trials. Hence, the cytotoxic activity of the enzyme L-glutaminase was determined on various human leukemic cell lines (K562, U937 and Jurkat), MCF-7, OV1063 and HCA-7. L-glutaminase from this research, showed good amount of cytotoxic activity on all cell lines. Thus, the enzyme proves to be a potential candidate for therapeutic application as broad spectrum anti-cancer agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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