

Research Paper

Kinetic properties of *Streptomyces canarius* L- Glutaminase and its anticancer efficiency

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Abstract

L-glutaminase was produced by *Streptomyces canarius* FR (KC460654) with an apparent molecular mass of 44 kDa. It has 17.9 purification fold with a final specific activity 132.2 U/mg proteins and 28% yield recovery. The purified L-glutaminase showed a maximal activity against L-glutamine when incubated at pH 8.0 at 40 °C for 30 min. It maintained its stability at wide range of pH from 5.0-11.0 and thermal stable up to 60 °C with Tm value 57.5 °C. It has high affinity and catalytic activity for L- glutamine (Km 0.129 mM, Vmax 2.02 U/mg/min), followed by L- asparagine and L-aspartic acid. *In vivo*, L-glutaminase showed no observed changes in liver; kidney functions; hematological parameters and slight effect on RBCs and level of platelets after 10 days of rabbit's injection. The anticancer activity of L-glutaminase was also tested against five types of human cancer cell lines using MTT assay *in vitro*. L-glutaminase has a significant efficiency against Hep-G2 cell (IC₅₀, 6.8 µg/mL) and HeLa cells (IC₅₀, 8.3 µg/mL), while the growth of MCF-7 cells was not affected. L-glutaminase has a moderate cytotoxic effect against HCT-116 cell (IC₅₀, 64.7 µg/mL) and RAW 264.7 cell (IC₅₀, 59.3 µg/mL).

Key words: L-glutaminase, anti-cancer, cytotoxicity, MTT assay.

Introduction

Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products like enzymes (Thadikamala and Reddy, 2011). Actinomycetes are considered to be preferred enzymes sources due to their production of extracellular enzymes. Many enzymes produced by actinomycetes and have been used as drugs like wise L-glutamine amidohydrolase (E.C. 3.5.1.2), commonly referred to as L-glutaminase (Sunil Dutt *et al.*, 2014). Since the discovery of its anti-tumor properties, L-glutaminases have been in prime focus (Lazarus and Panasci, 1986). Nowadays, L-glutaminase is used as enzyme therapy for cancer especially for acute lymphocytic leukemia (Robert *et al.*, 2001; Kyoko *et al.*, 2004).

Where, high rate of glutamine consumption is a characteristic nature of some types of cancerous cells (Lazarus and Panasci, 1986). Based on this character experimental

therapies have been developed to deprive L-glutamine to tumor cells (Iyer and Singhal, 2008). Inhibition of the tumor cell uptake of glutamine is one of the possible ways to stop the growth and this is the best accomplished by the use of L-glutaminase. This in fact, results in a selective starvation of the tumor cells because unlike normal cells lack properly functioning glutamine biosynthetic machinery (Tanaka *et al.*, 1988; Dang, 2010). Microbial therapeutic enzymes have a broad variety of specific uses as oncolytics, thrombolytics or anticoagulant, and largely as anticancer (Sabu, 2003; Sabu *et al.*, 2005).

The production of enzyme was influenced by a variety of physicochemical and nutritional factors. The factors affecting the production in recent years had received attention as of its great demand in clinical application and also in food industries. It is known that, the factors involved in the process of production, would not only enhance the quantity, but also quality of enzyme because of which it becomes more suitable for a specific application. Optimization of parameters can in turn influence enzyme

synthesis and cell yield (Okami, 1986). The strain of actinomycetes was used for glutamic acid production under optimum growth conditions (Divya Teja *et al.*, 2014). From the compatibility perspective in mass production and as well as beneficial application aspect extracellular enzyme producer as choice of source is always attractive (Pandey, 2003). The objective of this study was to utilize *Streptomyces canarius* FR with good ability to produce extracellular L-glutaminase and characterize the purification. Biochemical, kinetics and *in vitro* anticarcinogenic properties of L- glutaminase will be also examined.

Materials and Methods

Collection, isolation and identification of *S. canarius* FR

Streptomyces canarius FR isolated from Tell Basta, Zagazig, Sharkya Governorate, Egypt was identified according to identification keys (Kämpfer, 2006). The identification was confirmed by the analysis of 16S rRNA gene sequence (Altschul *et al.*, 1997).

The strain was preliminary tested for L-glutaminase production by streaking on minimal glutamine agar medium (MGA) plates. Components of MGA (g/L) include: KCl 0.5; MgSO₄·7H₂O 0.5; KH₂PO₄ 1.0; FeSO₄·7H₂O 0.1; ZnSO₄·7H₂O 1.0; glutamine 5 and phenol red 0.012. The plates were incubated at 30 °C for 5 days. Formation of pink zones around the microbial growth indicated the positive reaction (Balagurunathan and Subramanian, 1993; Balagurunathan *et al.*, 2010).

Optimization of culture conditions for L-glutaminase production

Streptomyces canarius FR was cultivated in L-glutaminase production modified Czapek's medium contained (g/L): L-glutamine 10; D-glucose 5; MgSO₄·7H₂O 0.5; KCl 0.05; KH₂PO₄ 1.0. Enzyme production was tested under different cultured conditions; different incubation periods (0-8 days); different temperatures (25-55 °C); different pH-values (pH 4-10) under shaking and static conditions. The culture was harvested and centrifuged at 10,000 rpm for 30 min the obtained cell free filtrate was used as crude enzyme according to Dura *et al.* (2002).

Activity assay and Protein determination of L-glutaminase

The activity of glutaminase enzyme is determined by estimating the amount of NH₃ liberated from glutamine (Borek *et al.*, 2004). Protein concentration was determined by Lowery *et al.* (1951) using bovine serum albumin (Sigma chemical Co.) as a standard.

L- Glutaminase purification

Two liters from the nutritionally optimized submerged 5 days culture of *S. canarius* FR growing in L-

glutaminase producing medium was prepared. The precipitated protein was collected by centrifugation at 10,000 rpm at 4 °C and dissolved in a minimum volume of phosphate buffer (0.01 M, pH 8.0) (Sabu *et al.*, 2005). L- glutaminase was fractionated by salting out (50-80% ammonium sulphate saturation). The collected precipitate was dissolved in phosphate buffer (0.01 M, pH 8.0) and then dialyzed against the same buffer for 24 h at 4 °C with continuous stirring and occasional changes of the buffers. The dialyzed was fractionated by ion-exchange chromatography (DEAE-Cellulose). After column equilibration the enzyme was eluted by gradient NaCl (50-200 mM) dissolved in phosphate buffer (0.1 M, pH 8.0) with 0.001 M EDTA. The activity, homogeneity (SDS-PAGE) and protein contents of the fractions were determined as above. The most active homogenous fractions were gathered and loaded to pre-equilibrated column of Sephadex G100 gel-filtration chromatography) using the same buffer for elution. For each fraction activity was assessed as above. The most active fractions were pooled and concentrated by dialysis against buffer (Nagendra Prabhu, 1997).

SDS-PAGE Analysis

The molecular weight of L- glutaminase from culture of *S. canarius* was carried out using SDS-PAGE according to Lammeli (1970). PageRuler Unstained Protein Ladder, Fermentas marker was used.

Biochemical and kinetic properties of the purified L-glutaminase

The biochemical properties of purified *S. canarius* L-glutaminase as optimum pH, pH stability, reaction temperature, thermal stability salt tolerance, metal ions and substrate specificity enzyme were determined as described by Amena *et al.* (2010) and Sabu *et al.* (2005).

The thermal stability of enzyme was examined after preincubation of the enzyme at 50-80 °C using 0.1 M phosphate buffer (pH 8.0) for different time (10-90 min). The relative activity was determined after the incubation of reaction mixture at 37 °C for 30 min. The thermal inactivation rate (Kr min) was calculated by the first-order kinetic model (Whitaker, 1972); $\ln(A_t/A_0) = k_t T$, where A₀ and A_t are the specific activity zero and t time. T1/2 was calculated from the linear equation for each temperature. The Tm was calculated from the linear equation of different preincubation temperature at 60 min.

Stability of L-glutaminase was examined after preincubation of the enzyme for 2 h at pH from 4.0-11.0. Acetate (0.2 M), phosphate (0.2 M) and glycine-NaOH buffers were used to covering pH range (4-5), (6-8) and (9-11) respectively. After adding glutamine (40 mM) the reaction mixture was incubated at 37 °C for 30 min. The activity of the enzyme was determined for each pH.

To assay the metal ions effect, the purified enzyme was pre-incubated with each metal ion separately for

30 min before adding glutamine (40 mM). The enzyme relative activity was determined immediately after incubation at 37 °C for 30 min.

The kinetic parameters of L-glutaminase as V_{max} , K_m and K_{cat} were estimated using different concentrations of glutamine, asparagine and aspartic acid, separately (10-100 mM). Michaelis-Mentel constant (K_m) and maximum velocity (V_{max}) were calculated from Lineweaver-Burk plot. Catalytic efficiency (K_{cat}) was expressed by the specific activity per mol enzyme.

Cytotoxicity of L- glutaminase

Cytotoxic effect of the L-glutaminase was evaluated using 5 New Zealand rabbits as experimental group and one rabbit as control. Experimental group were injected intravenous with 1 mL of L- glutaminase (132.2 U/mg protein/1.5 ± 0.1 kg) every 5 days for two weeks. After the two weeks, blood samples were collected 10; 25; 40 and 50 day of the last injection. Plasma were collected and stored at -20 °C. RBC, WBC, platelets, hemoglobin ALT, AST, GGT, total protein, albumin, cholesterol, glucose and creatinine were determined as described by Birt (1967) and Reitman and Frankel (1957). Hemolytic activity of the purified L-glutaminase was evaluated using a blood agar assay (Tay *et al.*, 1995).

Histopathological examination:

Histological examination for the experimental group and control was carried out on liver and kidney, 50 days of the last injection, according to Roy and Maity (2007).

Anticancer efficiency

Anticancer efficiency of L-glutaminase as antitumor was measured against: Human hepatocarcinoma cell line (HepG2); Human breast adenocarcinoma cell line (MCF-7); human colorectal carcinoma cells (HCT-116); Human cervical carcinoma cell line (HeLa) and Raw murine macrophage cells (RAW 264.7. MTT Cell Viability Assay was used as described by Hansen *et al.* (1989). (% Cell viability = O.D of treated cells / O.D of control cells X 100. The five human cell lines were purchased from ATCC, USA). The efficiency of L-glutaminase was determined using sigmoidal dose response curve-fitting models (Graphpad Prizm Software, version 3).

Statistical Analyses

All statistical analyses were carried out using SPSS ver. 11 on pc i5

Results and Discussion

Identification of *S. canarius* FR strain

The present study showed that, the strain is *S. canarius* FR used (after it has been identified by studying their morphological and biochemical characterization).

PCR amplification of 16S rDNA gene confirmed the identity (98%) of *S. canarius* FR. The partial nucleotide sequence of amplified gene was submitted in GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/update.htm>) under accession number KC748492. The results derived from the sequence analysis of the 16S rDNA gene show a G + C content of 58.9 mol % which is in good agreement with the data published for *Streptomyces* by Kannan and Vincent (2011). The tested strain was characterized by formation of a pink zone around colonies using MGA medium due to breakdown of amide bond in L-glutamine and ammonia liberation (Ranjekar and Sridhar, 2002).

Optimization of L-glutaminase production

The submerged fermentation method for producing L- glutaminase was showed that, the L- glutaminase produced, is in its optimum phase (at 30 °C for 5 days and pH 7.5 under shaking condition at 120 rpm) (Data was not shown). Dura *et al.* (2002) and Sabu (2003) stated that submerged fermentation was the routinely used methods for L-glutaminase production from various microorganisms. The result obtained by Krishnakumar *et al.* (2011) is in the agreement with present study. They showed that production of L-glutaminase from *Streptomyces* sp. SBU1 was at 30 °C after 96 h of incubation and initial pH 9.0. Balagurunathan *et al.* (2010) stated that, the optimum conditions for marine *Streptomyces olivochromogenes* L-glutaminase production were determined at pH 7, temperature 30 °C and 3.5% salinity for 5 days under shaking condition at 120 rpm. Divya Teja *et al.* (2014) reported that strain of actinomycetes gave maximum production at pH 7, temperature 30 °C, time 96 h and salinity 3.5%. On the other hands, the optimum conditions (pH and Temperature) for L-glutaminase family is varied from one member to another Usha-Kiranmayi *et al.* (2013) studied one of glutaminase family he found that, the maximum production of L- asparaginase from *Pseudonocardia endophytica* VUK-10 was found in the culture medium with pH 8 and temperature 30 °C incubated for four days. Moreover, Suresh Kumer *et al.* (2013) stated that the response surface methodology predicted that a production containing Temperature 36.31 C, pH 7.34, Time 67.63 h, Galactose 40.20 g/L and L-glutamine 19.09 g/L to be optimum for the production of *Serratia marcescens* L-Glutaminase. Kiruthika and Saraswathy (2013) revealed that the maximum yield of *Vibrio azureus* L- glutaminase production (247 U/mL) was achieved in a seawater based medium at pH 8, 37 °C, 1% inoculum concentration and 2% glutamine concentration for 24 h.

Purification of L-glutaminase

L-glutaminase was purified from 5 days *S. canarius* culture to assess their yield and purity. The crude L-glutaminase was undergoes fractional precipitation by salting out (50-80% ammonium sulfate). The enzyme overall

purification profile from *S. canarius* culture was summarized in Table 1. The crude extract contained 23.0 mg of protein and showed a total glutaminase activity 170 units with specific activity of 7.4 U/mg protein. At all purification steps, the specific activity increased compared to crude. The maximum specific activity 132.2 IU/mg protein with a yield of 28% was attained after Sephadex G100 purification. The partial purified was increased more than 17 fold compared with crude. These results were in the agreement with that reviewed by Mohana Priya *et al.* (2011). Also, Kumar *et al.* (2012) stated that *Bacillus* sp. LKG-01 (MTCC 10401) L-glutaminase activity was purified 49-fold from cell-free extract with 25% recovery with specific activity 584.2 U/mg protein after gel filtration. On the other hand, Elshafei *et al.* (2014) recorded that an intracellular L-glutaminase from *Penicillium brevicompactum* NRC829 was purified to homogeneity (162.75 fold) with an apparent molecular mass of 71 kDa. So the method used in the present study proved to be a good method in producing and purification of L-glutaminase.

Determination of molecular weight for enzyme:

The molecular homogeneity of the purified L-glutaminase from fermentation condition, as well as their purification steps were evaluated by SDS-PAGE. Single band at 44 kDa was appeared after final purification step (Column Sephadex G₁₀₀) (Figure 1). Similarly, the molecular mass of the native enzyme from *Stenotrophomonas maltophilia* NYW-81 was estimated to be 41 kDa by gel filtration (Wakayama *et al.*, 2005). And, Singh and Banik (2013) revealed that L-glutaminase extracellularly produced by *Bacillus cereus* MTCC 1305 was purified to apparent homogeneity with a fine band. The molecular weight of native enzyme and its subunit were found to be approximately 140 and 35 kDa, respectively, which indicates its homotetrameric nature.

Biochemical properties of the purified L-glutaminase

Optimal pH and pH stability

The L-glutaminase maintained its activity over a range of pH 5.0 -9 with optimum at pH 8.0 (Figure 2 A). The activity significantly decreased at both low and high pH values. The pH stability of the L-glutaminase also showed a similar trend, the enzyme was stable at wide range pH 5.0-11.0 with being most stable at pH 7.0-9.0.

Practically, at acidic pH values a higher rate of enzyme inactivation was appeared, comparing to alkaline side, assuming the enzyme basic identity. The negative effect on enzyme activity at lower and higher pH values, suggesting the effect on ionization state of enzyme, modifying the enzyme surface charge, dissociation of subunits/ coenzyme, consequently disrupt its binding with substrate. Consistently, the activity of L-glutaminase from *S. canarius* showed alkaline optimum pH 7- 8, this neutral pH stability of enzyme, being a favored criterion for enzyme action in vivo. Similarly, *Streptomyces gulbargensis* L-asparaginase was more stable at the alkaline pH than at the acidic one (Amena *et al.*, 2010; Kumar *et al.*, 2012).

Optimal temperature and thermal stability

The optimum temperature for the L-glutaminase activity was determined by incubation of the reaction mixture at various degrees (25-50 °C) using L- glutamine as substrate in 0.1 M potassium phosphate buffer (pH 8.0). The highest enzyme specific activity (53.3 U/mg protein) was obtained at 40 °C. Above and below this temperature the enzyme activity decreased (Figure 2 B). Regarding to thermal stability the enzyme had a catalytically thermal stability below 60 °C, with a slightly decreasing in its activity at 70 °C, while completely lost its activity at 80 °C (Figure 2 C). The enzyme half-life times ($T_{1/2}$) was 45.7, 38.8, 29.3 and 17.3 min at 50, 60, 70 and 80 °C, respectively. Also, the T_m of enzyme was 57.5 °C, assuming the retaining of about 50% of its initial activity by heating for 60 min (Figure 2 D). Theoretically, thermal inactivation rates (K_r) are 0.0184, 0.0227, 0.0544 and 0.0449 S^{-1} at 50, 60, 70 and 80 °C, suggesting the dissociation of co-enzyme or denaturation of subunits by heating per unit time. These results were in consistent with those reported for Amena *et al.* (2010) and Kumar *et al.* (2012). Also, Singh and Banik (2013) recorded that the purified L- glutaminase enzyme produced by *Bacillus cereus* MTCC 1305 showed maximum activity at optimum pH 7.5 and temperature 35 °C. The enzyme retained stability up to 50 and 20% even after treatment at 50 and 55 °C, respectively, for 30 min. Furthermore, Elshafei *et al.* (2014) recorded that the purified *Penicillium brevicompactum* L- glutaminase showed its maximal activity against L-glutamine when incubated at pH 8.5 at 50 °C for 30 min indicating the thermo-stability nature of this enzyme.

Table 1 - Summary of purification steps of *Streptomyces canarius* L- glutaminase.

Purification steps	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	170	23	7.4	1	100
70% Amm. Sulph.ppt	145	17	8.5	1.2	85
DEAE-Cellulose	95	0.77	123.4	16.7	56
Sephadex G100	47.6	0.36	132.2	17.9	28

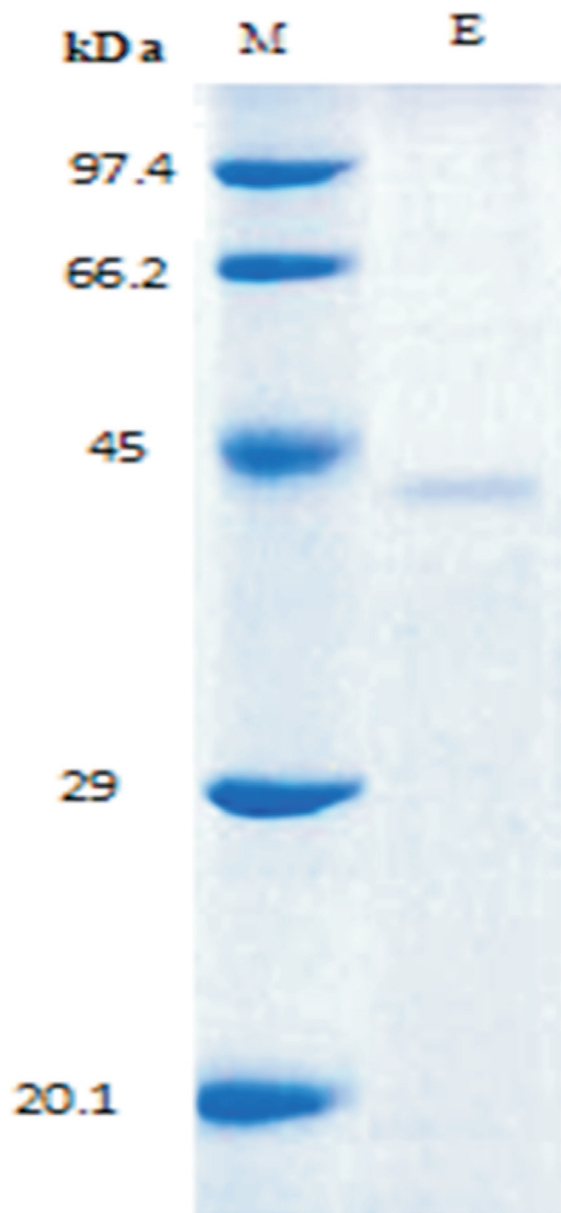


Figure 1 - SDS- PAGE of purified L-glutaminase from *S. canarius*. Lane M (marker protein), Lane E, the purified enzyme.

Salt tolerance of *S. canarius* L-glutaminase

The results represented in Figure (2 E) showed that, tolerance in glutaminase activity was observed by increasing the NaCl concentration up to 25%. This is a good tolerance value. Similarly, Wakayama *et al.* (2005) observed that the L-glutaminase produced from actinomycetes has a good salt tolerance.

Substrate specificity and kinetic properties of *S. canarius* L-glutaminase

The kinetic parameters for L-glutaminase towards substrates were determined from the Lineweaver-Burk Plot

(Figure 2 F and Table 2). The enzyme had relative high affinity and catalytic activity for L- glutamine (K_m 0.129 mM, V_{max} 2.02 U/mg/min), followed by L-asparagine (K_m 0.137 mM, V_{max} 1.75 U/mg/min) and low affinity to L-aspartic acid (K_m 5.38 mM, V_{max} 0.68 U/mg/min). Also, the highest catalytic efficiency (K_{cat} turnover number) for the enzyme was assessed for L-glutamine ($0.75 \times 10^{-3} s^{-1}$) followed by L-asparagine ($0.68 \times 10^{-3} s^{-1}$) and L-aspartic acid ($0.25 \times 10^{-3} s^{-1}$). The high affinity of *S. canarius* L-glutaminase to L-glutamine as substrate was detected previously by Senthil-Kumar and Selvam (2011) and Kumar *et al.* (2012) for *Streptomyces radiopugnans* MS1 and *Pseudomonas* sp.BTMS-51, respectively. Also, Singh and Banik (2013) revealed that the substrate specificity test of *Bacillus cereus* MTCC 1305 L-glutaminase showed its specificity for L-glutamine. Moreover, the small K_m (0.129 mmol) of the tested *S. canarius* L-glutaminase indicated high affinity of the enzyme to glutamine meaning that the rate will approach V_{max} more quickly (Lehninger *et al.*, 2005). Also, Elshafei *et al.* (2014) recorded that the highest activity of *Penicillium brevicompactum* L- glutaminase was reported towards its natural substrate, L-glutamine, with an apparent K_m value of 1.66 mM.

Influence of metal ions on L-glutaminase activity:

The impact of various inhibitors and activators on catalytic potency of the prepared L- glutaminase was evaluated by pre incubation of the enzyme with each compound for 30 min without substrate. Results in Table 3 showed that, Ca^{+2} , Ba^{+2} , Fe^{+3} , Zn^{+2} , Cu^{+} , Hg^{+2} and Cd^{+2} considered to be inhibitor. On the other hand, Mn^{+2} , Na^{+} and Co^{+2} were shown to be activator. These results were in agreement with Senthil-Kumar and Selvam (2011) for *S. radiopugnans* MS1 L-asparaginase. Also, Singh and Banik (2013) reported that monovalent cations (Na^{+} , K^{+}) and phosphate ion activated the *B. cereus* MTCC 1305 L-glutaminase activity, while divalent cations (Mg^{2+} , Mn^{2+} , Zn^{2+} , Pb^{2+} , Ca^{2+} , Co^{2+} , Hg^{2+} , Cd^{2+} , Cu^{2+}) inhibited its activity.

Cytotoxicity effect of L-glutaminase

The cytotoxicity effect of purified *S. canarius* L-glutaminase was explored based on the hematological and blood chemistry pictures for the New Zealand rabbits (Table 4). From the biochemical profiles, generally, there is no observed effect on liver, renal functions, glucose, lipids and other electrolytes, with slight inducing effect on the activity of ALT, AST and level of Random S. Glucose. Depending on these results, the L- glutaminase had relatively no negative effect on liver functions, where AST and ALT was the most potential indicator for liver dysfunction, this in the agreement with that obtained by Pratt and Kaplan (2000).

Also, the cellular toxicity of the purified *S. canarius* L-glutaminase was evaluated by estimation of the degree of

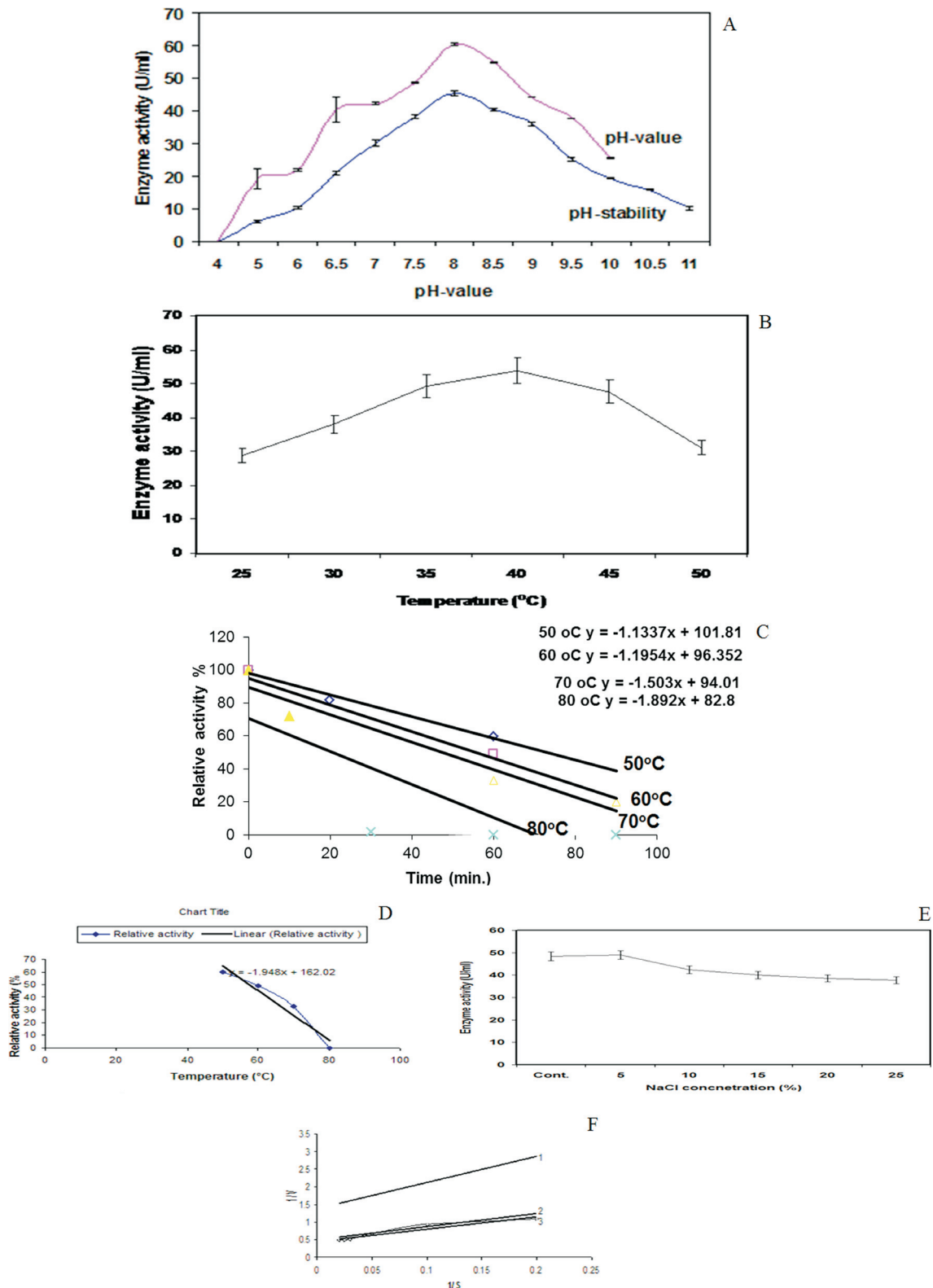


Figure 2 - Characterization of *S. canarius* L-glutaminase. (A) pH value and pH stability profile. The enzyme was preincubated for 2 h at various pHs (4.0 -11.0), then measuring the residual deaminating activity; (B) The optimal temperature for activity was assessed by measuring the enzyme activity at different temperatures between 25 and 50 °C; (C) Thermal stability profile. After incubation of enzyme in different temperature (50-80 °C) at various periods (10-90 min), the residual activity was determined by the standard assay method; (D) Thermal inactivation profile. T_m is temperature degree at which the enzyme retains half of its initial activity at 60 min. (E) Different NaCl concentrations from 0-25%; (F) K_m values (Lineweaver-Burk plot) were calculated by fitting the $1/S$ of substrate vs. the $1/V$ of activity of the enzyme. (1) L- aspartic acid $y = 7.4538x + 1.3854$; (2) L-asparagine $y = 3.4933x + 0.4514$; (3) L-glutamine $y = 3.7046x + 0.5076$.

Table 2 - Kinetics of *S. canarius* L- glutaminase.

Substrates (mM)	Km (mmol ⁻¹)	Vmax (U mg ⁻¹ protein min ⁻¹)	Kcat (s ⁻¹)
L- glutamine	0.129	2.02	0.75 x 10 ⁻³
L- asparagines	0.137	1.83	0.68 x 10 ⁻³
Aspartic acid	5.38	0.68	0.25 x 10 ⁻³

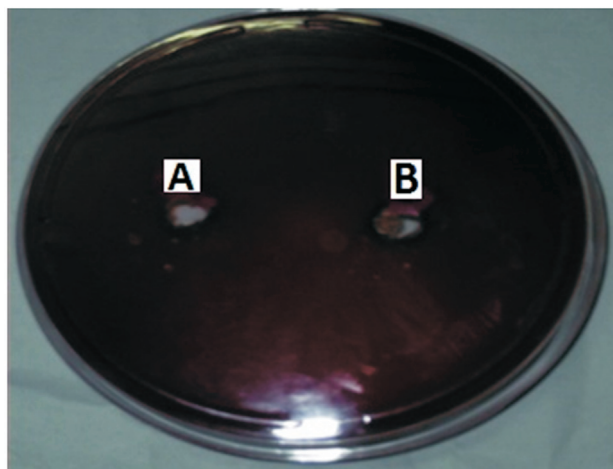
The kinetic parameters were determined by incubation of the enzyme (132.2 U/mg protein) in potassium phosphate buffer (pH 8) with various concentrations of substrate (10-100 mM) under the standard assay conditions, then measuring the deaminating activity of the enzyme. Maximum velocity (Vmax) was expressed by activity of enzyme in μmol of NH₃ compounds formed per minute per mg protein enzyme. Km is the substrate concentration (mM) at half of maximum velocity. Kcat is the maximum velocity of the enzyme per mol per second.

platelet aggregations and hemolytic activity as described by Wei *et al.* (2007). All the hematological parameters (Table 4) were in normal range along the experimented period, however, the red blood cells, hemoglobin, white blood cells and the platelets slightly decreased within normal range after 10 days of L-glutaminase injection. The L- glutaminase displayed no hemolytic activity to human blood (Figure 3). Platelet aggregation and hemolytic activity are the most relevant biochemical assays (Pratt and Kaplan, 2000). The lack of ability to aggregate human platelets and lyses of human RBCs are unique supportive criteria from therapeutic point of view. Similar results approved the non cytotoxicity of microbial glutaminase (Baskerville *et al.*, 1980).

Table 3 - Effect of inhibitors and activators on L- glutaminase activity.

Metal ions (5 mM)	Relative activity (%)
None (control)	100.67 abc \pm 0.15
Ca ⁺²	75.33 cd \pm 0.76
Na ⁺²	105.00 ab \pm 0.50
Ba ⁺²	56.70 d \pm 5.29
Mg ⁺²	81.33 bc \pm 60.48
Hg ⁺²	20.00 e \pm 0.50
Co ⁺²	122.00 a \pm 0.50
Fe ⁺³	53.00 d \pm 0.87
Mn ⁺²	125.00 a \pm 0.50
Cu ⁺²	50.00 d \pm 0.50
Zn ⁺²	87.00 bc \pm 0.92
Cd ⁺²	17.00 e \pm 0.50
I.s.d	27.49
F	12.669
Sig.	.000

The enzyme activity was determined in the absence and presence of different ions at concentration 10 mM after a 30 min exposure to each ion. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with the effectors to that of the untreated enzyme.

**Figure 3** - Hemolytic activities of *Streptomyces canarius* L- glutaminase, 100 μL of purified enzyme (A) and 200 μL purified enzyme (B).

Histological studies:

Figures (4 and 5) show photomicrograph of liver and kidney section histological of treated and non treated rabbits after 50 days of the last injection. It can be seen from photomicrograph of renal and liver tissues, there is no observed abnormalities in these structure. Since, the kidney showing normal glomeruli surrounded by normal renal tubules lined cubical epithelial cells, whereas in treated tissue showed round distal convoluted tubules lined by cubical epithelium and longitudinal collecting duct lined by columnar epithelium (Figure 4). Photomicrograph of treated rabbit liver showing variable sized central veins surrounded by cords and rows of hepatocytes, comparing with negative control (Figure 5). These results were in similarity with that obtained by Baskerville *et al.* (1980) and Roy and Maity (2007).

Anticancer efficiency *in vitro*

Using MTT assay in the present investigation, the effect of the purified enzyme on the growth of Hep-G2, MCF7, HCT-116, HeLa and RAW 264.7 cells were studied after 48 h of incubation. As shown in Figure (6), the tested enzyme had a high efficiency against Hep-G2 cell (IC₅₀, 6.8 $\mu\text{g}/\text{mL}$) and HeLa cells (IC₅₀, 8.3 $\mu\text{g}/\text{mL}$), while the growth of MCF-7 cells was not affect by the treatment. In the other hand, the treatment of HCT-116 and RAW 264.7 cells with the tested enzyme indicated that there was a moderate cytotoxic effect as concluded from their high IC₅₀ calculated values: 64.7 $\mu\text{g}/\text{mL}$ and 59.3 $\mu\text{g}/\text{mL}$, respectively, compared with the known anticancer drug paclitaxel, which its IC₅₀ values for these cell lines ranged from 0.5-1.2 $\mu\text{g}/\text{mL}$. Similarly, Devi and Azmi (2012) showed that the purified L-asparaginase from *Erwinia carotovora* MTCC 1428 used for killing of Hep-2C cell line. Also, *E. carotovora* MTCC 1428 asparaginase was showed better *in*

Table 4 - Hematological and biochemical parameters of rabbits in response to *S. canarius* L- glutaminase.

Parameters	Control (at zero time)			After 10 days			After 25 days			After 40 days			After 50 days		
	Control	Sample	t-test	Control	Sample	t-test	Control	Sample	t-test	Control	Sample	t-test	Control	Sample	t-test
Serum Bilirubin: Total	0.81	0.77	6.29*	0.74	0.63	12.85**	0.75	0.66	9.0*	0.72	0.64	2.28 n.s			
Direct	0.22	0.19	4.330	0.20	0.64	-936	0.21	0.17	11.000	0.20	0.17	2.598 n.s			
Serum Proteins: Total	4.50	4.64	11.930**	4.58	4.35	39.837**	4.94	5.09	-8.660*	4.90	4.81	3.118 n.s			
Albumin	3.55	3.58	2.177 n.s	3.50	3.18	19.4**	3.52	3.48	1.857 n.s	3.45	3.38	2.291 n.s			
Globulins	3.12	3.17	4.10	3.32	3.45	-8.51*	3.34	3.37	-2.219 n.s	3.38	3.34291	1.571 n.s			
S.Alk.Phosphatase	239	243.00	254.67	245.33	274.00	43.00-**	247.33	251.67	3.606- n.s	245.00	249.00	-3.464 n.s			
GGT	11	11.67	15.00	13.17	14.50	-8.0*	12.63	13.00	-78 n.s	12.10	12.50	-2.62 n.s			
S.GOT (AST)	22	29.00	43.00	32.33	41.00	-8.549*	31.00	39.00	**10.474-	30.00	34.00	-2.619n.s			
S.GPT (ALT)	66	68.00	80.67	66.00	69.00	-1.44n.s	64.00	67.00	-1.29n.s	63.00	65.00	-.756n.s			
Serum cholesterol: Total	45	53.00	56.00	49.00	46.00	1.964n.s	47.00	47.00	.000n.s	52.00	51.00	.577n.s			
Blood Urea	0.75	0.80	1.05	0.88	1.03	0.78 n.s	0.93	0.99	2.268-n.s	0.90	0.99	-7.794*			
S. Creatine	135	138.00	125.33	140.00	134.00	2.598n.s	145.00	138.00	2.782 n.s	142.00	140.00	.866n.s			
Random S. Glucose	93	100.33	115.00	107.00	118.00	-19.053**	100.00	106.00	10.392**	104.00	102.00	1.0n.s			
Red Blood cells (RBC)	6.12	6.29	5.00	6.37	5.93	44.00**	6.53	6.17	23.568**	6.50	6.47	1.299n.s			
Hemoglobin	11.7	11.33	10.00	11.40	11.20	1.23 n.s	11.70	11.00	2.646 n.s	11.90	11.20	6.062*			
White blood cells (WBC)	9.8	9.50	8.90	9.90	7.60	9.959*	9.80	9.80	.000 n.s	9.70	9.00	1.732n.s			
Platelets	382	379.00	360.00	378.00	373.33	1.257n.s	376.33	374.67	1.7814n.s	383.00	372.67	2.23n.s			

*, ** and NS indicate $p < 0.05$, $p < 0.01$ and not significant, respectively.

Five New Zealand rabbits were i.v. injected by 1 mL of three successive doses of *S. canarius* L- glutaminase (132.2 U/mg protein/1.5 ± 0.1 kg) during two weeks. Blood samples were collected after 10, 25, 40 and 50 day of the last injection. Control sera (zero time) without enzyme injection were used.

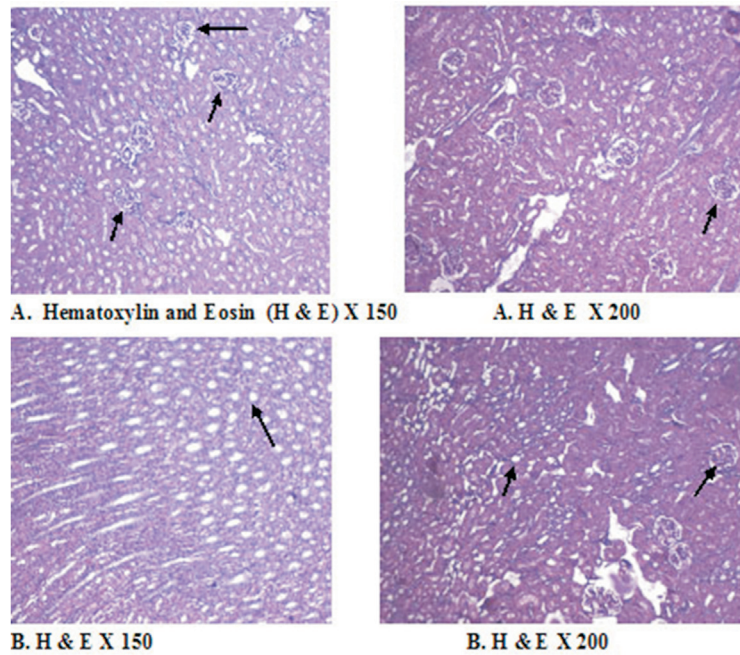


Figure 4 - Photomicrograph (at Hematoxylin and Eosin X 150 and X200) of renal section of, (A) negative control showing normal glomeruli (↑) surrounded by normal renal tubules lined cubical epithelial cells. (B) treated with *S. canarius* L-glutaminase showing round distal convoluted tubules (↑) lined by cubical epithelium and longitudinal collecting duct lined by columnar epithelium.

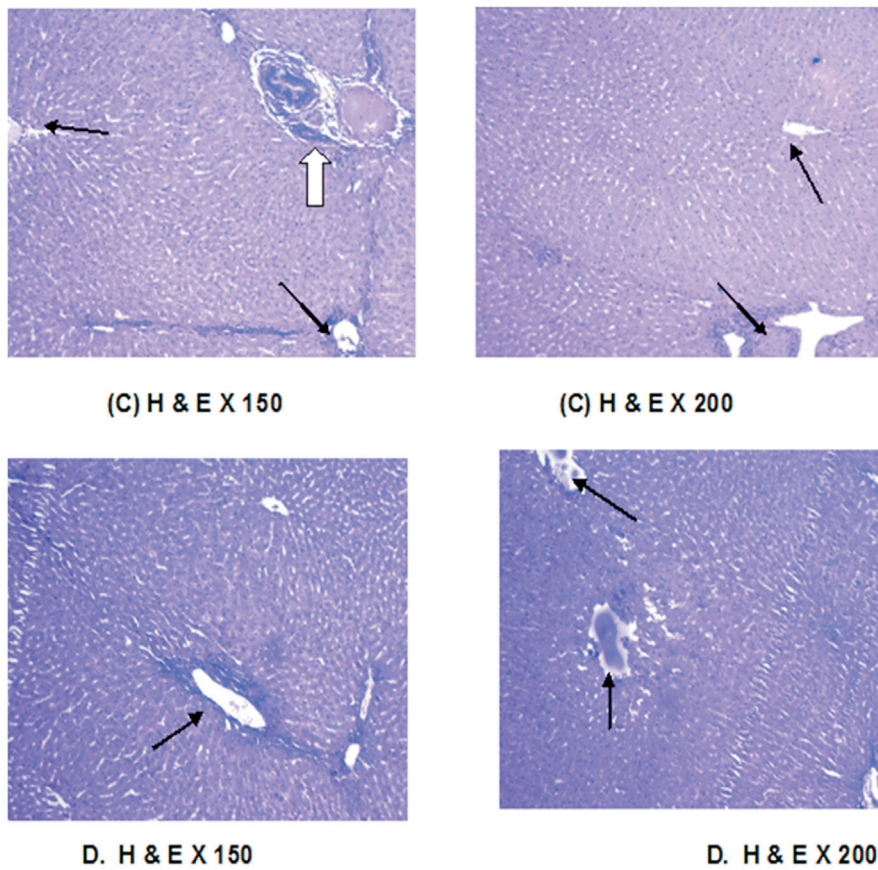


Figure 5 - Photomicrograph (at H & E X 150 and X200) of liver section of (C) negative control showing normal architecture formed of central vein (↑) and portal tract (ñ) surrounded by cords and rows of normal hepatocytes. (D) Treated with *S. canarius* L-glutaminase showing variable sized central veins (↑) surrounded by cords and rows of hepatocytes.

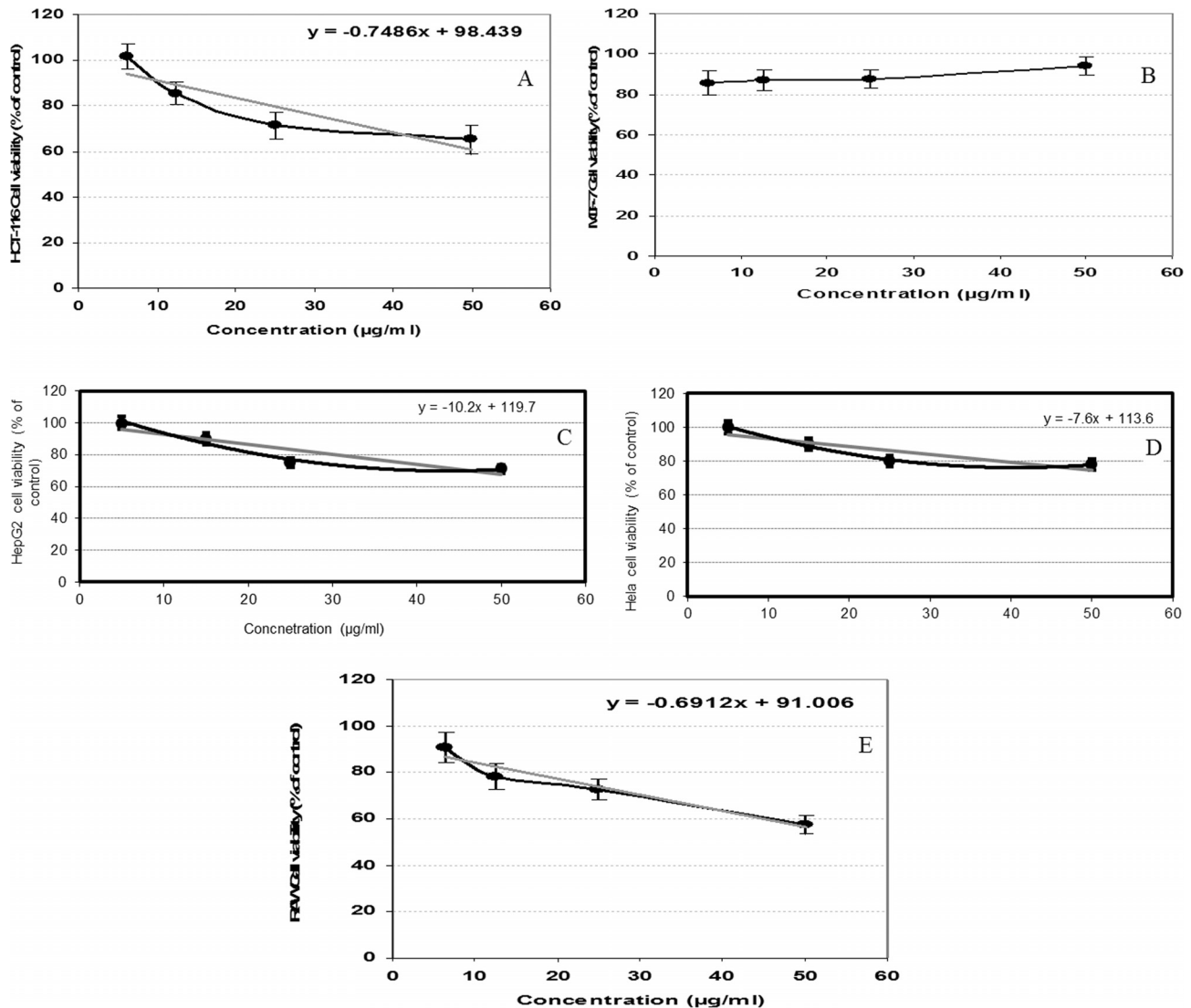


Figure 6 - Cytotoxic effect of *S. canarius* L-glutaminase on tumor cell lines using MTT assay (n = 4), HCT-116 (A), MCF-7 (B), HepG2 (C), HeLa (D) and RAW 264.7 (E). Cells exposed to different concentrations of the drug for 48 hours. All data are expressed as the mean value of cell viability (% of control) \pm S.E.s

in vitro toxicity on Hep-2C cell lines (84% survival) in comparison to commercial L-asparaginase preparation (90% survival) obtained from *E. coli*. Also, Singh and Banik (2013) reported that the gradual inhibition in growth of hepatocellular carcinoma (Hep-G2) cell lines was found with IC₅₀ value of 82.27 μ g/mL in the presence of different doses of L-glutaminase (10-100 μ g/mL). Moreover, Elshafei *et al.* (2014) recorded that the purified enzyme produced by *Penicillium brevicompactum* was inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2), with IC₅₀ value of 63.3 μ g/mL. Meanwhile, Nathiya *et al.* (2012) indicated a crucial role of *Aspergillus flavus* KUGF009 L-glutaminase in breast cancer (MCF7). Furthermore, Roberts *et al.* (1970) found that L-glutaminase from *Pseudomonas* sp. 7A is administered to

inhibit HIV replication in infected cells. The enzyme brings about inhibition of tumor (melanoma) and DNA biosynthesis in affected cells. Glutaminase and asparaginase enzymes have produced prolonged remissions of certain experiment tumors (Wriston and Yellin, 1973). During treatment, plasma glutamine and asparagines are depleted. The degree of amino acid depletion depends on the kinetic properties of the enzymes, its biological half life in the animal, and the rate of input of the amino acid into circulation (Wriston and Yellin, 1973).

In conclusion, *S. canarius* (KC460654) had remarkable capacity to produce L- glutaminase. The purified enzyme showed a unique specificity to glutamine, broad pH stability, and high thermal stability. Also, the purified en-

zyme is being promising candidate for application as anti-tumor agent in the future work.

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References

- Altschul SF, Madden TL, Schäffer AA *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 17:389-402.
- Amena S, Vishalakshi N, Prabhakar M *et al.* (2010) Production, purification and characterization of L-asparaginase from *Streptomyces gulgargensis*. *Braz J Microbiol* 41:1517-8382.
- Balagurunathan R, Radhakrishnan M, Somasundaram SR (2010) L- glutaminase sediments - selective isolation, semi Quantitative assay and characterization of potential strain. *Aust J Basic Appl Sci* 4:698-705.
- Balagurunathan R, Subramanian A (1993) Studies on marine *Streptomyces nigrifaciens* (P9) I. Taxonomy and standardization of antibiotic production. *Ciencias Marinas* 19:435-443.
- Baskerville A, Hambleton P, Benbough JE (1980) Pathological features of glutaminase toxicity. *Br J Exp Pathol* 61:132-138.
- Birt JH (1967) International committee for standardization in hematology. 13:6-18.
- Borek D, Michalska K, Brzezinski K *et al.* (2004) Expression, purification and catalytic activity of *Lupinus luteus* asparagines β -amidohydrolase and its *Escherichia coli* homolog. *Eur J Biochem* 271:3215-3226.
- Dang CV (2010) Glutaminolysis: supplying carbon or nitrogen. *Cell Cycle* 19:3884-3886.
- Devi S, Azmi W (2012) One step purification of glutaminase free L- asparaginase from *Erwinia carotovora* with anticancerous from activity. *Int J Life Sci pharma Res* 2:2250-2480.
- Divya Teja D, Sri Devi V, Harsha N *et al.* (2014) Production of L-glutaminase from marine ecosystems and optimal conditions for maximal production by actinomycetes. *International Journal of Advanced Research* 2:485-491.
- Dura MA, Flores M, Toldra F (2002) Purification and characterization of L-glutaminase from *Debaryomyces* spp. *Int J Food Microbiol* 76:117-126.
- Elshafei AM, Hassan MM, Ali NH *et al.* (2014) Purification, kinetic properties and antitumor activity of L- glutaminase from *Penicillium brevicompactum* NRC829. *British Micro Res J* 4:97-115.
- Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119:203-210.
- Iyer P, Singhal RS (2008) Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. *Bioresour Technol* 99:4300-4307.
- Kämpfer P (2006) The Family Streptomycetaceae. Part I, Taxonomy. In: M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt (eds) *The Prokaryotes. A Handbook on the Biology of Bacteria*. Vol. 3. 3rd ed. Springer-Verlag, Berlin, pp. 538-604.
- Kannan RR, Vincent SGP (2011) Molecular characterization of antagonistic *Streptomyces* isolated from a mangrove swamp. *Asian J Biotechnol* 3:237-245.
- Kiruthika J, Saraswathy N (2013) production of L- glutaminase ant its optimization from a novel marine isolate *Vibrio azureus* JK-79. *African J Biotech* 12:6944-6953.
- Krishnakumar S, Alexis R, Ravikumar S (2011) Extracellular production of L- Glutaminase by marine alkalophilic *Streptomyces* sp-SBU1 isolated from Cape Comorin coast. *IJMS* 40:717-721.
- Kumar L, Singh B, Adhikari DK *et al.* (2012) A temperature and salt-tolerant L-glutaminase from gangotri region of uttarakhand himalaya: enzyme purification and characterization. *Appl Biochem Biotechnol* 166:1723-35.
- Kumar SR, Chandrasekaran M (2003) Continuous production of L-glutaminase by an immobilized marine *Pseudomonas* sp BTMS-51 in a packed bed reactor. *Process Biochemistry* 38:1431-1436.
- Kyoko K, Hroaki N, An Y *et al.* (2004) Glutaminase, its gene and a method of producing it. U.S. Patent. Pat. No. 6830905 B2.
- Lammeli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, 227:680-685.
- Landualt G, Guiochen G (1964) Separation of amines by gas liquid chromatography using Telfon support. *J Chromatog* 13:565-567.
- Lazarus P, Panasci LC (1986) Characterization of L-theronine and Lglutamine transport in murine P388 leukemia cells in vitro. Presence of an N-like amino acid transport system. *Biochem Biophys Acta* 856:488-495.
- Lehninger AL, Nelson DL, Cox MM (2005) *Lehninger Principles of Biochemistry*. 4th ed. W.H. Freeman and Company, New York, pp 194-196.
- Lowery OH, Rosebrough AL, Farr NJ *et al.* (1951) Protein measurement with folin- phenol reagent. *J Biol Chem* 193:265-275.
- Mohana Priya P, Radhakrishnan M, Balagurunathan R (2011) Production and optimization of L-asparaginase from *Streptomyces* sp (TA22) isolated from Western Ghats, India. *J Chem Pharm Res* 3:618-662.
- Nagendra Prabhu G (1997) L-glutaminase production by marine *Vibrio costicola* under solid state fermentation. Ph.D. Thesis, Cochin University of Science & Technology, Cochin, India.
- Nandakumar R, Yoshimune K, Wakayama M *et al.* (2003) Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *J Molecular Catalysis B: Enzymatic* 23:87-100.
- Nathiya K, Nath SS, Angayarkanni J *et al.* (2012) In vitro cytotoxicity of L- glutaminase against MCF-7 cell line. *Asian J Pharm Clinical Res* 5:0974-2441.
- Okami Y (1986) Marine microorganisms as a source of bioactive agents. *Microb Ecol* 12:65-78.
- Pandey A (2003) Solid-state fermentation. *Biochem Eng J* 13:81.

- Pratt DS, Kaplan MM (2000) Evaluation of abnormal liver enzyme results in asymptomatic patients. *N Engl J Med* 342:1266-1271.
- Ranjekar MK, Sridhar KR (2002) Occurrence and extracellular enzymatic potential of actinomycetes of a Thermal spring, Southern India. *Asian J Microbiol Biotechnol Env Sci* 4:59-64.
- Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Amer J Clin Pathol* 28:56-63.
- Robert J, Allister JW, Sethuraman N *et al.* (2001) Genetically engineered glutaminase and its use in antiviral and anticancer therapy. U.S. Patent, No. 6312939.
- Roberts J, Holcenberg JS, Dolowy WC (1970) *Nature* 227:1136.
- Roy S, Maity P (2007) Modulation of Metastatic Potential of B16F10 Melanoma Cells by Acivicin: Synergistic Action of Glutaminase and Potentiation of Cisplatin Cytotoxicity. *Asian Pacific Journal of Cancer Prevention* 8:301-306.
- Sabu A (2003) Sources, properties and applications of microbial therapeutic enzymes. *Ind J Biotechnol* 2:334-341.
- Sabu A, Keerthi TR, Rajeev-Kumar S *et al.* (2000) L-glutaminase production by marine *Beauveria* sp. under solid state fermentation. *Process Biochem* 35:705.
- Sabu A, Nampoothiri KM, Pondevy A (2005) L-Glutaminase as a therapeutic enzyme of microbial origin. In: Baredo JL (Editor), *Methods in Biotechnology: Microbial Enzymes and Biotransformations*. Humana Press Inc, Totowa, pp 75-90.
- Salvatore D, Reagle D (2001) *Theory and Problems of Statistics and Econometrics*. 2nd Ed. McGraw Hill, New York.
- Senthil-Kumar M, Selvam K (2011) Isolation and Purification of High Efficiency L-asparaginase by Quantitative Preparative Continuous-elution SDS PAGE Electrophoresis. *J Microbial Biochem Technol* 3:73-83.
- Singh P, Banik RM (2013) Biochemical characterization and antitumor study of L-glutaminase from *Bacillus cereus* MTCC 1305. *Appl Biochem Biotechnol* 171:522-31.
- Sunil Dutt PLNSN, Siddalingeshwara KG, Karthic JI *et al.* (2014) Antitumor property l-glutaminase on from *Aspergillus oryzae* through submerged fermentation. *Int J Curr Microbiol App Sci* 3:819-823.
- Suresh Kumer S, Muthuvelayudham R, Viruthagiri T (2013) Statistical Optimization based Production of L- glutaminase by *Serratia marcescens* under submerged fermentation. *Res J Chem Sci* 3:43-53.
- Tanaka S, Robinson EA, Appella E *et al.* (1988) Structures of Amidohydrolases. Aminoacid sequence of a glutaminase-asparaginase from *Acinetobacter glutaminasificans* and preliminary crystallographic data for an asparaginase from *Erwinia chrysanthemi*. *J Biological Chem* 263:8583-8591.
- Tay ST, Devi S, Puthcheary SD *et al.* (1995) Detection of haemolytic activity of Campylobacters by agarose haemolysis and microplate assay. *J Med Microbiol* 42:175-180.
- Thadikamala S, Reddy SP (2011) Modeling the effect of L-glutamine, aeration and agitation regimes on production of L-glutaminase in stirred tank reactor using *Bacillus subtilis* RSP-glu. *International Journal of Industrial Biotechnology* 1:27-33.
- Usha-Kiranmayi M, Sudhakar Poda, Vijayalakshmi M (2013) Production and optimization of L-asparaginase by an actinobacterium isolated from Nizampatnam mangrove ecosystem. *J Environ Biol* 35:799-805.
- Wakayama M, Yamagata T, Kamemura A *et al.* (2005) Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. *J Ind Microbiol Biotechnol* 32:383-390.
- Wei XL, Wei JF, Li T *et al.* (2007) Purification, characterization and potent lung lesion activity of an L-amino acid oxidase from *Agkistrodon blomhoffii ussurensis* snake venom. *Toxicon* 50:1126-1139.
- Whitaker JR (1972) Principles of enzymology for food sciences. In: R.E. Feeney and J.R. Whitaker (eds) *Enzyme Inhibitors*. Marcel Dekker, New York, pp 255-282.
- Wriston JC, Yellin TO (1973) L-asparaginase: A review. *Advan Enzymol* 39:185-248.

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