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Anticancer activity of L- Glutaminase obtained from *Bacillus subtilis*

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Abstract:

This study is set out to explore and purify the L-glutaminase from *Bacillus subtilis*. Hepatocellular carcinoma has low worldwide prognosis and is one of the most prevalent forms of malignancies. Accumulating data shows the beneficial impact on the occurrence of diverse cancers of amino acid depleting enzymes. Here we investigated the effects of *Bacillus subtilis* L-glutaminase on Hep-G2 cell lines by in vitro antioxidants and anticancer activity. The enzyme was purified 22.9-fold from a cell-free extract with a final specific activity of 35.71 IU/mg and the recovery was 32.45%. DPPH radical scavenging activity of l-glutaminase IC_{50} value is 88.34µg/ml. MTT assay reveals IC_{50} value of L-glutaminase is 161.7µg/ml. These results together suggest that L-glutaminase plays a key function in liver cancer.

Key words: L-glutaminase, DPPH, Anticancer, Hep-G2 cell lines, MTT Assay, *Bacillus subtilis.*

1.INTRODUCTION:

L-glutaminase (L-glutamine amidohydrolase EC 3,2) is an important industrial hydrolytic enzyme that catalyzes L-glutamine hydrolysis to L-glutamic acid and ammonium [1]. In recent years L-glutaminase has attracted much attention as to its broad applications in pharmaceutical products as an antileukemic agent [2], and also as an efficient antiretroviral agent, etc. [3]From the literature, it's evident that only a couple of reports are available on the extracellular production of l-glutaminase from bacteria and hence we've used *Bacillus subtilis* strain for enzyme production .While l-glutaminase was mostly obtained from both plant and animal sources, due to economic development, efficiency, easy process modification and optimization techniques, the microbial source is generally preferred for industrial production[4-5]. One of the most common forms of cancers globally with poor prognosis is hepatocellular carcinoma (HCC)[6]. Due to underlying cirrhosis and a high rate of recurrence, the treatment of HCC is limited; the overall 5-year survival rate is 53.4% with hepatic resection, 42.0% with local ablation therapy, and 22.6% with catheter - based arterial embolization [7-8]. Efficient therapies with enhanced immune potential are urgently required to tackle the current morbidity and mortality associated with HCC without harming the host, as a result of the incidence of HCC[9-10]. The emergence of new drugs for HCC is therefore important. The majority of cancer cells have been shown to be auxotrophic (GS-) in nature and to rely on the exogenous supply of L-glutamine for their development and growth. L-glutaminase degraded this amino acid results in the destruction of cancerous cells[11]. Although the enzyme's biologic activity is different from one source to the other. We have analyzed the in vitro antioxidant and anticancer potential of the enzyme L- glutaminase in the current research.

2. MATERIALS AND METHODS

2.1 Chemicals

All the analytical grade chemicals used in this research work were purchased from Himedia, Mumbai, India.

2.2 Screening of L- Glutaminase by plate method assay

The *Bacillus subtilis* was screened for L-glutaminase production using minimal mineral glutamine medium containing g/l: l-glutamine, 0.5 glucose, 20.0; NaNO3, 2.0; KH2PO4, 1.0; MgSO4•7H2O, 0.5 and KCl 0.5. and 0.09%(v/v) phenol red as a indicator, the enzyme production was performed under optimized condition. The pH of the medium was set to 7.0 and for 48 hours, flasks were incubated at 37ºC. The formation of pink colour zones across the colonies was seen as a positive result for the production of L-glutaminase.[12]

2.3 Enzyme production by submerged fermentation method

L-Glutaminase production was carried out by submerged fermentation[13]. For production, a 250mL Erlenmeyer flask containing 50mL of sterilized medium was used. . In the above mentioned medium, a loop full of 24-hour-old culture of *Bacillus subtilis* was inoculated. At 120 rpm at 37º C for 24 hours, the flask was put in an incubating orbital shaker. The control acted as an un-inoculated medium. For 15 min at 4º C, the bacterial cell mass was separated by centrifugation at 5000 rpm. To evaluate the enzyme activity, the liquid supernatant was used as a crude enzyme source.

2.4 Estimation of L- Glutaminase Enzyme activity

L-Glutaminase was assayed according to (Imada et al., 1973) [14]. The reaction mixture containing 0.5 ml of 0.04 M L-glutamine, 0.5 ml of 0.5 M phosphate buffer (pH 7.8), 0.5 ml of an enzyme and 0.5 ml distilled water was added to make up the total volume to 2 ml. The tubes were incubated for 30 minutes at 30°C. By adding 0.5 ml of 1.5 M Trichloroacetic acid, the reaction was stopped. The blank was prepared by adding enzyme after the addition of TCA. 0.1 ml of the above mixture was added to 3.7 ml of distilled water and 0.2 ml of Nessler's reagent. . The OD was measured at 450 nm with a spectrophotometer after incubation of the mixture at 20 ° C for 20 minutes. The activity of the enzyme was expressed in International Unit.

2.5 Purification of L-glutaminase:

Purification of enzyme was carried by using Ammonium sulphate precipitation method[15]. Using solid (NH4)2SO44, the proteins present in a sample were salted out. To get 10-90 percent of the concentrations subsequently, fine powder of ammonium sulphate salt was added to the sample. The solution was left as such after adding salt at each point without shaking for 2hrs for maximal precipitation. The protein thereby isolated has been centrifuged for 40 minutes and collected. After (NH4)2SO4 fractionation, the precipitate thus obtained was dissolved in PBS (0.2 M) (pH 6) and desalinated extensively at 4 °C for 24 hours using the same buffer. The function of the enzyme and the dialysate protein content were also calculated [16]

Ion exchange chromatography was used for further purification. For this Sephadex G-100 column (1.5 x 50 cm) pre-balanced with a 0.05 M boric acid borate buffer pH 8.0 at a 20 ml / h flow rate was used to treat the partially purified enzyme fraction by ammonium salt precipitation[17]. For enzymatic activity and protein content, fractions were collected and analyzed. The most active fractions were mixed, dialyzed against the 0.01 M boric acid borate buffer (pH8.0), and preserved at -20ºC.

Gel filtration chromatography: In the pre-equalized column Sephadex G-200 (2.0 x 50 cm) the filtered fraction of the previous stage was filled with a buffer of 0.05 M boric acid (pH 8.0) to a flow speed of 10 ml/h[18]. L-glutaminase activity and protein content were obtained and analysed for these fractions. The most active fractions were concentrated and preserved at -20ºC.

2.6 Estimation of antioxidant activity of l-glutaminase by DPPH method:

2.6.1 *DPPH antioxidant activity:*

Free radical scavenging activity of L-glutaminase was calculated using a fast, relatively inexpensive method involving the use of reactive oxygen species 2, 2-diphenyl-1-picrylhydrazile (DPPH) [19]. DPPH is a violet colour free radical[20]. The antioxidants in the sample scavenge the free radicals and make them yellow in colour[21]. The change in colour from violet to yellow is directly proportionate to the radical scavenging activity[22]. In brief, sample stock solutions (1.0 mg/ml) were dissolved into ethanol at final concentrations of 50, 40, 30, 20, 10 µg/ml. In 2.5 ml sample solutions of different concentrations, 1 ml of 0.3 mM DPPH ethanolic solution was added and allowed to react at ambient temperature. The degree of absorption reduction in the UV-Vis spectrophotometer was reported at 518 nm (Shimadzu UV-Vis 2450) after 30 minutes.

The percentage of scavenging activity was calculated as:

$$AA\% = 100 \frac{[(Ab\ sample - Ab\ blank) \times 100]}{Ab\ Control}$$

The percentage of radical scavenging activity was compared to the corresponding extract concentration in order to obtain the IC50 value.

2.6.2 *Determination of cell viability by MTT Assay:*

For in vitro anti-proliferative activity against Hep-G2 cell lines, the purified enzyme thus obtained was tested by MTT assay. Cancer cell lines were maintained in RPMI – 1640 medium in CO2 incubator at 37°C with 98% humidity and 5% CO2 gas environment[23]. Epithelial Hep G2 of Human Caucasian hepatocyte carcinoma cancerous cell line was used. Purified l-glutaminase produced by *Bacillus subtilis* was evaluated by MTT assay to check anti-proliferative activity against Hep-G2 cell lines [24]. Stock samples were diluted with medium RPMI at required l-glutaminase concentrations ranging from 0.01 to 100 IU/ml. 100 µL of cells were added in 96 well plates at the density 5×10^5 cell/ml and incubated at 37°C in 5% CO_2, 95% air for 24 hrs. The cells were then treated with varying sample concentrations in total volume (200 µl/well) for 24 hours. After 21 hrs, the cells were centrifuged at 2000 rpm for 10 minutes and re suspended at 180 µL of RPMI medium to rinse treated samples. In each well, a volume of 20 µl of MTT solution (5 mg/ml) was added and incubated for another 3 hours at 37 °C 180 µL. The medium was then aspirated from each well. The formed formazan crystals were dissolved in 180 µL of dimethyl sulfoxide (DMSO). Formazan optical density (OD) was detected by a dual wavelength UV spectrometer at a reference wavelength of 570-650 nm. The cytotoxicity percentage was compared with the untreated cell as a control and estimated as below with the equation:

$$\text{Percentage cytotoxicity (\%)} = \frac{\text{OD of test sample}}{\text{OD of control}} \times 100$$

The plot of cytotoxicity percentage versus sample concentration was used to measure the lethal concentration of 50 percent of the cells (IC_{50}). The percentages of cell viability were presented graphically using Microsoft excel computer program.

3 RESULTS AND DISCUSSION:

3.1 Screening of L- Glutaminase by plate method assay

Bacillus subtilis was screened using minimal mineral glutamine medium with phenol red indicator for L-glutamianse production. The active glutamianse producing *Bacillus subtilis* colony changed the colour of the medium from yellow to pink, because of change in pH of medium as L glutaminase catalyzed the glutamine and librated ammonia. This strain is positive for extracellular L-glutaminase production tested further quantitatively for L glutamianse activity.

3.2 Estimation of L- Glutaminase Enzyme activity

Bacillus subtilis showed maximum enzyme production (101.4U/ml) at pH 7 after 48 hours. Similarly result reported that maximum activity was obtained at 24 hours of incubation from the bacterial isolates [25]. The growth rate and enzyme synthesis of the culture have been identified to be the two key characteristics that are primarily affected by incubation time [26].

3.3 Purification of L- Glutaminase

The crude extract indicates the presence of a high number of impurities, which were extracted step by step during the purification process. The presence of 202.1mg of total protein with a specific activity of 1.13IU/mg was found in the crude extract. The partial purification of the crude extract of L-glutaminase affected by the precipitation of ammonium sulphate (80 percent) showed that most of the enzyme activity was retained in the precipitate. This could have been found by decreasing the total protein in the ammonium sulphate precipitation step from 202.1 to 47.21 mg. The specific activity has increased to 12.3. A significant increase in specific activity to 35.71 IU/mg with 32.45 percent of recovery and 22.9 times purity was seen by further purification by ion exchange and gel filtration chromatography. The results of the purification of the enzymes are given in Table 3.

Separation and characterization of L-glutaminases from *Bacillu sps.* and *Bacillus subtilis* have been investigated[27-28]. Purification of L-glutaminase from cell-free extracts of *Debaryomyces sp. CECT11815* was achieved by ammonium sulphate treatment followed by anion exchange chromatography and gel filtration.[29]. Purification of glutaminase by *Aspergillus flavus* at acidic pH 4.0 while this activity decreased up to 50% at natural pH 7 [30]. In most glutaminases, particularly those from mammalian sources [31] and from several microbial sources, the lack of stability is a common feature [32].

3.4 DPPH antioxidant activity:

One of the most commonly used methods for screening the antioxidant function of drugs is the DPPH radical scavenging activity. Antioxidant compounds are those which, by inhibiting the initiation or propagation of oxidising chain reactions delay or inhibit the oxidation of lipids or other molecules[33]. The DPPH test demonstrated *Bacillus sp.* Compared to the well-known antioxidant ascorbic acid amino acid, SC_{50} 8.7 µgm/ml, R36 had low scavenging activity with a high SC_{50} value of 325.4 µgm/ml, The L-glutaminase IC_{50} value is 87.43 µgm/ml, while the L-ascorbic acid IC_{50} value is 80.52 µgm/ml [34]. Present studies have shown that the scavenging activity by DPPH methods increases with the rise in concentration. The IC50 value stringer decreases the potential for antioxidants. IC_{50} values lower than 250 µg/ml are usually considered to be important antioxidants[35], the results of the DPPH free radical scavenging process are shown in Table 2. The absorption of the DPPH solution changed as different enzyme concentrations were added to the solution. The l-glutaminase IC_{50} value is 88.34 µg/ml, while the l-ascorbic acid IC_{50} value is 76.71 µg/ml (Figure 1).

Table 1.: Purification steps of L- Glutaminase

Step	Total Enzyme Activity (IU)	Total Protein (mg)	Specific Activity (IU/mg)	% Recovery	Purification Fold
Crude Extract	299.6	202.1	1.13	100	1
Ammonium sulphate ppt	198.3	47.21	12.3	86.9	7.9
IEC	111.3	7.21	19.87	39.61	12.65
GFC	102.7	3.54	35.71	32.45	22.9

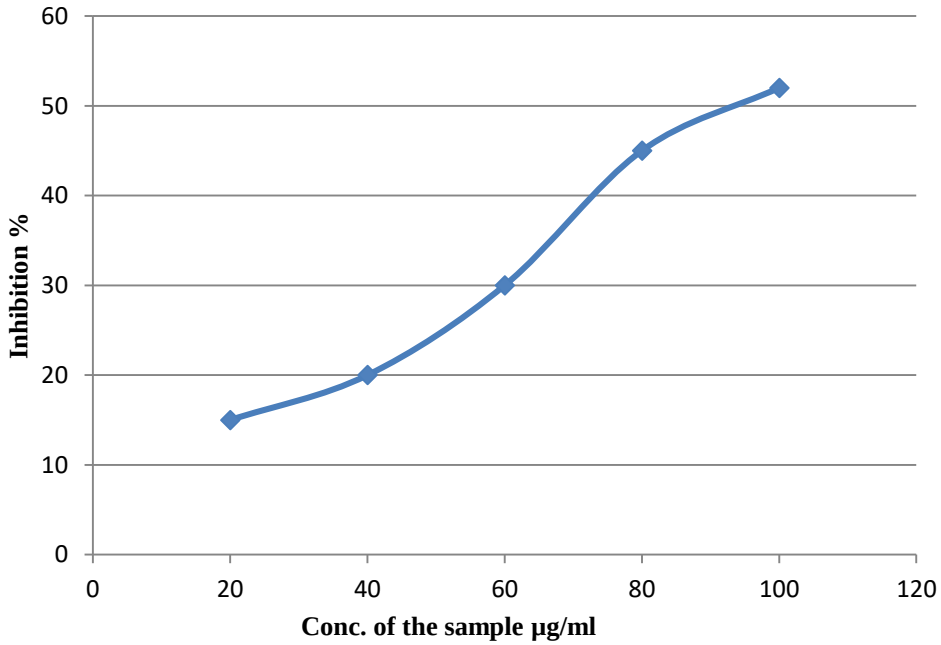

Fig.1: DPPH scavenging activity of L-glutaminase

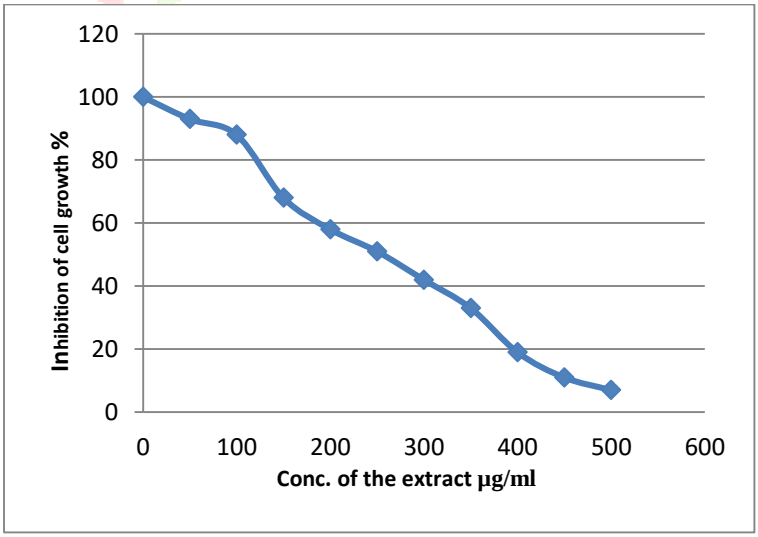

Figure 2: Effect of L- Glutaminase on Hep-G2 Cell line

3.5 Determination of cell viability by MTT Assay:

The cytototoxic effect of *Bacillus subtilis* L-glutaminase on the growth of Hep-G2 [Human hepatocellular carcinoma] cell line was checked by using in vitro MTT assay bioassay showed that crude enzyme extracts have anti-proliferative activity in the growth of cell lines. (Figure 2). Incubation of Hep-G2 with gradual doses of purified L-glutaminase from *Bacillus subtilis* leads to gradual inhibition of cell growth, concluding with a low IC_{50} value of 161.7 µg/ml (Figure 2). Cytotoxicity studies have shown that the L-glutaminase produced by the *Bacillus subtilis* is toxic to the cells. In this connection, the cytotoxicity of L-glutaminase from *Aspergillus flavus KUGF009* to Hep-G2 cell lines by the MTT assay (IC50 250 µg/ml)[36], where as in another study L-glutaminase from *Penicillium brevicompactum NRC 829* inhibited the growth of human cell line (Hep-G2) with an IC_{50} value of 63.3µg/ml [37].

4 CONCLUSION:

There is an indication of scope in the present study to explore *Bacillus substilis* as a source of L-glutaminase, an enzyme which has recently gained industrial and pharmaceutical significance. This enzyme also has a positive anti cancer and antioxidant attribute that has shown a proportionate relationship with antioxidant molecule concentration and scavenging activity. This profound protective effect of L-glutaminase may explain its extensive use in possible health benefits. Further studies are under progress to find out the exact mechanism of its anticancer activity.

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