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In Vitro Cytotoxicity Efficiency of Extracellular L-Glutaminase Produce by Mesophilic Bacteria, *Kurthia Gibsonii* on Lncap, MDA-MB 231 and Hepg-2 Cell Lines

Bhalchandra B. Waykar[1*], Trupti B. Namekar[2], Rajendra A. Satpute[3], Dinesh S. Kharate[4]

[1,2]Animal Physiology Labrotary, Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad-431004, Maharashtra State, India
[3]Department of Botany, Government Institute of Science, Aurangabad-431004, Maharashtra State, India
[4]Department of Zoology, Sant Ramdas Art's, Commerce and Science College, Ghansawangi, Jalna-431209, Maharashtra State, India

**Corresponding author's E-mail: bbwaykar@gmail.com*

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Abstract

Mesophilic bacteria from soil habitat have been reported to produce extracellular L-glutaminase. The present study was carried out to anticancer screening of L-glutamiase producing bacteria (Kurthia gibsonii) from soil sample of cattle feeding site from Satara parisar, Chhatrapati Sambhajinagar. Results showed, among three soil samples of cattle feeding farms, Kurthia gibsonii was isolated. From that exhibited the highest L-glutaminase activity. Moreover, the in vitro cytotoxic activity of L-glutaminase against the (Lymph Node Carcinoma of the Prostate) LNCaP, (an epithelial, human breast cancer cell line) MDA-MB 231 and hepatocellular (HepG-2) carcinoma cell lines at different concentration (0.47, 0.94, 1.88, 3.75, 7.50, 15.00, 30.00 and 60.00 µg/ml) by the MTT assay and compared with the standard Doxrubcin. The antitumor effect against human liver carcinoma cell line revealed that L-glutaminase produced by Kurthia gibsonii showed potent cytotoxic activity of tested cell line in a dose-dependent manner with an LC50 value of 4.1 µg/ml.

Keywords: *Mesophilic Bacteria, Cell line*

1. Introduction

L-glutaminase catalyzes the hydrolytic degradation of l-glutamine to L-glutamic acid and ammonia (1). Glutaminase catalyzes the hydrolytic degradation of L-glutamine. L-glutaminase shows an amide enzymatic activity that has a significant role in nitrogen metabolism in all living cells at cellular level (2). L-glutaminase has been considered to play a significant role in enzyme therapy for cancer treatment, especially in acute lymphocytic leukemia. It was also found to be effective against human immunodeficiency virus (3). biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need for separate measurement of glutamic acid (4). L-glutaminase enzyme is ubiquitous in nature and reported in animals, plants, bacteria, actinomycetes, yeast, and fungi (5,6). Attempts are being made to replace enzymes, which traditionally have been isolated from animal tissues and plants to enzymes from microorganisms because microbial enzymes are cheaper to produce, more predictable, controlled, and reliable (7,8). Many bacteria synthesize extracellular and intracellular glutaminases such as *Bacillus* sp., *Pseudomonas*, *Actinobacterium* sp, and *E. coli*. (9). The focal sources of fungal glutaminases are *Aspergillus* sp. and *Trichoderma* sp. (10). Reports showed that the majority of microbes producing L-glutaminase have been isolated from soil and aquatic environment (11,12). L-glutaminases produced by terrestrial microorganisms have been reported to have some disadvantages such as unstable in extreme conditions, incompatible with human blood, and may induce a lot of side effects to patients. Thus, there is a great urgency to investigate other enzymatic sources (13). Halophilic microorganisms are a potential source of extremozymes called halozymes which are capable of functioning under high concentrations of salt, a wide range of pH values, and temperatures (14). Furthermore, over the past few decades, several efforts have been made to discover the potential abilities of these extremophiles and using them efficiently for therapeutic purposes. The important features of these enzymes that distinguish them from all other types of drugs are high affinity, specificity, and

catalytic efficiency (8). Hence, there is an increasing interest in the identification of marine microbial strains and developing the practical bio-processing technique to improve their productivity for therapeutic purpose. L-glutaminase (EC 3.5.1.2) is an amido hydrolase enzyme which catalyzes L-glutamine to L-glutamate and ammonia (15). It is useful in the treatment of cancer (16). L-glutaminase is widely distributed in bacteria, actinomycetes, yeast and fungi (15,17,18). L-glutaminase exhibits anticancer effect by depleting the L-glutamine from the cancerous cells, since these cells more avidly consume L-glutamine for their energy needs and proliferation than normal cells (19). It is also understood that the cancerous cells cannot synthesis their own L-glutamine and this is the Achilles heel that is exploited by these amino acid depleting anticancer agents (20, 21). Other than in pharmaceutical applications, L-glutaminase finds applications in food industries as a flavor enhancement agent especially for fermented foods and has also got applications as a biosensor and analytical agent and used for the production of fine chemicals like theanine (22,23). There are many cattle feeding farms were found around Chhatrapati Sambhajinagar districts. While various microorganisms were found in these feeding sites have been studied, to date there has been no report concerning the production and characterization of glutaminase produced by microorganisms isolated from cattle feeding farms. With the ultimate goal of improvement of information, we presently screened the L-glutaminase-producing bacteria from cattle feeding farms. In this study, we determined the in vitro cytotoxicity of L-glutaminase produced by Kurthia gibsonii; isolated from soil samples from cattle feeding farm.

2. Materials And Methods

Sample collection

L-glutaminase samples were collected *Kurthia gibsonii*. The process of isolation, idenfication, bacterial inoculum preparation was described in my previous paper.

Partial purification of l-glutaminase

The purification was carried out using 500 ml of crude enzyme extract. Finely powdered ammonium sulphate was slowly added into cell-free supernatant (crude enzyme) so as to reach 40% saturation. The whole content was stirred at 4 °C using a magnetic stirrer. The precipitated crude enzyme was removed by centrifugation at 10,000 × *g* at 4 °C for 20 min. Fresh ammonium sulfate was added to the supernatant to increase the saturation to 50%. The obtained precipitate was re-suspended in a minimal volume of 0.01 M phosphate buffer (pH 8). Precipitated protein was removed by centrifugation as described earlier. Once again, the fresh ammonium sulfate was added to the cell free supernatant to increase the concentration to 80%. The obtained enzyme precipitate was re-suspended in a minimal volume of 0.01 M phosphate buffer (pH 8) and precipitated protein was recovered by centrifugation. The enzyme precipitate obtained after ammonium sulphate precipitation was dialyzed against 0.01 M phosphate buffer (pH 8) for 24 h at 4 °C with continuous stirring and occasional changes of the buffers. The dialyzed fractions were collected and freeze-dried, and the lyophilized enzyme was used for the MTT assays (24).

Preparing cell line

A vial of each cell lines was taken out from liquid nitrogen storage and thawed rapidly to room temperature. The contents in the vials were added to 9 ml of complete medium and centrifuged at 125g for 5 minutes. After centrifugation, the supernatant was discarded and pellet was mixed with 10 ml of complete medium and suspended in a T-25 flask and incubated at 37°C with 5% CO2. When the cell confluence reached ~80%, the cells were centrifuged at 125g for 5 minutes; pellet was mixed with 15 ml of complete medium and transferred to two T-75 flasks. When the cell confluence reached around 80-90%, cells in the flask were used for the assay.

Test system

Cell line	MDA-MB 231
Passage number	P4
Media	Eagle's Minimum Essential Medium
Source	ATCC HTB-26

Cell line	HePG2
Passage number	P3
Media	Eagle's Minimum Essential Medium
Source	ATCC HB-8065

Cell line	LNCAP
Passage number	P3
Media	RPMI-1640
Source	ATCC CRL-1740

MTT assay

200 µl cell suspension (in complete culture medium with 10% FBS) was seeded in a 96-well plate (20,000 cells per well), without the test agent and allowed to grow for 24 hours. After 24 hours of incubation, spent media in the wells of 96-well plate were replaced with appropriate concentrations of the test compounds and incubated for 48 hours at 37°C in a 5% CO2 atmosphere. After the incubation period, the plates were removed from incubator; spent media was removed followed by addition of MTT reagent to a final concentration of 0.5 mg/ml (0.2 µm filter sterilized). The plates were wrapped with aluminum foil to avoid exposure to light, and placed in the incubator for 3 hours. After incubation MTT reagent was removed and 100 µl of DMSO was added. Absorbance was measured on spectrophotometer (Tecan™ Infinite 200 Pro) at 570 nm.

Experimental design

Group/Treatment	Description	N/assay
Medium control	medium without cells	3
Negative control	medium with cells minus experimental drug/compound	3
Vehicle control	medium with cells and vehicle	3
Test compounds	0.47, 0.94, 1.88, 3.75, 7.5, 15, 30 and 60 µM	3

Data analysis

The percent viability of cells in the untreated (negative control) group was set to 100% and the % viability of cells in the treated groups was estimated relative to the negative control. The % viability was plotted against the concentration and evaluated for dose response. Based on the dose response relationships, an appropriate model was fit to estimate the Imax and IC50.

Percentage viability was calculated using the following formula:

$$\% \text{ Viability } = \frac{100 \times \text{OD570e}}{\text{OD570b}}$$

Where,

OD570e is the mean value of the measured Optical Density of the dilutions of test item;

OD570b is the mean value of the measured Optical Density of the negative control

Anticancer activity

The anticancer activity of the produced L-glutaminase was studied against (Lymph Node Carcinoma of the Prostate) LNCaP, (an epithelial, human breast cancer cell line) MDA-MB 231 and hepatocellular (HepG-2) carcinoma cell lines. The cells were propagated in 24-well plate (BD Biosciences, San Jose, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heatin activated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50 µg/ml gentamycin. The cell suspension (105cells/ml) was seeded in every well and incubated at 37°C for 48 hours in 5% CO_2 for the formation of a confluent monolayer. The cell viability was measured using MTT assay. The MTT assay is based on the reduction of yellow 2,5-diphenyl tetrazolium bromide to purple formazan by actively growing cells. The monolayer of cells in 24-well plates was incubated alone or with an enzyme at different concentration (0.47, 0.94, 1.88, 3.75, 7.5, 15, 30 and 60 µg/ml) for 48 hours. After incubation, 20 µl MTT stock solution (5 mg/ ml in phosphate-buffered saline or PBS, pH 7.5, filtered through 0.22-µm cellulose acetate filter; Sigma, St. Louis) was added to each well, incubated for 4 h at 37 °C then the solution was decanted. To stop succinate-tetrazolium reductase activity and solubilize formazan crystals, 100 µl of propanol was then added to each well. The viable cells yield was determined by a colorimetric method. The percentage of viable cells was calculated as follows: Cell proliferation (%) = (OD experimental group/OD control group) × 100. The 50% inhibitory concentration (IC50), enzyme concentration causing 50% inhibition of intact cells was calculated from the graph plotting percentage of cell viability against enzyme concentration. The lower the IC50 value indicates high antitumor capacity.

Statistical analysis

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

3. Results and Discussion

In the present study, In-vitro anticancer activity of L-glutaminase produced by *Kurthia gibsonii* was studied against the (Lymph Node Carcinoma of the Prostate) LNCaP, (an epithelial, human breast cancer cell line) MDA-MB 231 and hepatocellular (HepG-2) carcinoma cell lines at different concentration (0.47, 0.94, 1.88, 3.75, 7.50, 15.00, 30.00 and 60.00 µg/ml) by the MTT assay and compared with the standard Doxrubcin.

Table 1. Inhibition concentrations (IC50) for cytotoxicity activity of L-glutaminase

Test compounds	Cell line	IC50 value (µM)
Sample A	LNCAP	30.11
Sample B	MDA-MB 231	18.15
Sample C	HePG2	12.09

L-glutaminase produced by *Kurthia gibsonii* showed a potent cytotoxic activity of all cell lines in a dose-dependent manner as shown in Table 3. The results showed that LNCaP, HepG-2 and MDA-MB 231 cell proliferation were significantly inhibited by L-glutaminase with IC50 values of 30.11, 12.09, and 18.15 µg/ml, respectively (Table 4). The produced L-glutaminase exhibited weak toxicity to the hepatocellular cell line with IC50 of 50 µg/ml. A blank experiment was conducted containing the same extract concentrations, but heat-inactivated to confirm that this antitumor activity is related to the produced L-glutaminase not to other bacterial proteins. Thus, l-glutaminase produced by *Kurthia gibsonii* was found to be a potent cytotoxic agent against LNCaP, HepG-2 and MDA-MB 231 cell lines.

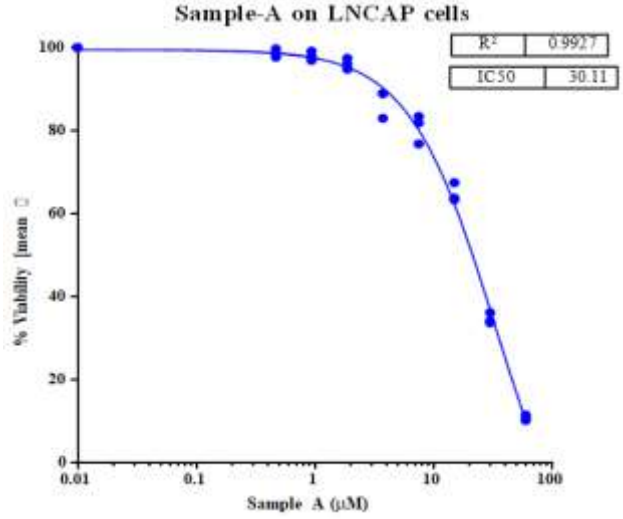
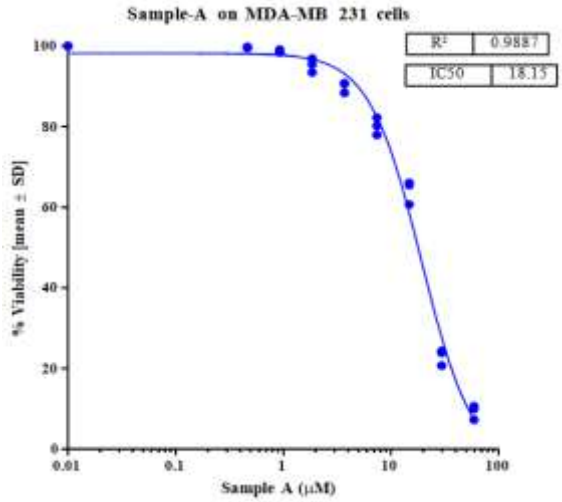


Figure 1. The Half maximal inhibitory concentration (IC50) of L-glutaminase



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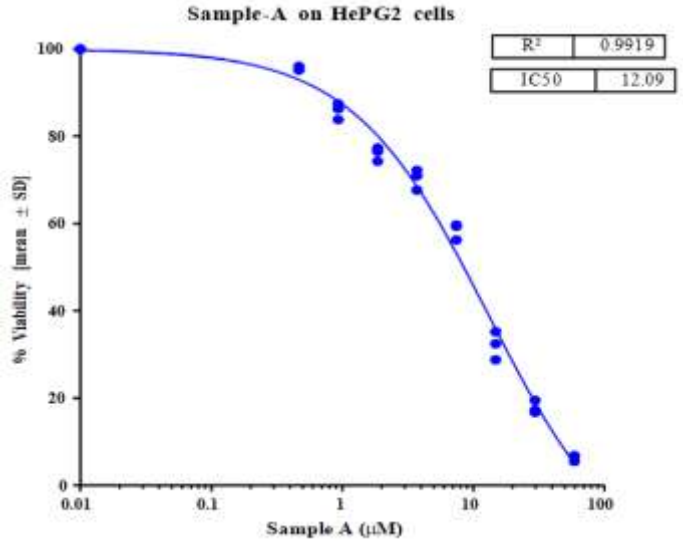

Figure 1. The Half maximal inhibitory concentration (IC50) of L-glutaminase

Cancer is one of the most dangerous diseases. It is the second bigger disease of human beings (25). Although several kinds of treatments are available, enzyme therapy is reported to be the more effective one. Cancer therapy using enzymes relies on the low molecular weight protein, specific in their action and has less or no toxic effects. In addition, the enzymatic approach was reported to be more promising in cancer therapy (26). Cancer cells, especially, cannot synthesize l-glutamine as they lack the proper functioning glutamine biosynthetic machinery (L-glutamine syntheses) and therefore require large amount of l-glutamine for their rapid growth. These cells depend on the exogenous supply of l-glutamine for their survival and rapid cell division, as it is a primary tool for donation of its nitrogen, which aid in protein, nucleic acid, lipid formation and participate in oxidative metabolism. Furthermore, glutaminase is already present in mitochondria, but it must be at the level that allows sequential and fast degradation of glutamine (27). Hence, the use of L-glutaminase deprives the tumor cells of l-glutamine and causes selective death of l-glutamine dependant tumor cells (28,29). The glutamine-deprivation therapy with L-glutaminase that hydrolyzes l-glutamine to l-glutamic acid and ammonia, not only selectively inhibits tumor growth by the blocking of the de novo protein synthesis, but also increase in the superoxide level of oxidative stress that promotes the death of the cancer cells (30). Thus, it can act as a possible candidate for enzyme therapy. For example, L-glutaminase has been receiving more attention as an antileukemic agent for treatment of acute lymphoblastic leukemia (ALL) and other types of cancer (31). L-glutaminase enzyme produced by halotolerant isolates can be used for increasing the level of glutamine catabolism and stopping cancer development. In addition, the chemical nature of seawater could provide microbial sources producing enzymes that could have fewer side effects when used in therapeutic applications (32). Thus, marine bacteria have recently attracted attention for the L-glutaminase production (33). Halomonas meridian was first reported as an L-glutaminase producer that is used as an anti-colon cancer agent (34). In the present study, the produced L-glutaminase by the isolated soil bacterial strain *Kurthia gibsonii* showed potential anticancer activity against the tested cell lines with IC50 values of 30.11, 12.09, and 18.15 µg/ ml for LNCaP, HepG-2 and MDA-MB 231, respectively. Furthermore, it is worth mentioning that the produced L-glutaminase exhibited weak toxicity to the hepatocellular cell line that confirmed that this enzyme possesses great selectivity to cancer cells. In the same trend, many studies investigated the cytotoxicity of L-glutaminase from various microorganisms. For instance, the purified L-glutaminase by *Alcaligenes faecalis* KLU102 inhibited the growth of Hela cells with an IC50 value of 12.5 µg/ml (26). Moreover, researcher mentioned that L-glutaminase purified from a bacterium was able to stop a breast carcinoma with IC50 of 256 µg/ml (35). Also, some workers indicated that L-glutaminase has a noteworthy efficiency contrary to Hep-G2 cell (IC50, 6.8 µg/ml) and a reasonable cytotoxic result against HCT-116 cell (IC50, 64.7 µg/ml) (36). In addition, l-glutaminase produced by *Aspergillus oryzae* showed significant cytotoxic activity against MCF-7 with IC50 of 283.288 ug/ml (37). Furthermore, the cytotoxicity effect of L-glutaminase produced by *Bacillus subtilis* OHEM11 indicated significant safety on Vero cells with high anticancer activity against NFS-60, HepG-2, and MCF-7 cancer cell lines (38). Overall, all these data confirmed that L-glutaminase possesses great selectivity to cancer cell and can display potential application in cancer chemoprevention and chemotherapy.

4. Conclusion

In this study we isolated bacterial strain *Kurthia gibsonii*. Moreover, the specific cytotoxicity activity against the tested cell lines with IC50 values of 30.11, 12.09, and 18.15 µg/ ml for LNCaP, HepG-2 and MDA-MB 231, respectively. Furthermore, it is worth mentioning that the produced L-glutaminase exhibited weak toxicity to the hepatocellular cell line that confirmed that this enzyme possesses great selectivity to cancer cells. It revealed that soil of cattle feeding farm has diverse bacterial strains and its habitat nature allowed the bacteria expressed the cytotoxic activity. The present isolation of *K. gibsonii* from cattle feeding site indicated that this bacterium is suitably adapted to the environment of excretion and to point of forming a microbiota in the fecal product.

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