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L-asparaginase Produced from Cow's Milk Isolate of *Lactobacillus plantarum* Shows Potent Anti-cancer Activity on Cervical Cancer Cells

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Cervical cancer is a leading cause of cancer death in women, occurring in more than a quarter of the developing countries of the world, with a slightly higher incidence in India. Many microbes producing *L-asparaginase* (ASNase) are used for the treatment of various cancers. However, previous studies have documented that long-term use of enzymes produced from these commercial strains triggers hypersensitivity in patients. Therefore, there is a need to find new microorganisms that produce L-asparaginase with anti-cancer properties, which can be used commercially in enzyme production. In the present study, of the 7 isolates, a single isolate of *Lactobacillus plantarum* had the highest enzyme production capacity. Purified enzyme can be obtained from fermented production medium by dialysis, thus showed a dose-depended cytotoxic effect on HeLa cells, as determined by MTT assay. The IC_{50} of the enzyme value was 0.75 IU µg/mL. This result indicates that *L. plantarum* producing L-asparaginase may be used for cancer treatment.

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Keywords: L-asparaginase; LAB; L. plantarum; Cancer; Cervical cancer; HeLa cells.

1. INTRODUCTION

Cancer is known to be one of today's most common and lethal diseases. Cancer is the second most common disease in India, and its largest mortality rate is about 300,000 deaths each year [1]. This is due to poor prevention, diagnosis, and treatment of the disease. At the national level, the average total cancer care expenditure was around Rs 1, 16, 218. In private hospitals, the total cost of cancer care was estimated to be Rs 1, 41,774, whereas it was comparatively lower at Rs 72,092 in public hospitals [2].

In addition to being expensive, Common interventions, such as chemotherapy and radiotherapy, can cause serious side effects, such as mucositis, Cardiotoxicity, reproductive dysfunction, pneumonia, neuropathy, and skin irritation. Aware of these challenges, researchers are trying to develop novel targeted biological strategies. Amino acid deprivation therapy (AADT) is a promising strategy characterized by the use of amino acid depleting enzymes to promote the treatment of auxotrophic tumors. During this enzyme treatment, protein synthesis was suppressed [3]. Therefore, the depletion of amino acids by AADT therapy induces the death of the malignant cells.

The enzymes used in AADT usually have a microbial origin because they are easily available, have high productivity, and are easy to handle to improve their performance. The L-asparaginase is the first material used for AADT and, the most effective drug against T-cell acute lymphoblastic lymphoma. Latterly, some other enzymes are utilized for the AADT, namely arginine deiminase, arginase, glutaminase, methionase, lysine oxidase, and phenylalanine ammonia lyase. Nevertheless, L-asparaginase is widely used as a drug for cancer especially which was used for the anti-leukaemic agent [4].

Currently, the therapeutic property of L-asparaginase was widely used for leukemia therapies, which are derived from various bacteria. This enzyme showed different pharmacological and biochemical characteristics and also different side-effects on the normal human cells because also microbes producing enzyme had different structural, physicochemical and kinetic properties [5]. Therefore, there is a continuing need to screen newer organisms to obtain strains that can produce new and high-yield L-asparaginases with fewer adverse effects.

Certain probiotic bacteria, especially those belonging to lactic acid bacteria (LAB), offer potential as chemoprotective agents and are capable of an anti-carcinogenic effect. This effect may be species- or strain-specific [6]. Among the bacterial ASNase resources, ASNases from LAB were very rare.Research has not yet been done on the treatment of multiple cancers, especially with the L-asparaginases enzyme produced by LAB bacteria; therefore, it is clear that further research is needed to quantify the beneficial effects of preventing human cancer with L-asparaginases. In the present study, our goal was to control the HeLa cancer cells with LAB of *Lactobacillus plantarum.*

2. MATERIALS AND METHODS

2.1 Collection of Milk Samples and Isolation of Lactic Acid Bacteria

Ten fresh milk samples were collected into the sterile plastic container from the nearby village of Erode, Tamilnadu, India, and shifted to the microbiology laboratory within 45min and kept refrigerated (2-8°C) condition until microbiological analysis. For isolation of Lactic acid bacteria, each sample was serially diluted upto 10^{-6} and 0.1 mL of samples were spread into MRS agar media (Himedia, India).The plates were incubated at 37°C for 48 h, under anaerobic conditions using desiccators with anaerogaspack(Himedia).Typical LAB characteristics colonies were randomly picked up and purify by streaking into MRS agar plates followed by macroscopic and microscopic examinations. Furthermore, each isolate was subjected to biochemical and sugar fermentation test [7].

2.2 Screening of L-asparaginas-eproducing Isolates

The selection of L-asparaginase-producing isolates was carried out by the plate assay developed by Gulati*et al*. [8]. All isolates of LAB were inoculated into M9 basal medium containing Asparagine andphenol red (0.009%). After incubating at 37 °C for 76 hrs, the pink zone around the colonies was measured, and an enzyme index was calculated using the equation:

Enzyme index = Pink zone (mm)/Colony diameter (mm).

2.3 Enzyme Assay

The L-asparaginase activity was determined after fermentation and centrifugation of liquid media by the Nessler method. The activity was expressed in enzymatic units (U) defined as the amount of enzyme that releases one µmol of ammonium per time unit (U = µmol /h) [9].

2.4 Identification of Potential Isolates by 16sr RNA Sequence

Based on the screening and assay results, a potential isolate was selected and subjected to a 16sr RNA sequence for identification of isolate. The amplified gene was subjected to sequence and compared with the sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16s rRNA sequence was blast using the NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment (MUSCLE 3.7).

2.5 Anticancer Activity of L-asparaginase

The anticancer activity of L-asparaginases on HeLa cells was determined by the MTT assay [10]. Cells (1×10^5/well) were plated in 100 µl of medium/well in 96- well plates (Tarson, India). After 48 hours of incubation, the cell reaches the confluence, then added different concentrations of enzyme (1,2,3,4, and 5U/mL) with 0.1% DMSO which were incubated for 48h at 37°C. After removal of the sample solution and washing with phosphatebuffered saline (pH 7.4), then added 20 µl of 0.5% of 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl—tetrazolium bromide cells (MTT) phosphate buffered saline solution. After 4 hrs incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm with reference at 655 nm. Measurements were performed 3times, and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically and without sample containing cells as blanks. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by 100% relative viability.

Cell survival was calculated by the following formula:

$$\text{Cell Viability \%} = \frac{(\text{Test OD/ Control OD})\ \text{X } 100}{\text{Cytotoxicity \%} = 100 - \text{Viability \%}}$$

3. RESULT AND DISCUSSION

The currently commercially available ASNase produced by bacteria may cause side effects during the treatment process, which indicates the need to determine a new source of these enzymes. In the present study, totally 45 isolates of LAB were isolated from different cow milk samples. It is an alliance of 5 LAB species namely, *Lactobacillus casei*(20.00%), *Lactobacillus plantarum* (37.77%), *Lactobacillus fermentum* (4.44%), *Lactobacillus brevis* (2.22%),and *Lactobacillus acidophilus* (35.55%).

The rapid plate assay is a simple and semi-quantitative method for detecting L-asparaginase produced by isolates by directly observing the plate. Out of 45 isolated LAB screened for L-asparaginase activity, 13 isolates showed positive results exhibiting pink zones in a rapid plate assay. Among them, 7 of were showed predominant zone on the plate and those isolates were considered as active strains and furthermore subjected for asparaginase activity through enzyme assay. Results obtained showed that one of the seven tested isolates gave higher enzyme activity and therefore it was selected for further process for identification. The results obtained by the enzyme assay are more accurate than the results obtained by plate assay at the end of this test, and similar results can be found in a previous study [11]. According to 16Sr RNA sequence analysis, the bacterium was identified as *Lactobacillus plantarum* (Fig.1).In 2019, Phetsri*et al*., [12] observed the ASNase producing *Lactobacillus plantarum.*

The ASNase is an important therapeutic enzyme for the treatment of various cancers, especially certain types of leukemia and lymphoma [4]. ASNases produced by microbes, especially *E. coli,* and *E. carotovora*, cause a variety of side effects. Accordingly, there is a continuing need to screen newer organisms to obtain strains that can produce new and high-yield of L-asparaginases with fewer side effects. Currently, researchers are turning their attention to lactic acid bacteria for the production of L-asparaginase production and utilized for cancer treatment. In the present study, *Lactobacillus plantarum* producing L-asparaginase was subjected to anticancer activity against HeLa cell lines by MTT assay.

The survival rate (%) of Hela cell lines was used as an indicator of cell death after cells were treated with different concentrations of ASNase produced by *L. plantarum* for 72 h. The anti proliferation of the enzyme to Hela cells is dose-dependent because the gradual increases in the dose of ASNase enzyme will cause gradually inhibit proliferation (Fig. 2). At the concentrations tested, the purified ASNase enzyme selectively inhibited cancer cell replication while having no effect on non-carcinogenic vero cell lines (normal cells). After treatment, morphology was observed under the inverted microscope, in which the cells showed signs of detachment from the surface of the well, indicating cell death.

Fig.3 represents morphological changes in cervical cancer cells such as shrinkage, detachment, membrane blebbing, and distorted shape induced by sample ASNase treatment as compared with control. Control showed normal intact cell morphology and their images were capture by light microscope (10X). The *L. Plantarum* ASNase showed significant anti proliferation activity toward Hela cells (IC_{50} value of 0.75 IU/mL). The activity of the enzymes in our study was in accordance which was reported earlier [13]. It was reported that *P.aeruginosa* producing L-asparaginase has inhibited the growth of Hela cells. In 2018, Moharib *et al* [14] reported that plant producing L-asparaginase was effectively suppressed the human liver cancer cell and colon cancer cells, whereas, HeLa cells were not inhibited. In contrast, many studies have shown that L-asparaginase produced from microbial sources had anticancer effects on HeLa cell lines [15,16,13].

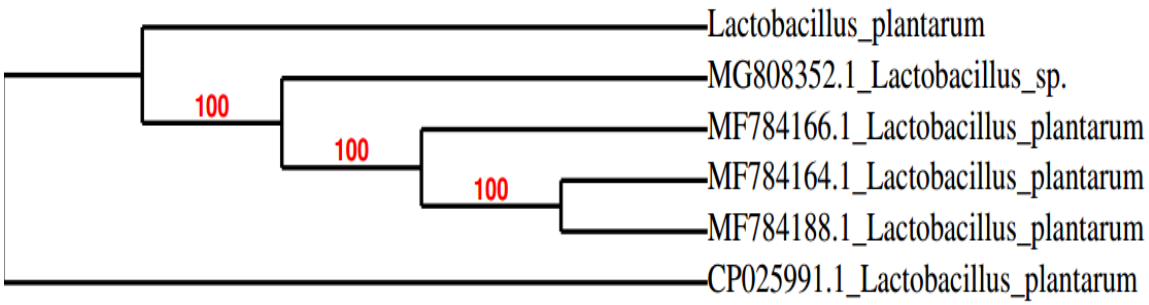

Fig. 1. Phylogenetic tree analysis of *Lactobacillus plantarum*

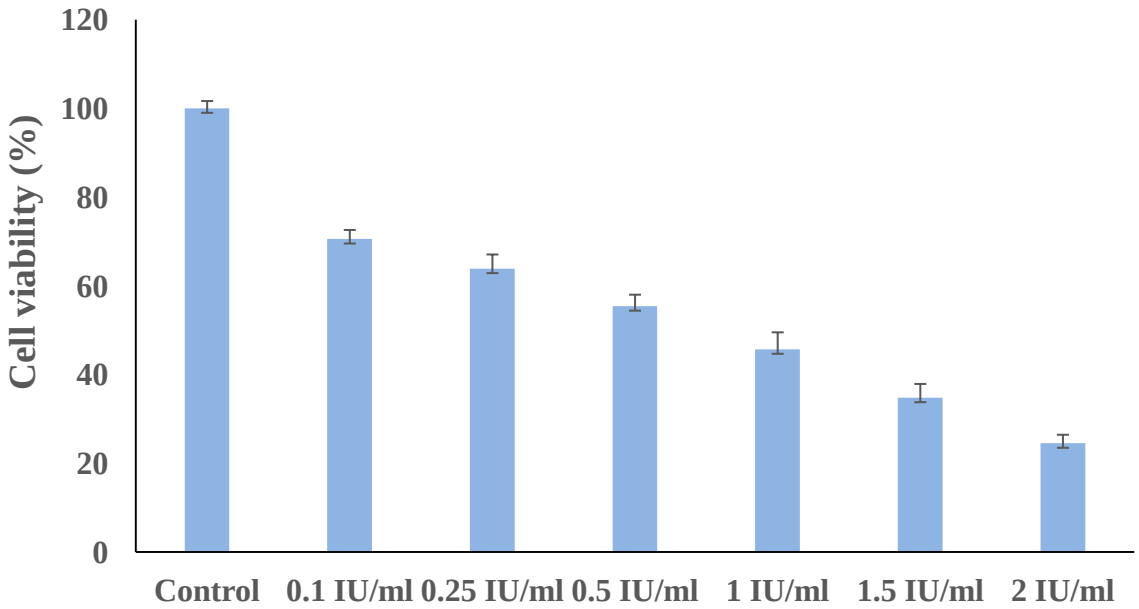

Fig 2. Anti -proliferative effect of different concentration of L-asparaginase on HeLa cells

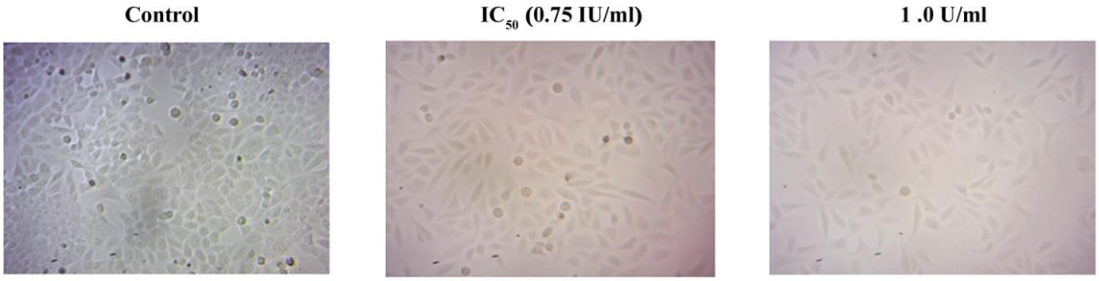

Fig. 3. Anti-proliferative effect of L-asparaginase on HeLa cells

A previous reported an IC_{50} of 0.171 IU of L-asparaginase on HeLa cells [17]. In the present study, 0.75 IU of concentration IC_{50} against Hela cells (Fig.3).This value was low compared to the previous study of Naggar and Shweihy [18], they were observed 2.16±0.2 IU of the enzyme for $1C_{50}$ value against Hela cell lines. However, Moharib [14] reported that L-asparaginase has higher apoptosis activity against HepG-2 and HCT-116 than HeLa and MCF7 carcinoma cell lines.

The mechanism of action of L-asparaginase is different in each cancer cell, in ovarian cancer cells, asparaginase is reported to induce autophagy by induction of ATG12, beclin-1, and cleavage of LC3. The *E.coli* producing L-asparaginase was suppressed the ribosomal protein synthesis at mRNA translational levels in acute myeloid leukemiaof cancer cells.In 2017, Feng *et al*., [19] demonstrated that Asparaginase like 1 was showed increased expression of Bax and decreased expression of Bcl-2, CDK2 and cyclin A2, which was proved that Asparaginase like 1 as anti-cervical cancer agent. However, the role of L-asparaginase in cervical cancer has not yet been reported.

4. CONCLUSION

ASNases are widely distributed in various Microorganisms, but ASNase from LAB has not extensive research. In particular, no studies have been conducted on the mechanism of action of L-asparaginase by lactic acid bacteria. Although the mode of action has not been studied in our current study, it does prove that this substance acts as an anti proliferating agent by in vitro study. However, further in vivo studies and trials are needed to assess L-therapeutic asparaginase's efficacy. Since this bacterium is already used as a probiotic drug, the enzyme produced from it is unlikely to cause any side effects.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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