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L-ASPARAGINASE PRODUCED FROM *BACILLUS* SPECIES IN CHEESE WHEY PRODUCTION MEDIUM SHOWS POTENT CYTOTOXIC ACTIVITY AGAINST HELA CELL LINES

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ABSTRACT

L-asparaginase (Systematic name L-asparaginase amidohydrolase) is used in treatment of acute lymphoblastic leukemia (ALL). Different species of bacteria and fungi have been reported to produce L-asparaginase. Currently, L-asparaginase produced from E.coli and Erwinia species have been used in ALL. Because of Allergies & liver toxicity associated with commercially available L-asparaginase, it requires further research to discover novel bacterial strains producing l asparaginase with less toxic effects. In the present study, L-asparaginase enzyme was produced from soil isolates of *Bacillus* species, by using cheese whey as substitute of asparagine in production media; Bacillus species isolated in this study are *Bacillus subtilis* (LC425423), *Bacillus aerophillus* (LC425427), *Bacillus endophyticus* (MG928501), respectively. L-Asparaginase enzyme produced from these three Bacillus species was purified by using dialysis technique, and the purified enzyme was tested for its anticancerous activity against HeLa cell lines by MTT assay. Surprisingly, the enzyme tested inhibited the cell growth effectively; the concentration of enzyme used to inhibit 50% of cell culture growth was 65.44, 131.35 & 60.78µg/ml. Therefore, the result obtained in this study indicates possible use of these Bacillus species in the industrial production and in anticancerous therapies. In addition, this study also has demonstrated that cheese whey can be used for cost effective production of L asparaginase enzyme.

KEYWORDS: L-asparaginase, Bacillus Species, HeLa-Cells, Acute Lymphoblastic Leukemia & Cheese whey

INTRODUCTION

L-asparaginase enzyme is a therapeutic enzyme, which catalyzes the hydrolysis of L-asparagine to result into aspartate and ammonia. L-asparagine is an essential amino acid required by cancer cells to proliferate, as they lack the enzyme asparagine synthetase which can synthesize asparagine from aspartic acid and glutamine. In condition of asparagine scarcity in blood, the metabolic processes of cancer cells are greatly affected there by resulting in cell death[1, 2]. The *E.coli* & Erwinia asparaginase enzyme used in ALL therapy are commercially known as ELSPAR, Erwinase respectively. ELSPAR is the most effective drug used in treatment with 90% of cure rate; the dose recommended is 200IU/kg/day for 28 days or 25000/m^2 weekly. However, side effects such as hypersensitivity, liver dysfunction, and pancreatitis are some of the outcomes from *E.coli* asparaginase. Whereas, Erwinia asparaginase is used in second line therapy in patients with developed allergies to *E.coli* asparaginases, which is less effective and have shorter life[3, 4]. Therefore, an alternate l asparaginase produced from different bacterial species enclosing potent anticancerous activity is much required to overcome the side effects of classical therapies. In addition, the production of l asparaginase from the novel strains should also be economically feasible. Cheese whey is a bi product of cheese production, widely used in many

productions such as ethanol, biogas, single cell protein etc. Cheese whey is chosen in industrial productions, since it is a waste product and a cheaper resource of proteins, minerals, essential and non-essential amino acids [5, 6]. Hence in the present study, we report novel Bacillus strains such as *Bacillus subtilis* (LC425423), *Bacillus aerophillus* (LC425427), *Bacillus endophyticus* (MG928501) producing l asparaginase enzyme in production media containing cheese whey. The enzyme produced was tested on HeLa cell lines, and it was found to be cytotoxic against HeLa cell lines (with IC50 of 65.44, 131.35 & 60.78 µg/ml). Therefore, this study is first of its kind to report production of l asparaginase enzyme using a cost effective production media containing cheese whey.

MATERIALS AND METHODS

Isolation of Bacillus species from soil: Soil samples were collected from Garden in and around at a depth of 30 to 40 cm. The samples were collected into a sterile screw capped tube. One gram of soil was separately suspended in 9 ml of physiological saline in a flask and placed on an orbital shaker at 100 rpm at room temperature 28° C ± 2° C for 1hr. At the end of shaking, the flasks were kept aside the flasks to settle the suspending matter. The soil samples supernatant were serially diluted up to 10^{-9} with physiological saline. 1 ml from 10^{-4} – 10^{-9} dilutions were plated on modified Brain heart infusion agar (BHA) medium by spread plate technique [7]. One litre of Brain heart infusion media was modified by supplementing with 6gms of KH_2PO_4, Aspergine- 10gms, 4ml of 1M $MgSo_4$, 2ml of 0.1M$Cacl_2$, 0.4ml of 0.009% phenol red indicator, the pH of the medium was adjusted to 7 by 1N Hcl. Control plates containing BHA medium without dye and without asparagine were also prepared. After inoculation, plates were then incubated at 37°C for 24 hrs[8].

Screening of L-asparaginase by Plate Method Assay

After incubation, 10^{-7} dilution plates were selected for screening, as this dilution showed isolated bacterial colonies. The colonies exhibiting pink zone were considered as L- Asparaginase positive colonies. Presence of pink zone around the colony indicates deamination of asparagine to yield aspartate and ammonia, release of ammonia is detected by phenol red pH indicator. Phenol red at acidic pH is yellow and turns pink at basic pH, therefore formation of pink zone indicates isolates exhibiting L-asparaginase activity. Isolated colonies were selected and pure cultures were obtained by repeated sub culturing followed by gram staining. The isolates were then subjected to secondary screening [8-10]

Qualitative Assay by Agar Well Diffusion (AWD) Assay

Agar well diffusion assay is a modified version of Ditch Plate Assay; this technique was initially designed by Heatley in 1944. Culture filtrates of selected isolates were qualitatively screened for L-asparaginase production using plate assay.

50 mL of asparagine tryptone glucose yeast extract broth medium were dispensed in 250 mL Erlenmeyer conical flasks, sterilized, inoculated, and incubated at for 48hrs 37°C in a rotatory incubating shaker at 150 rpm[11].

50 µl of culture broth was dispensed in each well of modified BHA medium containing 1% aspargine and 0.009% phenol red indicator (8 mm well were made in the center of each plate using sterilized cork borer), and palate with well containing inoculated media served as control. The plates were incubated at 37°C for 24 hrs. After the incubation was over, the plates were observed for the formation of pink zone formation around the wells. The zone diameter was recorded, the cultures showing greater enzyme production were selected for identification and further study [12, 13].

Enzyme Production by Submerged Fermentation Method

Inoculum Preparation

The strains showing the bigger pink zone in plate assay were tested for production. The loopful of culture of selected strains were first inoculated into 50ml of inoculum media. 1000ml of inoculum media contains Na_2HPo_4-6gms, KH_2Po_4-3gms, Nacl-0.5gms, glucose-1gm, 1M $MgSo_4$-2ml, 0.1M $Cacl_2$-1ml. the pH was adjusted to 7.2. The inoculated flasks with inoculum media was incubated at 37^0C for 16-18hrs[14].

Production of l asparaginase Enzyme by Using Whey

2ml of 18hrs, inoculum culture was inoculated into 50 ml of production media which was prepared with 5% of cheese whey, one liter of production media also contained glucose -10gms, K_2HPO_4-1gm, yeast extract-5gms, and tryptone -5gms. The pH of the production media was adjusted to 8. Flask containing tryptone yeast extract broth with 1% aspargine were kept as control[15, 16].

Purification of Enzyme by Dialysis

After production, the fermented broth was centrifuged at 10000 rpm for 10 min at 4°C. the liquid supernatant was used as crude enzyme source, which was purified by dialysis technique using nitro cellulose dialysis bag of 8cm[17, 18].

Determination of Enzyme Activity

0.5 ml of dialyzed enzyme, was added to 0.5 ml of 50 mM asparagine and 1ml potassium phosphate buffer (0.02 M) in each tube with pH 8 was taken and mixed well, the mixture was incubated in water bath at 37ºC for 15 minutes, after the incubation, 1ml of 1.5 M trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 12000 rpm for 10 minutes and the supernatant was collected. After that, the supernatant was transported to clear test tubes to determine the concentration of ammonia which is liberated from the enzyme action by the method of direct Nesslerization[1, 19].

Direct Nesslerization

The concentration of ammonia was estimated for each sample by mixing 4 ml of distilled water to 0.5 ml of sample to be estimated, then 0.5 ml of Nessler′s reagent was added, the mixture was shaken well, and incubated at 37ºC for 15 minutes, the absorbance was measured at wave length (450 nm). The blank was prepared by mixing 4.5 ml distilled water with 0.5 ml Nessler's reagent[1, 8].

Standard Curve of Ammonium Sulfate

Stock solution of ammonium sulphate was prepared by adding 2.5 mg of ammonium sulphate in 25ml of distilled water to give a concentration of 100 μg/ml of stock solution. Graduated concentrations of ammonium sulfate were prepared by adding graduating volumes of stock solution to suitable volume of distilled water to make up the final volume of 8ml**.** After that, the absorbance was measured at wavelength (450nm) and relation between ammonium sulfate concentration and absorbance was represented**.** Based on the standard graph of ammonium sulphate, the concentration of ammonia released from the samples was calculated and enzyme activity was expressed in international units. One unit of L-Asparaginase is the amount of enzyme which catalyzes the formation of 1μmol of ammonia per min at 37^0C [1].

Quantitative Estimation of Dialyzed Protein

The amount of protein content present in both crude and dialyzed supernatant was determined by Lowry's method.

Culture Identification

The cultures exhibiting maximum l asparaginase activity were identified on the basis of gram stain colony morphology & molecular Identification by 16srRNA typing. The accession numbers for the cultures strains were obtained from DDBJ and NCBI gene bank

Anticancerous Activity

The partially purified enzyme was tested for anti-cancerous activity on HeLa cell lines by MTT assay.

Maintenance of Cells

The Cancer cell line HeLa was procured from NCCS, Pune and the cells were maintained in DMEM supplemented with 10 % FBS and the antibiotics penicillin/streptomycin (0.5 mL^{-1}), in atmosphere of 5% CO_2 /95% air at 37 ^{0}C.

Procedure

Ability to showcase anticancer activity was evaluated by MTT assay in triplicates with six concentration check for each sample; positive (Cisplatin**)** and negative control were also kept with test samples. Firstly, cells were trypsinized and perform the tryphan blue assay to know viable cells in cell suspension. Cells were counted by haemocytometer and seeded at density of 5.0 X 10^3 cells / well in 100 µl media in 96 well plate culture medium and incubated overnight at 37 ^{0}C. After incubation, old media was discarded and fresh media was added, 100 µl with different concentrations of test compound in respective wells in 96 plates was also added. After 48 hrs., Discard the drug solution and add the fresh media with MTT solution (0.5 mg / $mL^{-1)}$ to each well, plates were then incubated at 37 ^{0} C for 3 hrs. At the end of incubation time, precipitates are formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria. The optical density of solubilized crystals in DMSO was measured at 570 nm on a microplate reader. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50 % values is generated from the dose-response curves for each cells using with origin softwaren [20-22].

$$\% \text{ Inhibition} = \frac{100\ (\text{Control} - \text{Treatment})}{\text{Control}}$$

RESULTS

Identification of bacillus strains

The bacillus cultures that exhibited the best l asparaginase production & enzyme activity were identified as *Bacillus subtilis,* (LC425423) *& Bacillus aerophillus* (LC425427), *Bacillus endophyticus* (MG928501) The phylograms for the two bacillus strains are as follows.

- ***Bacillus subtilis*** **(culture code 10)**

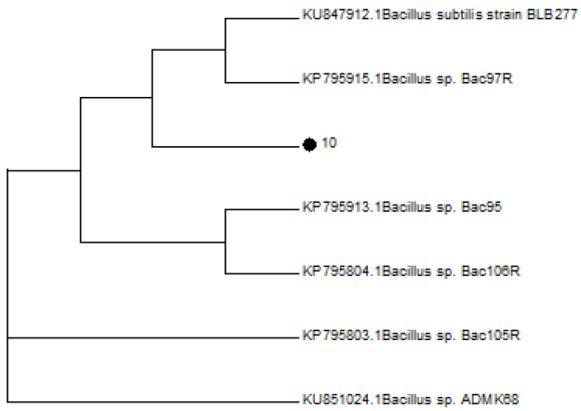


Figure 1: Phylogram of Bacillus subtilis (LC425423)

- ***Bacillus aerophilus*** **(culture code : C_4)**

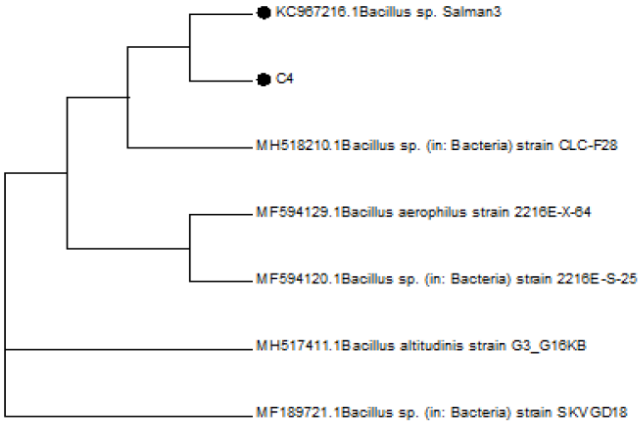


Figure 2: Phylogram of Bacillus aerophilus (LC425427)

- ***Bacillus aerophilus*** **(culture code : G_1)**

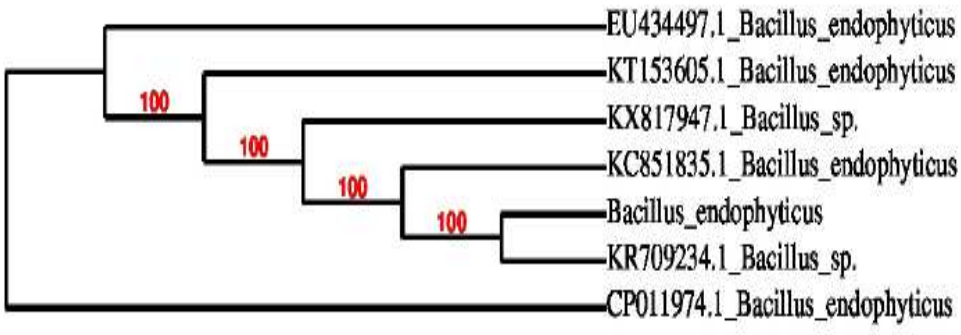
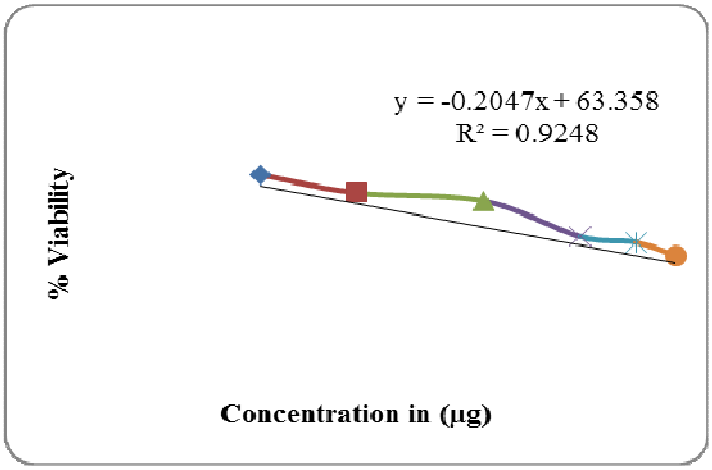


Figure 3: Phylogram of Bacillus Endophyticus (MG928501)

Enzyme Yield: The amount of enzyme produced by *Bacillus subtilis* (LC425423) was 525µg/ml. *Bacillus aerophilus* (LC425427) 454 µg/ml and *Bacillus endophyticus* (MG928501) 525 µg/ml respectively

Enzyme Activity: The enzyme activity of l asparaginase produced from *Bacillus subtilis*(LC425423), *Bacillus aerophilus* (LC425427), *Bacillus endophyticus* (MG928501) was 8.52, 14.3 & 7.19 (IU/ml)

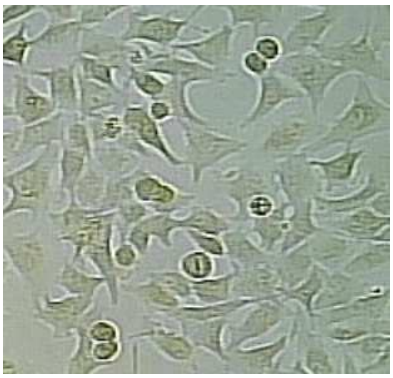
Anticancerous Activity of L asparaginase Produced from *Bacillus subtilis* (LC425423)



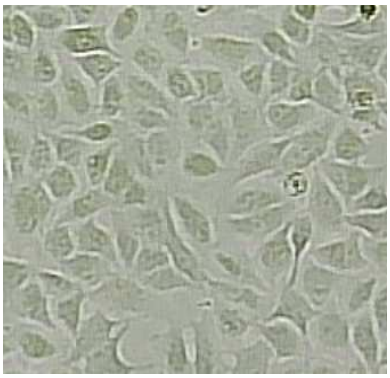
Graph 1: Representing Cytotoxic Effect of L asparaginase Produced from Bacillus Subtilis,(LC425423)

Table 1: Cytotoxic Properties at Different Concentrations of L Asparaginase Produced from Bacillus Subtilis (LC425423).

Concentration(ug)	Absorbance at 570nm			Average	Average-Blank	% Viability	IC_{50} (ug)
100	0.829	0.831	0.833	0.831	0.826	44.672	65.441
75	0.891	0.892	0.894	0.892	0.887	47.971	
50	0.918	0.919	0.921	0.919	0.914	49.432	
25	1.084	1.086	1.087	1.085	1.08	58.41	
10	1.122	1.123	1.125	1.123	1.118	60.465	
5	1.205	1.207	1.208	1.206	1.201	64.954	
Untreated	1.854	1.856	1.854	1.854	1.849	100	
Blank	0.005	0.006	0.005	0.005	0		

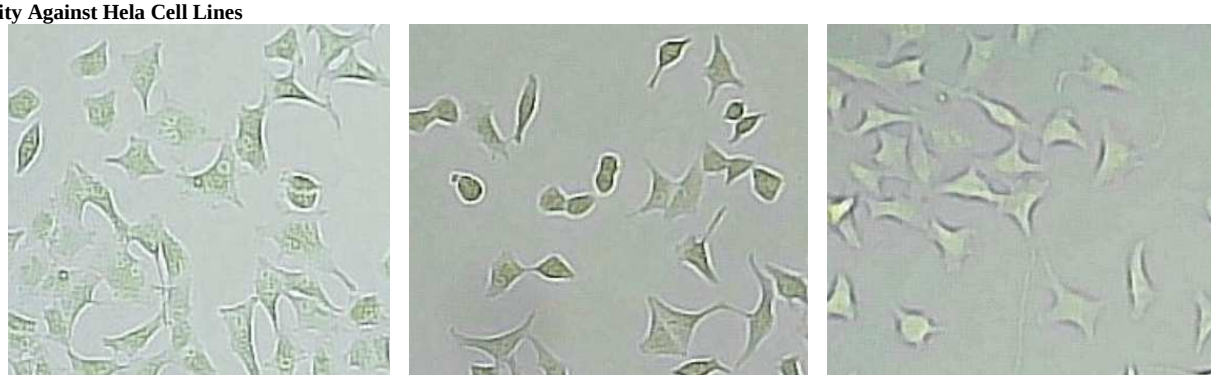


5µg



10µg

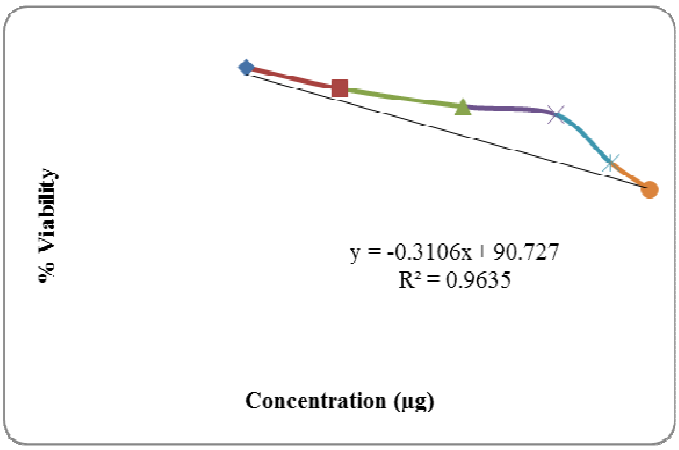
25µg



50µg **75µg** **100µ**

Figure 4: Cyto Toxic Effect of l asparaginase Produced from Bacillus Subtilis (LC425423) at Different Enzyme Concentrations

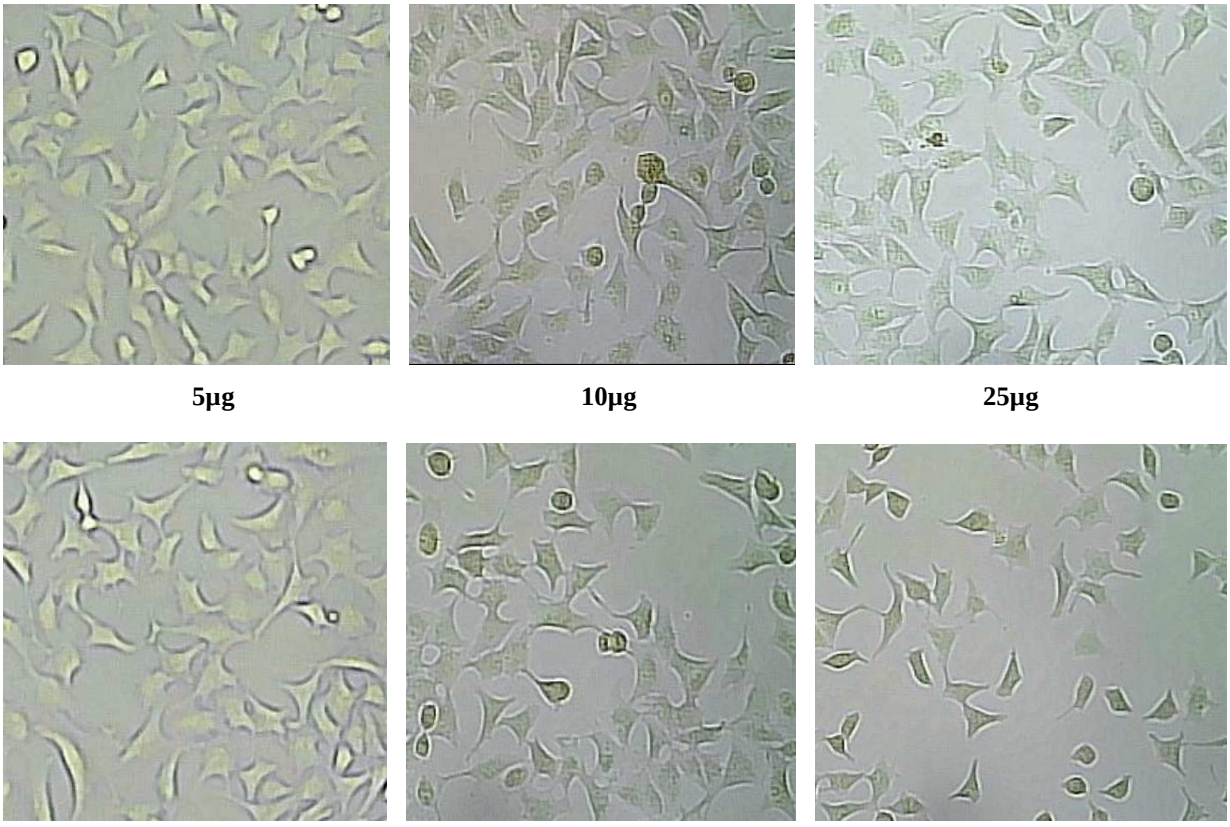
Anticancerous Activity of L asparaginase Produced from *Bacillus Aerophilus* (LC425427)



Graph 2: Representing Cytotoxic Effect of L asparaginase Produced from Bacillus Aerophilus (LC425427)

Table 2: Cytotoxic Properties at Different Concentrations of L asparaginase Produced from *Bacillus Aerophilus* (LC425427)

Concentration(µg)	Absorbance at 570nm			Average	Average-Blank	% Viability	IC_{50} (µg)
100	1.101	1.103	1.105	1.103	1.1	59.363	131.35
75	1.229	1.231	1.233	1.231	1.228	66.27	
50	1.462	1.464	1.465	1.463	1.46	78.791	
25	1.5	1.502	1.504	1.502	1.499	80.895	
10	1.588	1.59	1.592	1.59	1.587	85.644	
5	1.689	1.691	1.693	1.691	1.688	91.095	
Untreated	1.856	1.857	1.856	1.856	1.853	100	
Blank	0.003	0.004	0.003	0.003	0		

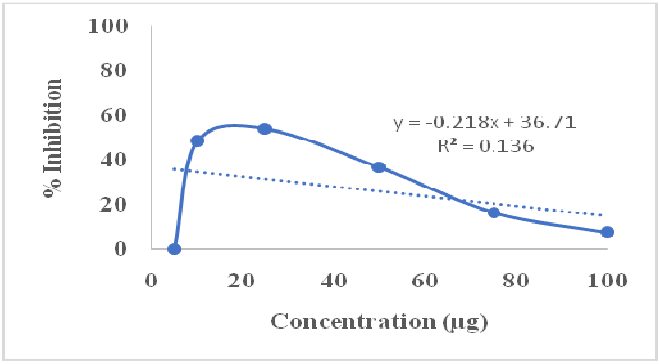


5µg 10µg 25µg

50µg 75µg 100µg

Figure 5: Cyto Toxic Effect of l asparaginase Produced from Bacillus Aerophilus (LC425427) at Different Enzyme Concentrations

Anticancerous Activity of L Asparaginase Produced from *Bacillus Endophyticus* (MG928501):



Graph 3: Representing Cytotoxic Effect of L Asparaginase Produced from Bacillus Endophyticus (MG928501)

Table 3: Cytotoxic Effect of Different Concentrations of Produced from Bacillus Endophyticus (MG928501)

Concentration (µg)	OD at 570	% Inhibition	IC_{50}
5	0.864	0	60.78
10	0.444	48.19	
25	0.394	54.02	
50	0.542	36.75	
75	0.717	16.33	
100	0.796	7.11	

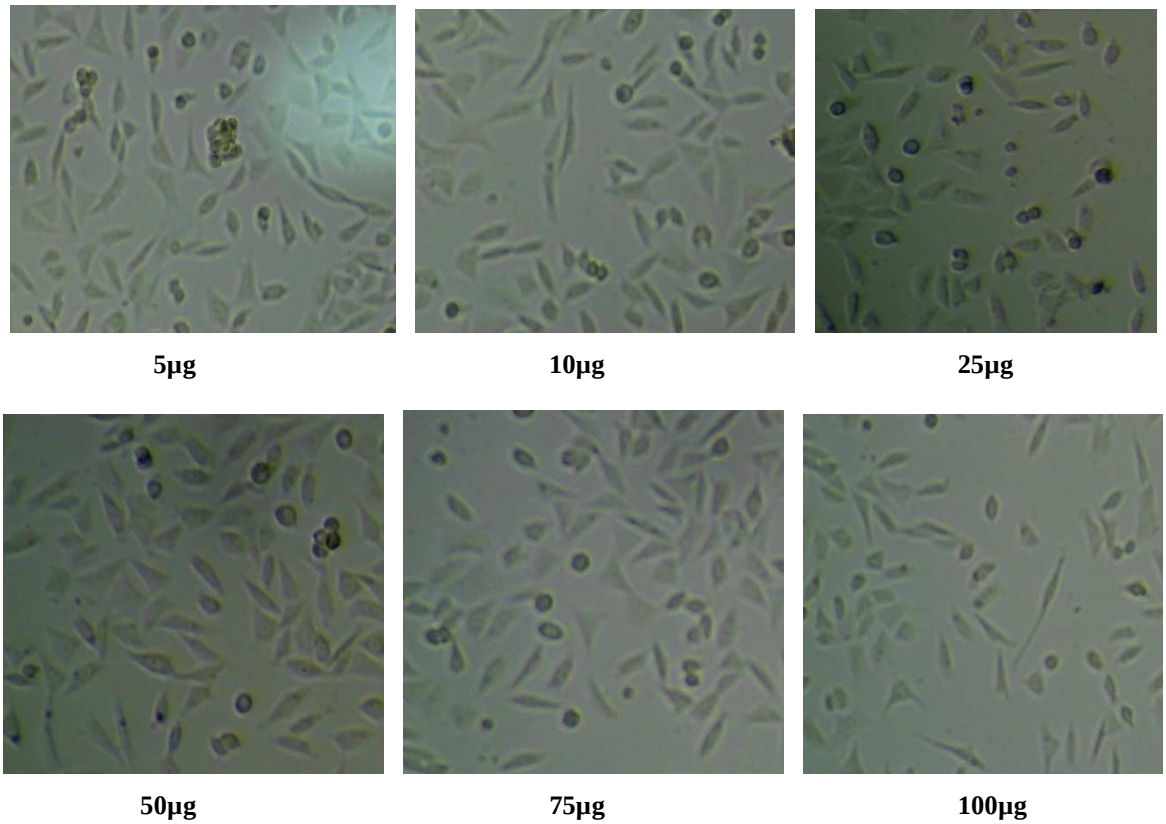


Figure 6: Cyto Toxic Effect of l asparaginase Produced from Bacillus Aerophilus (LC425427) at Different Enzyme Concentrations

DISCUSSIONS

Bacillus species can be easily isolated and cultured from soil, water and surrounding environments. Bacillus species such as *B subtilis* have been applied to produce a number therapeutic drugs such as fibrinolytic enzymes, amylolytic enzymes, antibiotics like bacitracin, and it is also being used in probiotics[23, 24]. A number of studies have reported the production of l asparaginase from different Bacillus species. For instance, in a study, *B. licheniformis* (RAM-8) was used to optimize the l asparaginase production, in which, the maximum production was 32.26 IU/ml[25]. Similarly, a recent study by pallavi et al. has tested the effects of parameters like carbon nitrogen, pH and temperature on mangrove isolate of *Bacillus firmus* –AVP 18. In this study, the maximum yield of the enzyme was noted in presence of dextrose (171.96IU/mil), beef extract (176.78 IU/ml), pH 9 (29.05 IU/ml) and temperature at 37 ^{0}C (24.26 IU/ml). In our study, the soil isolates of *Bacillus subtilis* (LC425423), *Bacillus aerophilus & Bacillus endophyticus* (MG928501) produced 525, 454 & 525 (µg/ml) of L asparaginase enzyme, respectively. The enzyme activity shown by partially purified enzyme from the said isolates was recorded as 8.52, 14.3 & 7.19 (IU/ml). The results are in accordance with a study reported by vidya moorthy et al. in which, Bacillus species DKMBT10 was reported to produce maximum yields of asparaginase (2.6mg/l) in presence of glucose and maltose. The activity of purified enzyme recorded in this study was 1.12 & 1.05 (units/mg)[10]. Furthermore, a study by Naglaa etal reported the use of agro industrial waste like corn steep liquor, black strap molasses etc. as nitrogen sources for production of l asparaginase from *B subtilis*(SW5) & *B.marisflavi* (SO5). In this study, it was also reported that the combination of nitrogen sources along with asparagine favored the enzyme production and enzyme activity[16]. However in our study, cheese whey and yeast extract was used instead of aspargine to

produce l asparaginase enzyme from the isolated Bacillus species; which indicates the possible use of cheese whey in production of l asparaginase. This combination was also reported to effectively increase the production of butanol from *C. acetobutylicum* NCIB 2951 by Maddox etal [26]. Besides this, our study is first to report l asparaginase production from *Bacillus endophyticus* and *Bacillus aerophilus* (LC425427) species. Furthermore, the enzyme activity in our was noted at pH 8, which indicates that the produced enzyme can be used anticancerous therapies; Since enzyme activity below pH 8 is considered to be therapeutically not effective in tumor patients[27]. However, the cytotoxicity against cell cultures of l asparaginase produced from Bacillus species have also been reported in recent studies, for example, a study by shakambari et al. it was reported that the lipid encapsulated l asparaginase enzyme produce from *Bacillus tequilensis* PV9W was cytotoxic on HeLa cell cultures with IC50 of 0.085 IU[28]. In another study, Shakambari et al. have reported the cytotoxic effect of la sparaginase produced from *Bacillus tequilensis* PV9W on HeLa cell cultures with IC50 of 0.036 ± 0.009 IU[29]. Similarly, Maysa E moharam *et al.* reported antitumor activity of l asparaginase produces from Bacillus sp R36 on Hep G2 and HCT-116 cell cultures with IC50 value of 112.19 µg/mL & 218.7 µg/mL respectively [30]. Similarly, Naglaa et al. also reported that the crude enzyme extract (5mg /100ml) from *B subtilis*(SW5)& *B.marisflavi* (SO5) effective antitumor activity in *Drosophila melanogaster* Strains [16]. In our study, the IC50 value of the enzyme produced from *Bacillus aerophilus* (LC425427) was 131.35 *Bacillus subtilis*,(LC425423) was 65.44 and *Bacillus endophyticus* (MG928501) 60.78 (µg/ml) the IC50 concentrations are comparatively lesser than the concentrations reported by Maysa E etal & Rani SA et al [30, 31]. Many studies have also shown the anti-cancerous activity of l asparaginase on HeLa cell lines produced from fugal species such as *Aspergillus. oryzae*, *Aspergillus flavus* [31, 32]. Nonetheless, our study is first of its kind to report the anticancerous activity asparaginase produced from three different Bacillus species on HeLa cell lines (human cervical cancer cell lines).

CONCLUSIONS

L- asparaginase is therapeutically and industrially important enzyme, therefore, the cost of industrial production can be reduced by using waste like cheese whey as nitrogen source. In addition, cervical cancer is the second most common cancer in females, therefore, new line of drugs are required for effective treatment. Since in many studies, including our study that have effectively demonstrated the in-vitro anticancerous activity of l asparaginase against human cervical cancer cell lines, the in vitro findings suggests the possible use of L asparaginase in therapy treating cervical cancers. However, further in vivo studies and trials are required, before considering it in therapy against cervical cancer.

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