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Anticancer Activity of L-asparaginase Produced from *Amycolatopsis japonica*

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Abstract— *The ability of L-asparaginase to inhibit the formation of cancer cells has aroused scientists' curiosity in biological realms. In cancer cells, L-asparaginase suppresses protein synthesis by hydrolyzing L-asparagine to L-aspartic acid and ammonia. As a result, it's a crucial therapeutic enzyme in the treatment of Acute Lymphoblastic Leukemia in combination with other drugs (ALL). This enzyme has recently been discovered to be useful in a number of scientific fields, including clinical research, pharmacology, and the food business. Purification, characterization, and assessment of the cytotoxic effect of Amycolatopsis japonica L-asparaginase were the goals of this study. Amycolatopsis japonica was isolated from the plant rhizosphere and L-asparaginase was recovered. With a molecular weight of 37.5 KDa, partially purified L-asparaginase from A. japonica had a total activity of 1968.98 U with 26.696 mg total protein and a specific activity of 73.75 U/mg, 6.42 purification fold, and 42.86 percent recovery yield. In the presence of EDTA, Mg^{2+}, pH8, 45°C, and 0.13 mM L-asparagine, L-asparaginase from A. japonica demonstrated good activity and stability, with Km and Vmax values of 0.13 mM L-asparagine and 0.43U/ mL, respectively. The cytotoxicity of L- asparaginase from A.japonica against a colon cancer cell line was high; with an IC50 value of 36 L. Amycolatopsis japonica could be a source of L-asparaginase, which could be a new target for cancer cells.*

Keywords— ***L-asparaginase, Cancer cells, L-aspartic, Amycolatopsis japonica, Characterisation, Purification***

I. INTRODUCTION

Several studies have focused on the production of enzymes by actinomycetes, particularly L-asparaginase [1]. Actinomycetes are unicellular organisms that develop branching filaments known as hyphae. Spores or fission are used to replicate asexually [2]. They are Gram-positive bacteria with DNA that contains a lot of guanine and cytosine. Actinomycetales is the order in which they live [3]. They are unique among bacteria in that they can build branched networks and have a variety of life cycles [4]. L-asparaginase is a hydrolytic enzyme that breaks down L-asparagine (an amino acid) into L-aspartic acid and ammonia in a physiologically irreversible manner [5]. This enzyme, which is made up of four identical subunits, catalyzes the reaction by grasping the active sites on L-asparagine.

L-asparagine is essential for tumor cells to obtain their protein in order to survive, although normal cells can grow without it because they have an enzyme called L-asparagine synthase that helps them manufacture the protein within the cells and absorb it from the outside. This distinguishes cancer cells from normal cells since they are unable to produce L-asparagine due to a lack of L-asparagine synthetase, relying on those found in

circulating plasma pools [6]. Meanwhile, when L-asparaginase is present, cancer cells are unable to obtain a critical growth factor (L-asparagine), which is required for their continued growth, limiting their growth because they are unable to manufacture L-asparagine in their cells. L-asparagine is continuously reduced when this enzyme is supplemented. The goal of this study was to generate, purify, characterize, and test the cytotoxicity of *A. japonica* L-asparaginase.

II. METHODOLOGY

2.1 Isolation of Actinomycetes

Actinomycetes *A. japonica* was isolated from the rhizosphere of a plant in this investigation. Hi-media provided the media components used in the study (Mumbai, India). Sigma provided the asparagine substrate Sephadex G-50 (Sigma-Aldrich, USA). All of the substances were reagent-grade analytical compounds.

2.2 Production of L-asparaginase from *Amycolatopsis japonica*

The bacterial cultured was stored on starch casein agar (SCA) slant at 4ºC and subculture on SCA plate, and then incubated at 27ºC for 3 days. One hundred millilitre of M9 medium broth (Na_2HPO_4; 6.0 g, K_2HPO_4; 0.9 g, NaCl; 0.5 g, L-asparagine; 10 g, 1M $MgSO_4.7H_2O$ - 2 mL, 0.1 M solution of $CaCl_2.3H_2O$; 1m L, 20% glucose stock; 10 mL, 0.005% phenol red, pH 6.5) in 250 mL conical flask was inoculated with 1.5x 10^8 CFU/mL of 72 hrs old colony suspension and incubated at 27 °C in a shaker incubator at 200 rpm for 7 days. At the end of the fermentation period, the medium was centrifuged at 10,000 rpm for 15 minutes and cell-free supernatant was taken as the crude enzyme [7].

2.3 Determination of the L-asparaginase Activity

The activity of the generated L-asparaginase enzyme was measured using the method of Saxena *et al*. [7], in which the rate of L-asparaginase hydrolysis was determined by measuring the ammonia emitted during Nessler's reaction. A 0.1 mL crude extract was mixed with 0.2 mL 0.05M Tris-HCl buffer (pH 8.6) and 1.7 mL 0.01 M L-asparagine in a 0.2 mL Tris-HCl buffer (pH 8.6). The reaction was stopped by adding 0.5 mL of 1.5 M Trichloroacetic acid to the mixture and incubating it for 10 minutes at 37ºC.After centrifuging for 5 minutes at 10,000 rpm, 0.5 mL of the supernatant was added to 7 mL distilled water and treated with 1 mL of Nessler's reagent. The absorbance was measured with a UV spectrophotometer at 480 nm after the color reaction had developed for 10 minutes. It was determined how much ammonia was liberated. The amount of L-asparaginase enzyme that liberated 1m of ammonia per minute under the assay conditions was determined as one international unit [7].

$$Enzyme\ activity\ (IU) = \frac{Amount\ of\ Ammonia\ Liberated}{Incubation\ time\ x\ mL\ of\ enzyme\ used}$$

2.4 Partial Purification of L-asparaginase

The crude enzyme was purified in three steps: ammonium sulphate salting, dialysis desalting, and Sephadex G-50 gel filtration chromatography [8]. Ammonium sulphate at concentrations of 0-40 percent, 40-80 percent, and 80-100 percent (12.28 g, 14.18 g, and 7.68 g, respectively) was dispensed into 50 mL crude enzyme in a 250 mL Erlenmeyer flask and gently magnetically agitated until it dissolved. These flasks were stored at 4ºC overnight before being centrifuged for 15 minutes at 10,000 rpm at 4ºC. The precipitate was dissolved in 0.5 M Tris-Hcl (pH 8.6) after the supernatant was decanted [9].

Each salt concentration range's dissolved precipitate was dialyzed. At this point, the dialysis bag was cut (5 cm long) and soaked overnight in Tris-HCl buffer pH 8.6, 10 mL of dissolved precipitate was added to the bag, sealed snugly, and soaked in 50 mL of the same buffer at 4 oC. The buffer was changed every 2 hours and then left at 4 oC overnight. The enzyme activity as well as the protein content was measured. By putting the dialyzed enzyme on Sephadex G-50 at 25 oC, it was further purified. After soaking 10 g of Sephadex powder in 100 mL of Tris-Hcl buffer pH 8.6 for 48 hours, a portion of it was put onto the column (15cm X 2cm). The dialyzsd enzyme was loaded on the column after it was equilibrated with the same buffer. It was eluted at a flow rate of 0.5 mL/min using the same buffer. The protein and enzyme activity of each fraction was measured using the Lowry assay at 660 nm and Nesslerisation at 480 nm on 3 mL fractions. The fractions with the highest activity were combined, freeze-dried, and used in further research.

2.5 Characterisation of L- asparaginase.

2.5.1 Effect of temperature on stability and activity of L-asparaginase

The effect of temperature on the activity and stability of L-asparaginase was investigated using the approach of Mohamed *et al*. [10]. 0.1 mL of enzyme was mixed with 0.2 mL of Tris-HCl buffer pH 8.6 and 1.7 mL of 0.05 M L-asparagine substrate in a 60-minute incubation at temperatures ranging from 10 to 75 degrees Celsius. The activity and stability of the enzyme were tested using the Nesslerisation method at 10-minute intervals for 60 minutes [11].

2.5.2 Effect of pH on activity and stability of L-asparaginase

The activity and stability of the enzyme were tested at various pH levels. The pH of the medium was varied during the assay method. Different buffers (Citrate buffer; pH4 – 5, Phosphate buffer; pH 6 – 7, Tris-Hcl buffer; pH 8, Carbonate buffer; 9 – 10) were employed to achieve a pH range of 4 to 10. The stability and activity of the enzyme were tested using the Nesslerisation method every 10 minutes for 60 minutes[11].

2.5.3 Influence of inducers and inhibitors on activity and stability of L-asparaginase

This was accomplished by adding 0.1 mL of a 1 percent inhibitor solution to the assay mixture, which included Ascorbic acid, Urea, Triton X-100, Sodium azide, Tween 80, EDTA, and Sodium deocyl sulphate, and checking the stability and activity of the enzyme every 10 minutes for 1 hour using the Nesslerisation method [11].

2.5.4 Influence of different metal ion on activity and stability of L-asparaginase

To determine the effects of metal ions on the stability and activity of the enzyme, 0.2 mL of 0.2 M of different metals in the chloride form (Magnesium chloride, Potassium chloride, Calcium chloride, Iron chloride, Mercury chloride, Sodium chloride, Zinc chloride, and Ammonium chloride) was added to the assay procedure. The activity was monitored using the Nesslerisation method at 10-minute intervals for an hour [12].

2.5.5 Effect of Substrate concentration on activity and stability of L-asparaginase

The assay mixture contained 1.7 mL of various concentrations of L- asparagine (4.0 mM, 8.0 mM, 1.2 mM, 1.6 mM, and 2.0 mM), and the effect of these concentrations on the stability and activity of the enzyme was determined using the Nesslerisation method at 10-minute intervals for 1 hour [13].

2.5.6 Influence of amino acids on L-asparaginase

The enzyme's specificity towards different substrates was determined by incubating the enzymes with 1.7 mL of 0.05 M of various amino acids such as L-aspartic acid, L-arginine, L-phenylalanine, L-asparagines, L-glutamine, and L-asparagine, and observing the stability and activity using the Nesslerisation method at 10-minute intervals for 1 hour [13].

2.6 Molecular Weight Determination of L-asparaginase.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to evaluate the molecular weight of the crude enzyme and to determined the purity of the purified enzyme. 10% gel was prepared. The gel was stained with Coomassie Brilliant Blue R-250. The protein ladder (cat 26614) was used as reference marker. The material used were; sample buffer(Tris-HCI (pH 6.8) buffer 0.4 mL, 10% SDS 2.5%, 2-mercaptoethanol 0.4 mL, Glycerol 2.0 mL, Bromophenol blue 0.002g, Distilled water 4.7 mL), Electrode buffer (Tris-HCI 6.05 g, SDS 2 g, Glycine 28.8 g, Distilled water 2.0 L), Separating (4x) gel buffer (Tris-HCl 18.3 g, distilled water 100 mL, pH 8.8), Stacking (4x) gel buffer (Tris-HCl 6.055 g, distilled water 100 mL, pH 6.8), (30%) Bisacrylamide (29.2 g acrylamide, 0.8 g of bis-acrylamide, 100 mL).

The procedure is as follows; separating gel that comprises of (Distilled water 19.5 mL, Bisacrylamide (30%) 10 mL, 4x separating gel buffer 10 mL , SDS (10%) 0.8 mL, Glycerol (10%) 0.35 mL, TEMED 20 µL, APS (2%) 0.6 mL) were mixed and gently poured in a vertical mould, the saturated butanol was added and the gel was allowed to polymerise. After 30 minutes, the butanol was removed and upper portion of gel was washed with deionized water. the stacking gel (Comprised of Distilled water 6.3 mL, Bisacrylamide (30%) 2 mL, 4x separating gel buffer 2.5 mL, SDS (10%) 0.2 mL, Glycerol (10%) 0.15 mL, TEMED 10 µL , APS (2%) 0.13 mL) was poured on the separating gel on the vertical mould and comb was placed in it, the gel was allowed to polymerised and the comb was removed. 30 µL of enzyme was mixed with 4X lamelli reducing buffer to give it colour, the sample was heated at 98^{o}C for 10 minutes and 15 µL of sample was loaded in the wells. Electrophoresis was carried out at 50 V until when the dye front reached the separating gel and the voltage was increased to 100 V. After the run is complete the gel was taken out and washed with water. Then commissive blue staining was carried out. The staining solution consisted of 90 mL water, 90 mL methanol, 10 mL acetic acid and 0.25 g Commassive blue dye. While the destaining solution consisted of 90 mL water, 90 mL methanol, and 10 mL acetic acid. The gel was placed in 100 mL of staining solution for 30 minutes (for staining the protein in the gel) and removed; it was then placed in the destaining solution for destaining the gel overnight[14].

2.7 *In vitro* anticancer activity of L-asparaginase from *Amycolatopsis japonica*

This was done to test the activity of the crude and partially purified enzyme on three cell lines: 3T3 (Normal cell line), AUB5 (breast cancer cell line), and $CaCo_2$ (intestinal cancer cell line) (Colon cancer cell line). Cell lines were obtained from the Panjwani Centre for Molecular Medicine and Drug Research, ICCBS, Karachi, and were kept in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acid, and cultured at 37 degrees Celsius in

a 5% CO2 incubator. DMEM was replenished every 48-72 hours until the confluence reached 80 percent. The MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide test was used to determine the cytotoxicity of substances against the three cell lines. At a density of 10000 cells per well, 1×10^5 cell/mL cell suspension (100 L) was planted on a round bottom 96 well plate and incubated at 37 oC in a 5% CO_2 incubator. The cell has grown entirely and connected to the wells after 48 hours of incubation. The media was withdrawn, and different concentrations of pure L-asparaginase (100, 50, and 25 µL) were combined with DMEM medium (100, 150, and 175 µL) to make a total volume of 200 L solution that was dispensed in their respective wells and incubated for 24 hours at 37°C in a 5% CO_2 incubator. Positive growth control was provided by an untreated well. After a 24-hour incubation period, all wells were examined under a fluorescence microscope (Nikon Eclipse TS100 Inverted microscope), photos were obtained, and the entire solution was withdrawn from each well. 2 mL MTT dye (5 mg/mL) was diluted 10 times with new DMEM media to yield 0.5 mg/mL MTT dye, and 200 L of this solution was dispensed in each well and incubated for 2 hours at 37°C in a 5% CO_2 incubator. The MTT dye-containing media was withdrawn after incubation, and 100 L of dimethyl sulfoxide (DMSO) was added to all wells to solubilize the produced formazan crystals, and absorbance was measured in a spectrophotometer at 570 nm (Multiskan GO, Thermo Scientific). The enzyme's cytotoxicity (percent) was determined by comparing it to an untreated positive growth control. The concentration that displayed 50% cytotoxicity was calculated using a plot of percent cytotoxicity/inhibition vs sample concentration (IC50). All of the experiments were carried out in triplicate [15].

$$\% \text{ cytotoxicity/inhibition} = 100 - \frac{\text{O.D of treated well} - \text{O.D of media control}}{\text{O.D of untreated control} - \text{O.D of media control}} \text{ X } 100$$

III. RESULTS AND DISCUSION

3.1 Purification

The crude L-asparaginase was purified from A.japonica culture filtrate in three steps: ammonium sulfate precipitation, dialysis, and Sephadex G-50 gel filtration. The purifying procedure is depicted in (Table 1). The total L-asparaginase activity of the crude enzyme from A. japonica was 4593.12 U, while the total protein content was 399.97 mg, with a specific activity of 11.48 U/mg. The treatment with 40-80% ammonium sulphate yielded 2696.41 U with 90.71 mg total protein, 29.72 U/mg specific activities, 2.58 purification fold, and 58.7% recovery yield. Total activity of 2307.2 U, 33.63 mg protein, and specific activity of 68.52 U/mg, 5.96 purification fold, and total recovery of 50.20 percent were obtained from dialyzed 40 - 80 percent ammonium sulphate fractions. The elution profile of the chromatogram is shown in Figure 1, total activity of 1968.96 U, 26.69 mg protein content, specific activity of 73.75 U/mg, 6.42 fold purification, and recovery yield of 42.86 percent were achieved after further purification of 40 – 80 percent dialyzed fraction by gel filtration using Sephadex G-50. All fractions that formed a single peak and had high L-asparaginase activity were lyophilized.

Despite the fact that different researchers utilized nearly identical purification methods for different enzymes, the fold and yields of purification varied. This could be because different proteins in the fermentation medium have different interfaces [16].

Table 1: Summary of the purification steps of the L-asparaginase produced by Amycolatopsis japonica

Purification step	Total activity (U)	Total protein (mg)	Specific U/Mg	Purification fold	% yield
Crude extract	4593.12	399.97	11.48366	1	100
Ammonium sulfate	2696.41	90.71	29.7255	2.588504	58.70
Dialysis	2307.2	33.63	68.52	5.96	50.20
Sephadex G- 50	1968.96	26.696	73.75	6.42	42.86

This finding is consistent with those published by Sahu *et al*. [17], who purified enzyme from Actinomycetes strain L9 with 18 fold and 1.9 percent recovery and a specific activity of 13.57 U/mg. From isolated L-asparaginase from Streptomyces tendae, Kavitha and Vijayalaksh [18] found a specific activity of 51.7 U/mg with a purity of 17.23 fold and a recovery of 30.5 percent. According to Narayan *et al*. [19], L-asparaginase from Streptomyces albidoflavus had a purity of 99.3 fold and a final recovery of 20%. Lopez *et al*. [20] purified Streptomyces longsporusflavus (F-15) L-asparaginase up to 30.5-fold with 19.1 percent recovery. L-asparaginase from Streptomyces sp. PDK2 has an 83-fold purity and 2.18 percent recovery, according to Dhevagi and Poorani [21]. Dias *et al*. [22] also reported

28.6 fold purification from A. oryzae with a yield of 6% recovery. Kumar *et al*. [23] used sephadex G-100 column chromatography to obtain 42.02 percent yield 20.91 U/mg specific activity from L-asparaginase generated from *P. carotovorum*. In addition, as each purification stage progressed, the enzymes' particular activity rose. Amena *et al*. [24] Heinen *et al*. [25] and Moharib [26] all report a similar tendency.

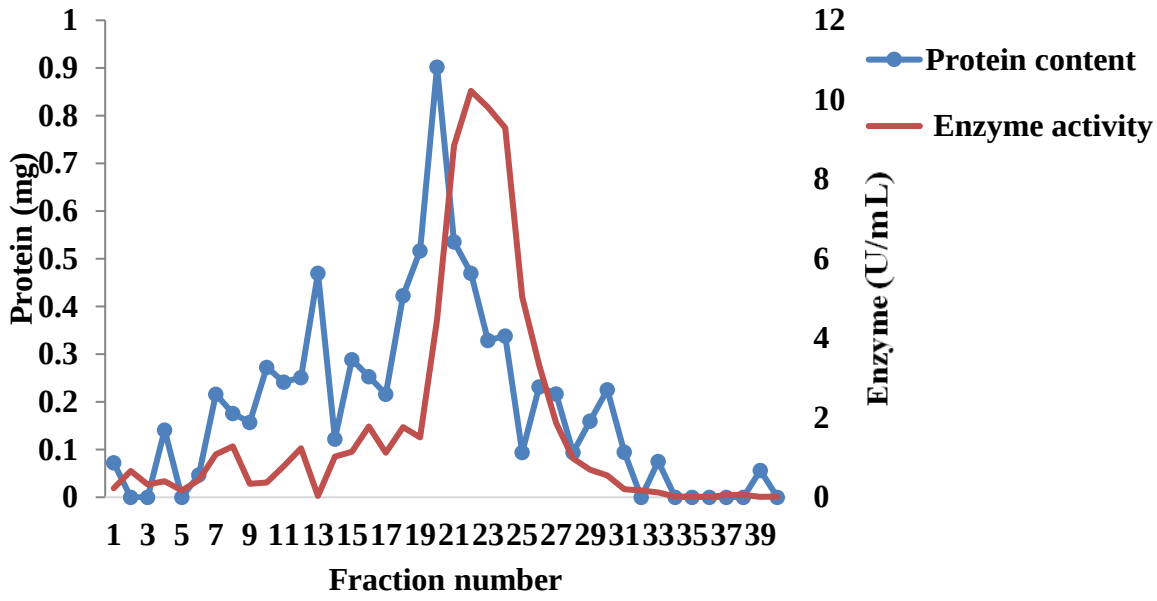

Fig: Enzyme activity and protein content elution profile of the chromatography separation of L-asparaginase from Amycolatopsis japonica on G-50 Sephadex column (40-80% ammonium sulphate dialyzed fraction)

3.2 Characterisation of L-asparaginase from *A. japonica*

3.2.1 Effect of pH on L-asparaginase activity and stability

The isolated A.japonica enzyme was active between pH 6 and 8, with the highest activity obtained at pH 8.0 (Figure 2). After 40 minutes of incubation at 37ºC, the enzyme demonstrated high stability from pH (6-9) and approximately preserved 50% of its activity in pH 8.0 (Figure 3). L-asparaginase is an amidase enzyme that has been found to be active and stable at both alkaline and neutral pH. At pH 8.0, the enyzme preserved 50% of its activity for 40 minutes. Because L-asparaginase stability at physiological pH has been reported as a determinant for anticancer efficacy, the enzymes could be a potential antitumor drug. Streptomyces sp. PDK7 L-asparaginase had the highest activity at pH 8-8.5, which was similar to this investigation [21]. At pH 7.0 and 8.0, Basha *et al*. [27] found that L-asparaginase activity from marine actinomycetes is at its peak.

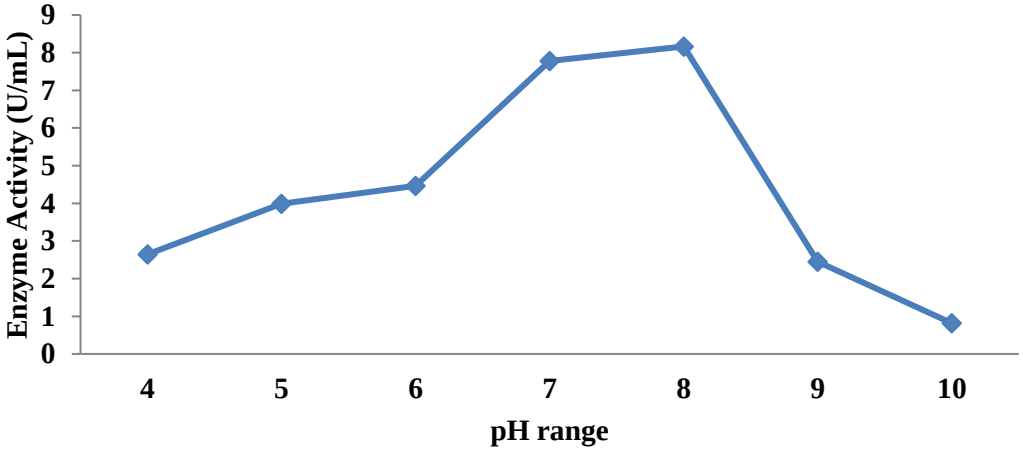

Fig: Effect of pH on activity of L-asparaginase from Amycolatopsis japonica

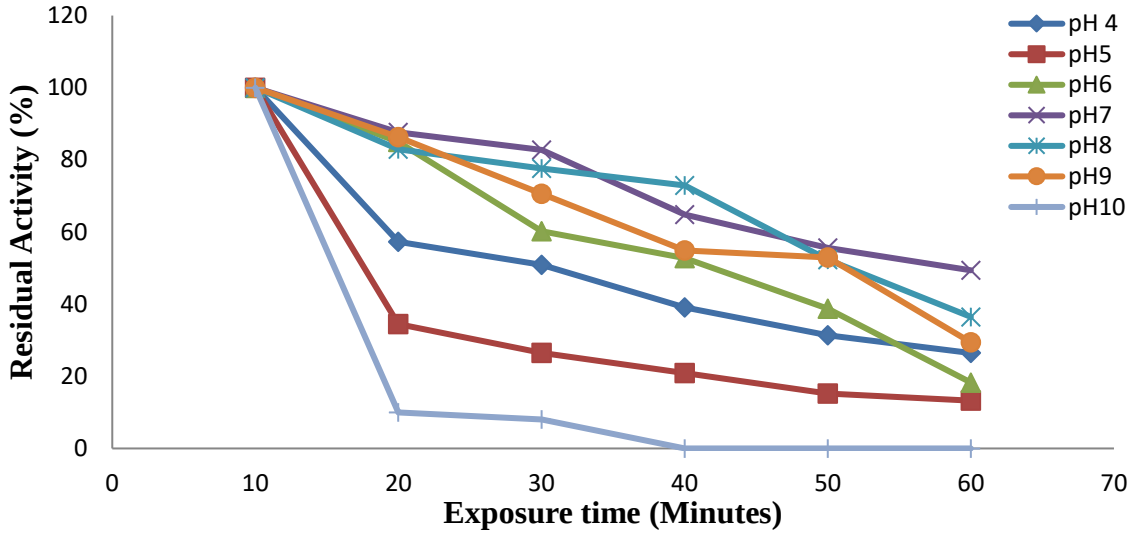

Fig: Effect of pH on stability of purified L–asparaginase from Amycolatopsis japonica

3.2.2 Effect of temperature on the activity and stability of purified L-asparaginase from *A. japonica.*

Temperature, inferentially, is one of the decisive elements that influence an enzyme's activity. Amycolatopsis japonica L-asparaginase showed good activity between 35°C and 55°C, with optimal activity at 45°C. After 40 minutes of incubation, it retained between 92 and 97 percent of its activity (Figure 4 and 5). Other studies have shown that the optimal temperature for L-asparaginase enzyme activity is between 35ºC and 45ºC. *Pseudomonas stutzeri* MB-405 and Erwinia sp. L-asparginase activity was found to be maximum at 37°C and 35°C, respectively, by Borkotaky and Bezbaruah, [28]. At 40°C, L-asparaginase from Streptomyces gulbargensis had the highest activity [29]. When L-asparaginase isolated from Streptomyces radiopugnans MS1 was pre-incubated at 40ºC for 60 minutes, Kumar *et al*. [23] found no significant loss of activity. Because of the properties of this enzyme, it can be fully removed from the body of a tumor patient who has received L-asparaginase in vivo. The decrease in enzyme activity with increasing temperature could be due to denaturation of the enzyme, which causes a change in the active site, resulting in inactivation of the enzyme at high temperatures.

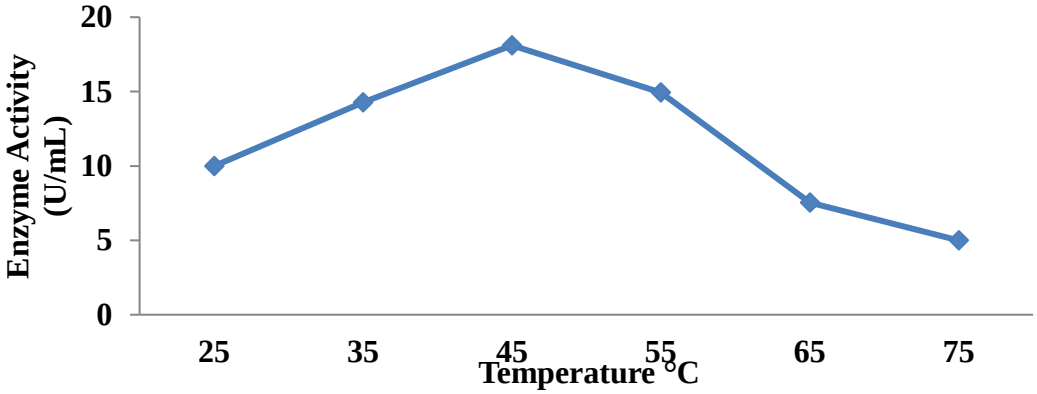

Fig.4: Effect of temperature on activity of L-asparaginase from Amycolatopsis japonica.

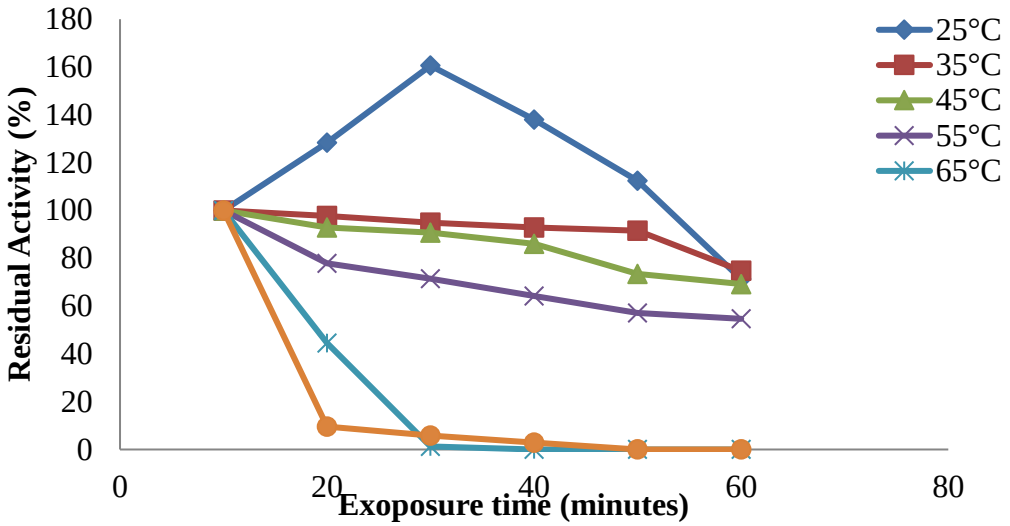

Fig.5: Effect of temperature on stability of purified L–asparaginase from Amycolatopsis japonica.

3.2.3 Effect of various metal ion on the activity and stability of purified L-asparaginase from *A. japonica.*

The effect of various metal ions on the activity of pure L-asparaginase was investigated. Mg^{2+} supported the highest activity of pure L-asparaginase from *Amycolatopsis japonica* among the metal ions examined, while Hg^{2+} supported the lowest activity of this enzyme (Figure 6). In the presence of Mg^{2+}, the activity of Amycolatopsis japonica enzyme was increased by 3%. In the presence of Hg^{2+}, the activity of *Amycolatopsis japonica* L-asparaginase was significantly reduced (Figure 7).

$HgCl_2$ was a strong inhibitor of Streptomyces brollosae NEAE-115 L–asparaginase, according to Noura *et al*. [11]. Kumar *et al*. [23] discovered that Hg^{2+} reduced nearly 80% of the activity of Pectobacterium carotovorum L-asparaginase. When Hg^{2+} and ZN^{2+} were present, Moharib [30] found that *V. Unguiculata* L-asparaginase activity was reduced. $MgCl_2$ enhances the activity of Vigna radiate purified L-asparaginase [31]. Mg^{2+} was found to be an activator of L-asparaginase in Pseudomonas aeruginosa strain SN004 in the presence of $HgCl_2$, according to Badoei [32].

In the presence of Hg^{2+}, there was enzyme inhibition which might be due to the essential vicinal sulfhydryl groups (SH group) of the enzyme for productive catalysis.

Mg^{2+} ions increase the enzyme activity suggests that these metals ion can serve as co-factor, which can help to activate the enzymatic reaction. Mg^{2+} was thought to be the activating metal; Mg^{2+} may activate the substrate, bound directly to the enzyme-substrate complex. Mg^{2+} locks the enzyme-substrate complex in place and then rapidly causes release of the reaction products [33]. This corresponds to fast dissociation rates for the enzyme-product complex rendering more favorable substrate binding sites. Metal ions play a crucial role in maintaining the active configuration of the enzymes at elevated temperatures by protecting them against thermal denaturation [34].

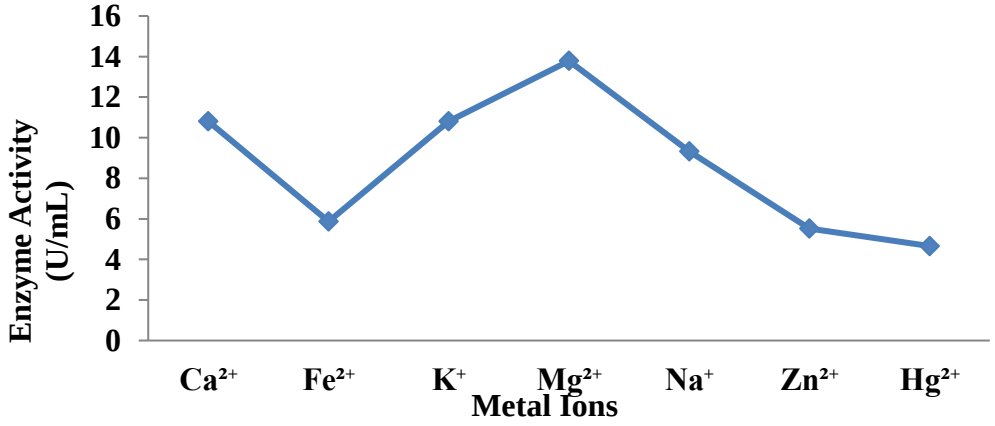

Fig: Effect of metal ions on activity of L-asparaginase from Amycolatopsis japonica

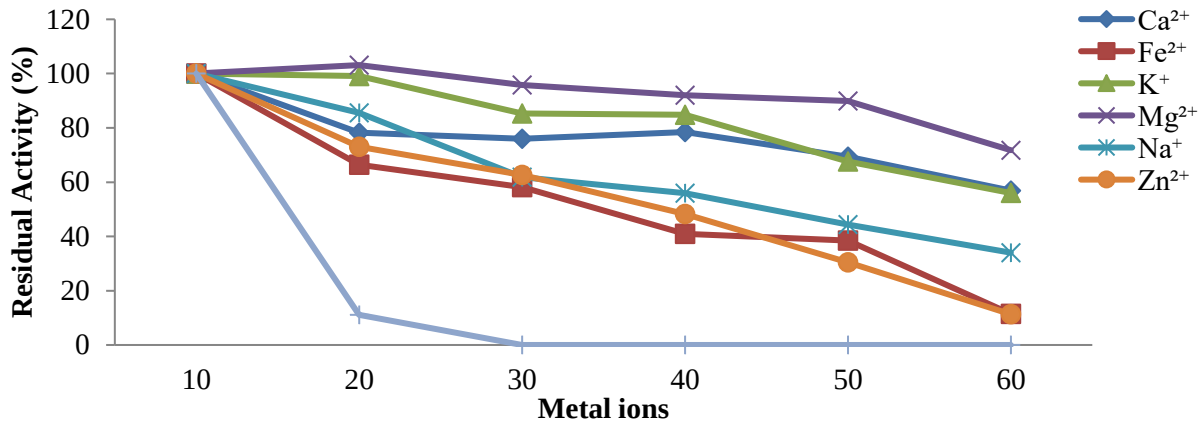

Fig.7: Effect of metal ions on stability of purified L–asparaginase from Amycolatopsis japonica

3.2.4 Effect of Inhibitors and Inducers on the activity and stability of purified L-asparaginase from *A. japonica.*

EDTA acted as inducers for the purified L-asparaginase from *Amycolatopsis japonica* with maximum activity of 18.73 U/mL, the lowest activity was found in the presence of ascorbic acid with enzyme activity of 4.89 U/mL. The enzyme showed good stability within 60 minutes of incubation and retained up to 60% of its activity in the presence of EDTA. This result obtained is in line with the work of Dias *et al.* [22] who reported slight decrease of around 30% of L–asparaginase from *Aspergillus oryzae* in the presence of EDTA. Noura *et al*. [11] recorded that Tween 80 acted as activators for L-asparaginase activity from *Streptomyces brollosae* NEAE-115 and reported EDTA to be an inhibitor of L–asparaginase from *Streptomyces brollosae* NEAE-115 as slight decrease of about 37.55% in the activity of the enzyme was observed. On the contrary, Moorthy *et al.* [35] and Jayachandra, [31] recorded inhibition of L-asparaginase activity from *Bacillus* sp. and *Vigna radiate* respectively by EDTA.

The fact that the activity of the enzyme was inhibited in the presence of some metal ion and was not inhibited in the presence of EDTA shows that the enzyme was not a metalloprotein. Direct and quick contact of enzyme with substrate sites seems to be increase by biosurfactant, and this might be why Tween 80 supported the activity of the enzyme [36]. Tween 80 enhanced substrate binding capacity and stability of enzymes under *in vitro* conditions [37]

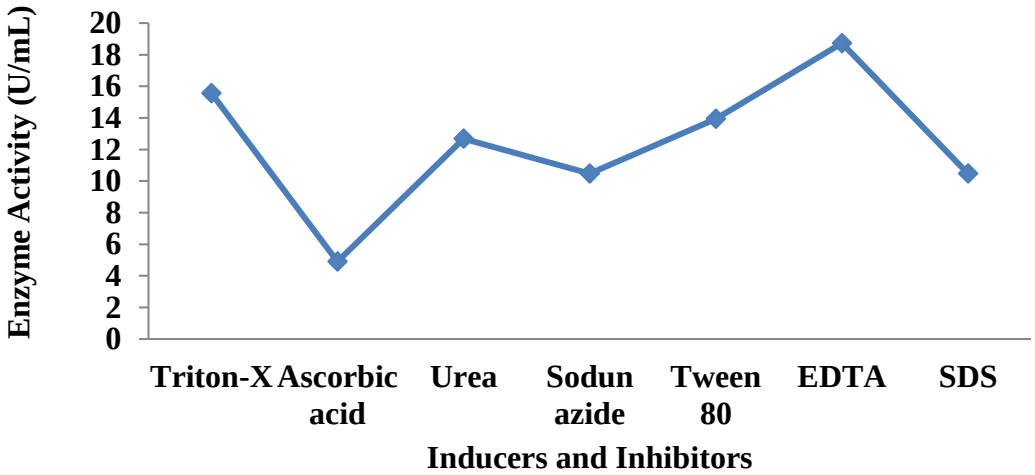

Fig.8: Effect of Inhibitors and Inducers on activity of L-asparaginase from Amycolatopsis japonica

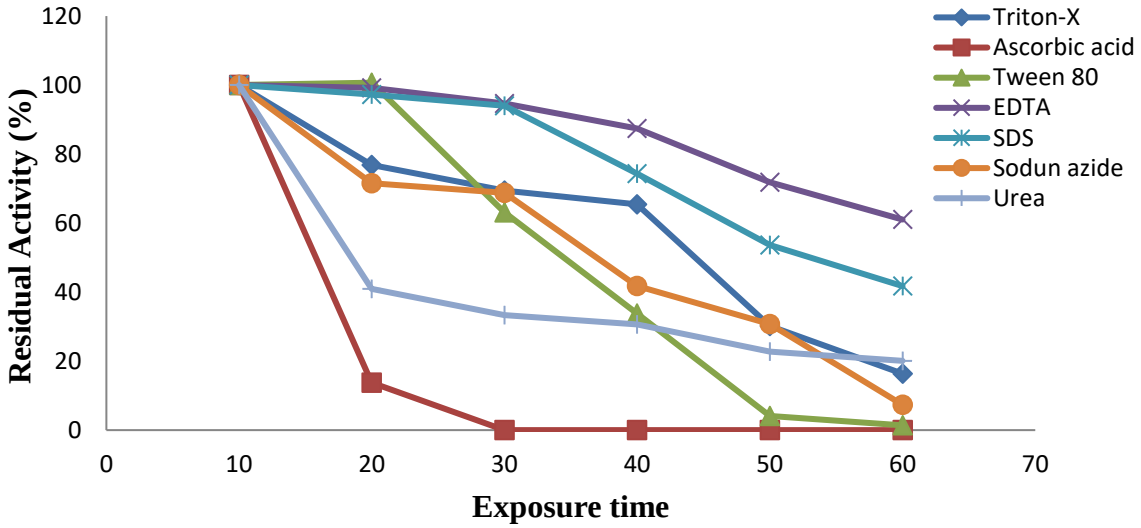

Fig.9: Effect of inhibitors and inducers on stability of purified L–asparaginase from Amycolatopsis japonica

3.2.5 Effect of amino acids on the activity and stability of purified L-asparaginase from *A. japonica.*

The purified L-asparaginase from *Amycolatopsis japonica* showed highest activity when L-asparagine was present with lowest activity in the presence of L-glutamine, and retained 60% of its activity in the presence of L-asparagine after 50 minutes of incubation (Figure 10 and 11)

The enzyme production is the complex chain reactions and is supported and induced by suitable substrates [38]. One of the properties of enzymes that make them useful as diagnostic tools is their specificity towards their substrate. The enzyme showed high specificity towards its natural substrate L-asparagine, very low specificity towards L-aspartic acid, while no activity towards L-glutamine.

This is in line with [38] who reported that *Penicillium* sp. preferred L–asparagine as substrate and contrary to Dunlope and Roon [39] who noted the increment in L-asparaginase production from *Penicillium sp* due to the addition of L glutamine or glutamate in the fermentation medium.

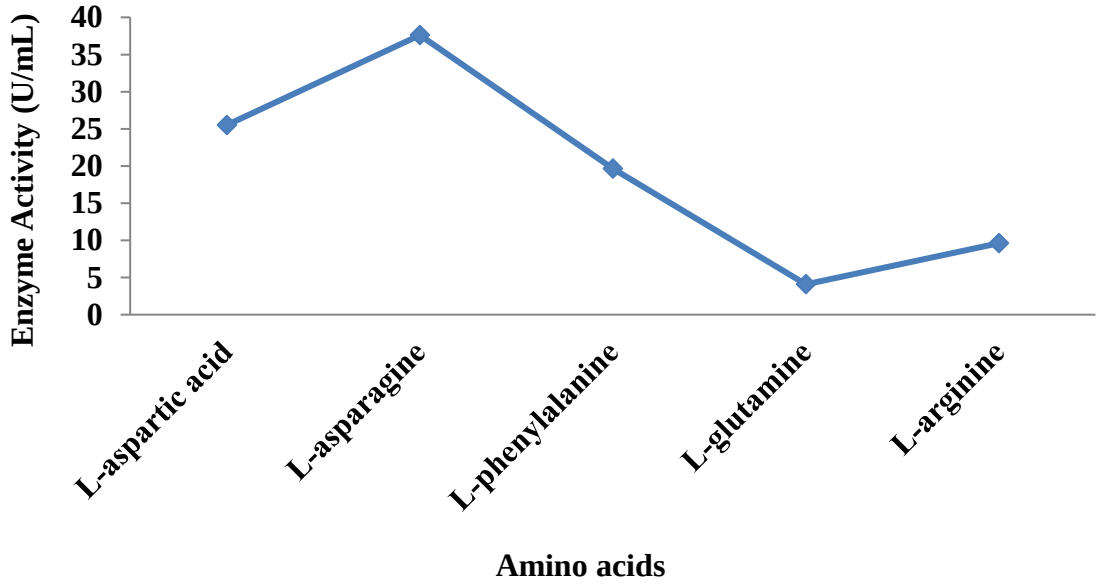

Fig.10: Effect of amino acids on activity of L-asparaginase from Amycolaptosis japonica from rhizospheric soil.

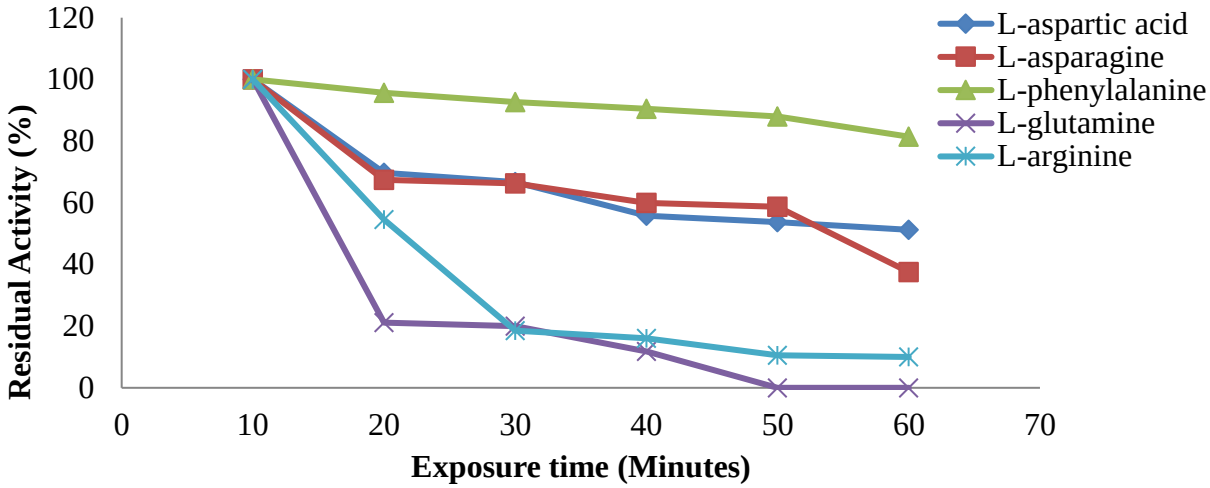

Fig.11: Effect of amino acids on stability of purified L–asparaginase from Amycolatopsis japonica

3.2.6 Effect of Substrate concentrations on the activity and stability of purified L-asparaginase from *A. japonica.*

L-asparaginase of *Amycolatopsis japonica* had Vmax value of 0.13 mM and Km value of 0.43 U/mL (Figure 12 and 13). Kotzia and Labrou, (2007) reported that *Erwinia chrysanthemi* L-asparginase had K_m value of (0.058 mM). K_m and V_{max} of purified L-asparaginase from *F. culmorum* ASP-87 were reported to be 3.1 mM and 0.77 µmol/ ml/min respectively. Km value of *P. brevicompactum* L-asparaginase was reported by Elshafei *et al.* ([41] to be 1.05 mM. Dias *et al.* [22] reported that L-asparaginase from *A. oryzae* CCT 3940 demonstrated high affinity for the substrate L-asparagine with Km and Vmax values estimated in 0.66 mol/L and 313 U/mL, respectively. Km value of 7.14 mM was recorded for L-asparaginase from *Erwinia carotovora* by Kamble *et al.* [42]. Higher Km values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported [43]. However, slightly higher Km values of 12.5 mM were reported in *Aspergillus aculeatus* [44].

Summarily, from the above values, there exist great variations in the kinetic parameters of L-asparaginase from different sources. However, substrate concentration is another environmental factor that affects enzymes activity. The rate of an enzyme-catalysed reaction increases with increase in substrates concentration. Therefore the maximum velocity (Vmax) and Michaelis Constant (Km) of enzymes are usually determine in order to know the best substrates loading for enzymes meant to be applied. Vmax refers to the rate at which an enzyme converts its substrates to products in the presence of excess substrates, while Km refers to the concentration of substrates at which an enzyme acts at half its maximum velocity. Km is also a measure of the apparent substrates affinity of an enzyme.

From this study, the enzyme has high affinity to it natural substrate, which can be reasons for its degree of inhibition against cancer cell lines. Raha *et al.* [45] noted that the effectiveness of an L- asparaginase enzyme against tumor is dependent on its affinity to its substrate.

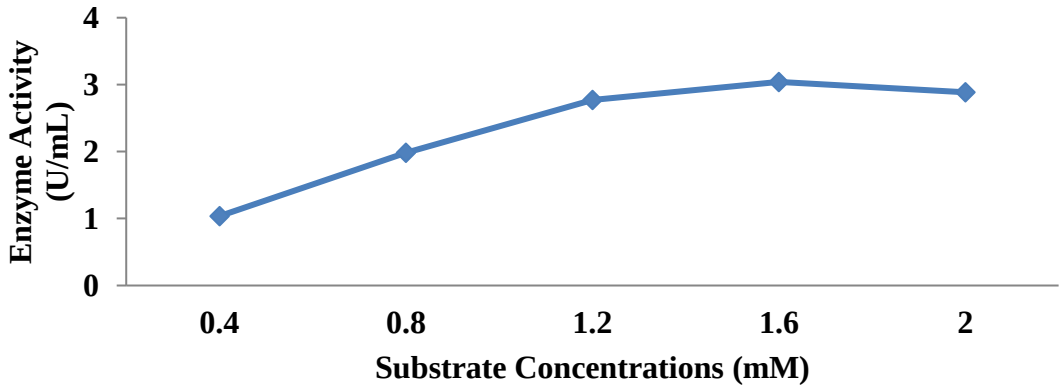

Fig.12: Effect of substrate concentration on activity of L – asparaginase from Amycolatopsis japonica

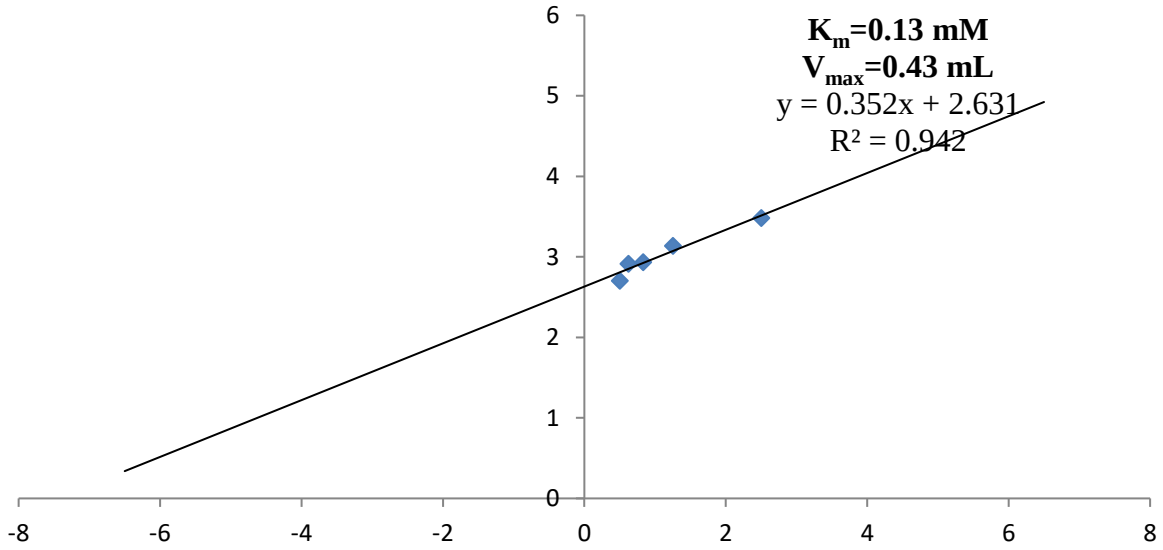

Fig.13: Line weaver - burk plot for the reaction kinetics of L-asparaginase from Amycolatopsis japonica

Cytotoxicity

3.3 In vitro anticancer activity of L-asparaginase produced.

L-asparaginase from *Amycolatopsis japonica* showed more inhibitory activity against colon cancer cell line (Caco-2) and breast cancer cell line (AU5) but less on normal rat fibroblast (3T3) cell line (Figure 14). The enzyme exhibited more effectiveness on growth inhibition on colon cancer cell line but less on breast cancer cell lines. The incubation of colon cancer cell line with gradual doses of L-asparaginase from this organisms led to a gradual inhibition in the cell growth with IC_{50} values of 36µL (Figure15)

Moharib, [30] recorded that, L-asparaginase from *Vigna unguiculata* seed have more anticancer effect against liver (HEPG2) and colon (HCT-116) but lower effective against cervical (HELA) and Breast (MCF7) cancer cell lines. Studied the *in vitro* cytotoxicity of *Bacillus* sp R36 L-asparaginase against different cell lines and reported that, the enzyme have more cytotoxic effect on liver cancer cell line (Hep G2) than colon cancer cell line (HCT- 116) [46]. Dias *et al*., 2016 studied cytotoxic activity of L-asparaginase produced from purified L-asparaginase from *A. oryzae* CCT 3940 on broad range of human tumor cell lines (786-0 (kidney), NCI-H40 (lung, non-small cell), PC-3 (prostate),Type, U251 (glioma), UACC-62 (melanoma), HT29 (colon) and K562 (leukemia) at different concentration and reported that the enzyme completely inhibited the cell proliferation of these cell lines and did not inhibit the non-carcinogenic human cell line growth at the concentrations studied.

The antineoplastic activity of the L-asparaginase produced by the isolated bacterial was performed based on the fact that lymphatic cells demand huge quantities of L-asparagine in order to have rapid malignant growth as these tumor cells lack or have very low expression levels of L-asparagine synthetase and depend on the extracellular pool of this amino acid unlike normal cells. The presence of an external L-asparaginase enzyme in the growth medium causes depletion of asparagine due to the catalysis of the supplemented enzyme and kills tumor cells by depriving them of an essential factor required for protein synthesis [47].

Tumor cells are destroyed by L-asparaginase without significant damage to normal cells. This explains less inhibitory activity of this enzyme on 3T3 rat fibroblast cell because cancer cells are L-asparagine dependent, an amino acid essential for lymphoblasts growth. Non-cancerous cell has the ability to manufacture L-asparagine and cannot be affected by L-asparaginase treatment, because they contain L-asparagine synthase, cancerous cells do not have L-asparaginase synthase, and so they cannot produce L-asparaginase on their own, thereby affected when treated with L-asparaginase [40].

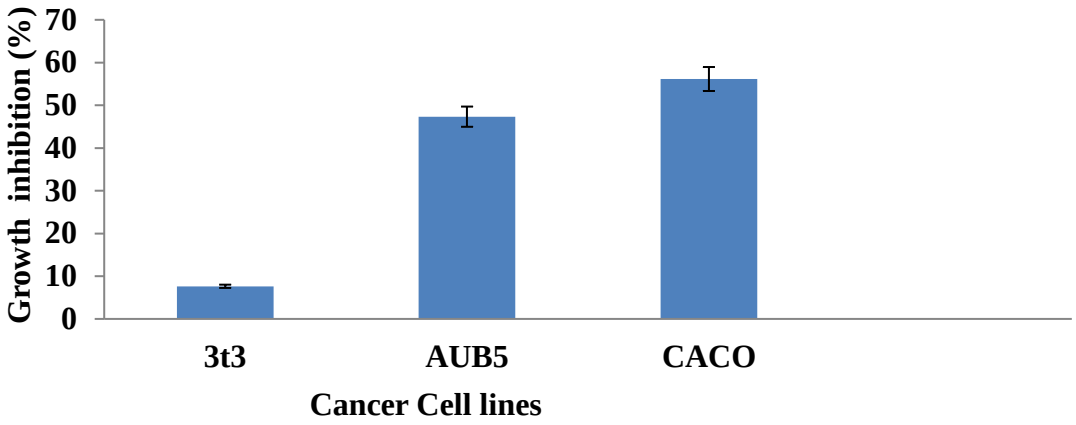

Fig.14: Cytotoxicity effect of L-asparaginase from Amycolatopsis japonica on three cell lines

Keys:

3t3: Rat fibroblast cell line

AUB5: Breast Cancer cell line

CACO2: Colon cancer cell line

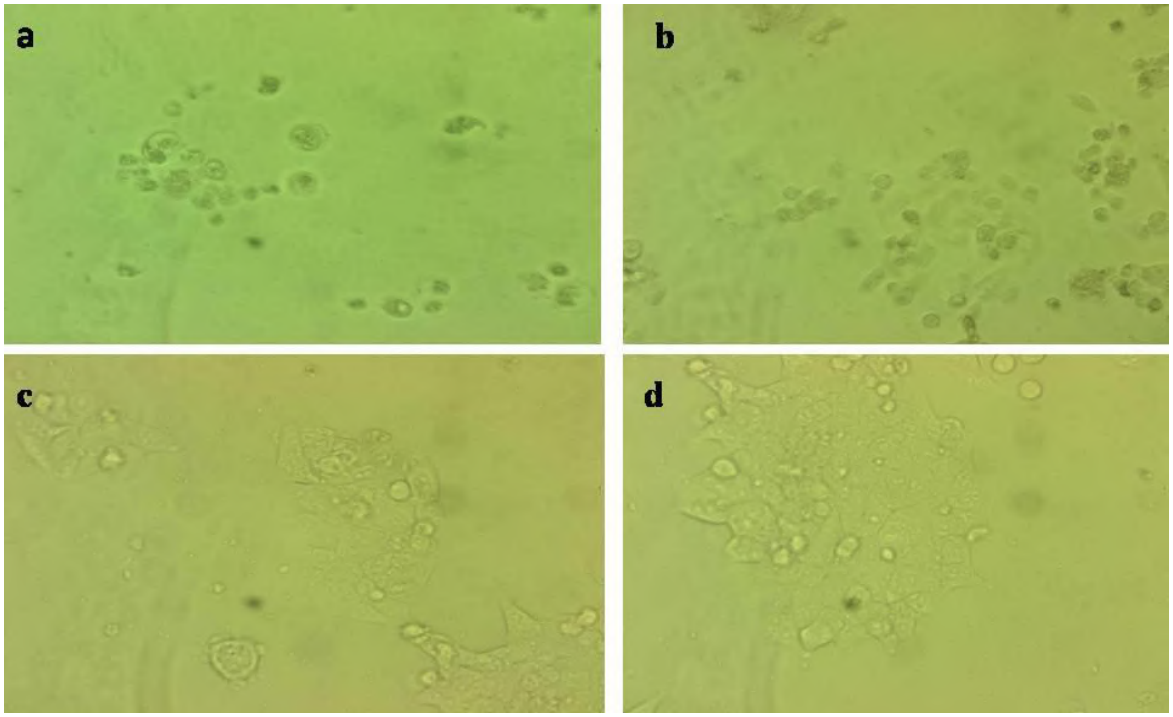

Plate 1: Microscopic picture of anticancer activity of L-asparaginase from Amycolatopsis japonica on colon cancer cell line at different concentrations.

Keys**:** **(**a) 100 µL; 83% dead cells, **(**b)50 µL; 78% dead cells with few live ones, **(**c) 25 µL; Live cells with 26% dead cells, (d) Control; Lives cells

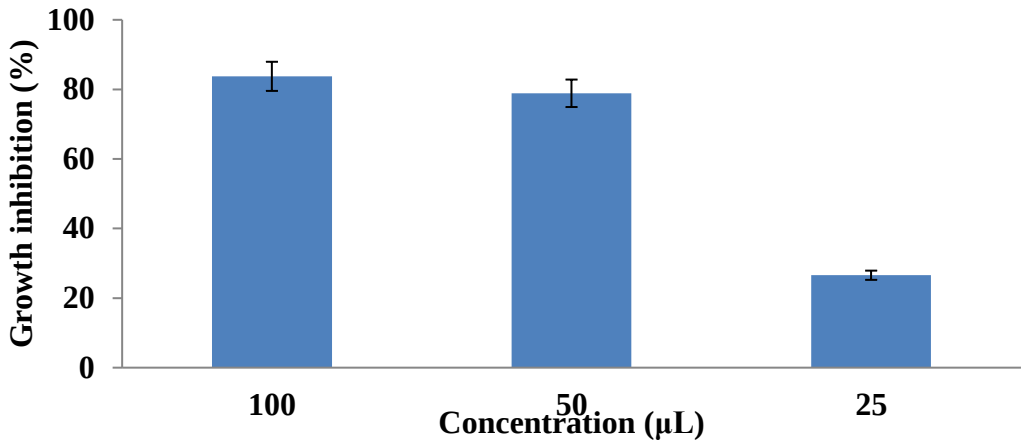

Fig.15: Cytotoxicity effect of different concentration of L-asparaginase from Amycolatopsis japonica on Colon cancer cell line

IV. CONCLUSION

From this result, it could be concluded that L-asparaginase from *Amycolatopsis japonica* exhibited anticancer potential and could be used as drug to complement the ones currently in use. To the best of my knowledge, this is the first report on anticancer activity of L-asparaginase from *Amycolatopsis japonica.*

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