

Purification and Characterization of Produced *L-Asparaginase* from *Streptomyces Avermitilis* Isolated from Marine Environment in Lagos, Nigeria

Courage G. Dimowo and Solomon E. Omonigho

Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

*Correspondence: Courage G. Dimowo. Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. Email: courage.dimowo@uniben.edu

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ABSTRACT

Asparaginase is an enzyme that is used as a medication and in food manufacturing. In medicine, it is used in the treatment of acute lymphoblastic leukemia, acute myeloid leukemia and non-Hodgkin's lymphoma, administered through the vein, muscle or under the skin. This study carried out purification and characterization of produced *L-asparaginase* from *Streptomyces avermitilis* isolated from marine environment. Samples were collected from Atlantic ocean Lagos, Nigeria and production of *L-asparaginase* was carried out in Erlenmeyer flask containing Czapek Dox broth supplemented with *L-asparagine* (1 g/L), as production medium for 3 days at 120 rpm and at 28 °C. A 5 % inoculum was used. Crude extract of produced *L-asparaginase* was obtained by centrifuging at 10,000 rpm for 5 min using cold centrifuge. Precipitation of crude extract was carried out using 60 % ammonium sulphate. Dialysis, ion exchange chromatography and gel filtration were used to carry out the complete purification process. Effect of substrate concentration, pH, temperature, incubation time and metal ions were studied to characterize the enzyme. Results revealed maximum velocity of purified *L-asparaginase* as 51.136 μmol/min/ml and Km value was 3.812 μmol. The highest enzyme activity was recorded at pH 6.0 and at a temperature of 40 °C. The purified enzyme had half - life of 50 minutes at 60 °C and was inhibited by urea. Metals such as Mn²⁺, Co²⁺, and Hg⁺, had significant increase on purified *L-asparaginase* activity. In conclusion, the purified enzyme showed viability under various conditions in the study carried out.

Keyword: Leukemia, Precipitation, Dialysis, Chromatography, Maximum velocity and viability

INTRODUCTION

Asparaginase is an enzyme that is used as a medication and in food manufacturing. In medicine, it is used in the treatment of acute lymphoblastic leukemia, acute myeloid leukemia and non-Hodgkin's lymphoma. It could be

administered through the intravenously, intramuscularly or subcutaneously. This enzyme acts by breaking down the amino acid known as asparagine without which, cancer cells cannot make DNA. In food manufacturing, it is used to

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decrease the acrylamide content of pastry food^[1]

In food industry, asparaginase is generally regarded as “safe” and it is marketed as Acryl away and PreventASE. Asparaginase is used as a Food processing aid to reduce the formation of acrylamide, which is known as a suspected carcinogen in food rich in carbohydrates such as snacks and biscuits^[1]

In 1978, this enzyme gained approval for medical use in the United States. It is on the WHO'S list of essential medicines. Common side effects of asparaginase when used by injection are allergic reaction, blood clotting problems, high blood sugar, Kidney problems, pancreatitis and liver dysfunction. Also, when used in pregnancy could cause harm to fetus^[2]

The enzyme, asparaginase is often produced from microbes including *Escherichia coli* or *Erwinia chrysanthemi*. However, in this study we purified and characterize the produced L- Asparaginase from *Streptomyces avermitilis* isolated from Atlantic Ocean in Lagos.

METHODOLOGY

Collection of samples

Marine sediments, soil and water samples were collected from 10 m deep from Lagos lagoon and Atlantic Ocean. Specifically soil sample was collected from the sea shore and samples were collected using sterile universal containers.

Isolation and Identification of *Streptomyces avermitilis*

Isolation of *Streptomyces avermitilis* was carried out by Pour Plate method, and pure culture was obtained by streaking as described by Senthil^[3]. Identification was carried out according to Bergy's (2000) manual of determinative bacteriology and by using Actinomycetes Atlas^[4]

Production of L – asparaginase

Production of L -asparaginase was carried out according to the method described by El – Naggar^[5] with *Streptomyces avermitilis* in Erlenmeyer flask containing Czapek Dox broth supplemented

with L-asparagine (1g/L), as production medium for 7 days at 120 rpm and at 28 °C. A 5 % inoculum was used containing 2.5×10^5 cells/ml during production; about 5 ml of the broth was withdrawn on day 0 and on day 3 to determine the L-asparaginase activity by Nesslerization^[6]. L-asparaginase Activity was calculated with the fomular below^[6];

Activity of L-asparaginase =
Absorbance / slope of graph x Time of incubation x
Volume of protein x DF

Purification of L – Asparaginase: Ammonium sulphate precipitation.

Crude extract of produced L- asparaginase was obtained by centrifuging at 10,000 rpm for 5 min using cold centrifuge (Labnet prism)TM the supernatants were collected in a clean sterile beaker and precipitated using 60 % ammonium sulphate. Thereafter, the precipitated solution was centrifuged again to get the pallets. The pallets were collected in a sterile container and mixed with buffer (Tris Hcl, 8.0) and then stored at 4 °C for further studies^[3]

Dialysis/Ion Exchange Chromatography.

Low molecular weight dialysis bag was used for dialysis. It was prepared as described by Mukherjee^[7] by boiling it for about 15 mins. The crude extract (pallets) were concentrated by dialysis against 20 mM tris HCL. Concentrated crude extract was applied onto a DEAE (Diethylaminoethyl) Sepharose column which was previously equilibrated with 20 mM Tris –Hcl buffer pH 8.0 for ion exchange chromatography. The enzyme was eluted by different concentrations of sodium chloride (0.1, 0.2, 0.3, 0.4, 0.5, 0.61.0 g) in the same buffer at a flow rate of 60 ml/hr and 5ml fractions were collected. The protein concentration was checked using ultra violet spectrophotometer (model UH4150AD UV Vis-NIR) at 280 nm and two peaks of proteins were obtained.

Gel filtration

Gel filtration was carried out according to the method of Balasubramanian^[8]. *L* asparaginase with the highest activity was poured onto a sephacryl S-200 column (90 x 0.6 cm) which was previously equilibrated with the same buffer at a flow rate 60 ml/hr and 3 ml fractions were collected. The protein concentrations were evaluated using ultraviolet spectrophotometer (model UH4150AD UV Vis-NIR) at 280 nm and enzyme assay on each of the fractions containing proteins was carried out.

Characterization of Purified *L*–asparaginase.

Characterization was carried out according to the method of Andrew^[9]. The purified *L*-asparaginase was characterized by determining the Michaelis-menten's kinetics (*K_m*) and maximum velocity (*V_{max}*), effect of pH and pH stability, effect of temperature, effect of incubation period, effect of metal ions and inhibitor.

Effect of Substrate concentration on *L* – asparaginase Activity.

Effect of substrate concentration on *L* –asparaginase activity was carried out according to the method of Basha^[10].

The value of *K_m* and maximum velocity (*V_{max}*) was determined by using *L*-asparagine as substrate in the range of 0 -20 mM. Kinetic parameters were determined using direct plot.

Effects of pH and pH stability

The optimum pH for the asparaginase activity was determined by assaying the activity at different pH values. The pH stability was tested by incubation of the enzyme at different pH values (3, 4, 5, 6, 7, 8, 9 and 10). The pH stability was tested by incubation of the enzyme at pH 5 - 9 for 24 hrs at 4 °C in the absence of substrate and the residual activity was determined under the standard assay condition.

Effects of Temperature

The optimum temperature for asparaginase activity was determined according to the method of Basha

^[10] by assaying the enzyme at different temperatures (0 to 100 °C). Heat stability was measured according to the method of Basha^[10] by incubating the enzyme alone at different temperatures for 1 hr. After heat treatment, the enzyme solution was cooled and the residual activity was assayed after adding the substrate *L*- asparagine

Effects of incubation period

The optimum incubation period for asparaginase activity was determined according to the method of Basha^[10] by assaying the enzyme at different incubation time (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mins) at 60 °C. This was carried out to determine the half - life of the purified *L*-asparaginase.

Effects of metal ions and inhibitor.

The effects of various metal ions on enzyme activity were determined according to the methods of Basha^[10] by pre incubating the enzyme alone with 10 mm metal ion for 15 mins prior to adding the substrate *L*-asparagine. The activity which was assayed in the absence of metal ion was taken as 100 % (control).

Data Analysis

All analyses were carried out using SPSS 16.0. Data were presented as Mean ± S.d (Standard Error of Mean). The differences between and within groups were tested using ANOVA Posthoc analysis. A probability of less than or equal to 0.05 were considered as significant.

RESULTS

Figure 1 explains Chromatogram of ion exchange in *L*-asparaginase purification. Highest protein concentrations were obtained from sample fractions collected (50- 100), also significant enzyme activity were from sample fractions collected labeled (50-100).

Figure 2 explains Chromatogram of Gel filtration in *L*-asparaginase purification. Significant protein concentrations correlated with fractions with highest *L*- asparaginase activity.

Figure 3a explains the effect of substrate concentration on *L*-asparaginase activity with maximum velocity at 51.136 $\mu\text{mol}/\text{min}/\text{ml}$ and K_m value of 3.812 μmol .

3b. explains the effects of pH on purified enzyme activity. The highest enzyme activity was at pH 6.0 having a value of 92.27 $\mu\text{mol}/\text{min}/\text{ml}$ of *L*-asparaginase activity and the lowest value at pH 3.0 having an activity value of 66.99 $\mu\text{mol}/\text{min}/\text{ml}$.

3c. explains the effects of pH stability on purified *L*- asparaginase activity. The purified enzyme activity was fairly stable at pH 5.0 -8.0 and its

stability reduced from 74.87 to 56.066 $\mu\text{mol}/\text{min}/\text{ml}$ at pH 9.

3d. explains the effects of temperature on purified *L*-asparaginase activity. Maximum activity of 34.1515 $\mu\text{mol}/\text{min}/\text{ml}$ was at 40 °C.

3e. explains the effect of incubation time on *L*-asparaginase activity with an initial concentration of 80 $\mu\text{mol}/\text{min}/\text{ml}$ and a half-life value of 50 mins.

3f. explains the effect of metal ions and urea on *L*-asparaginase activity. Maganese, copper and cobalt had the highest positive effect on *L* - asparaginase activity.

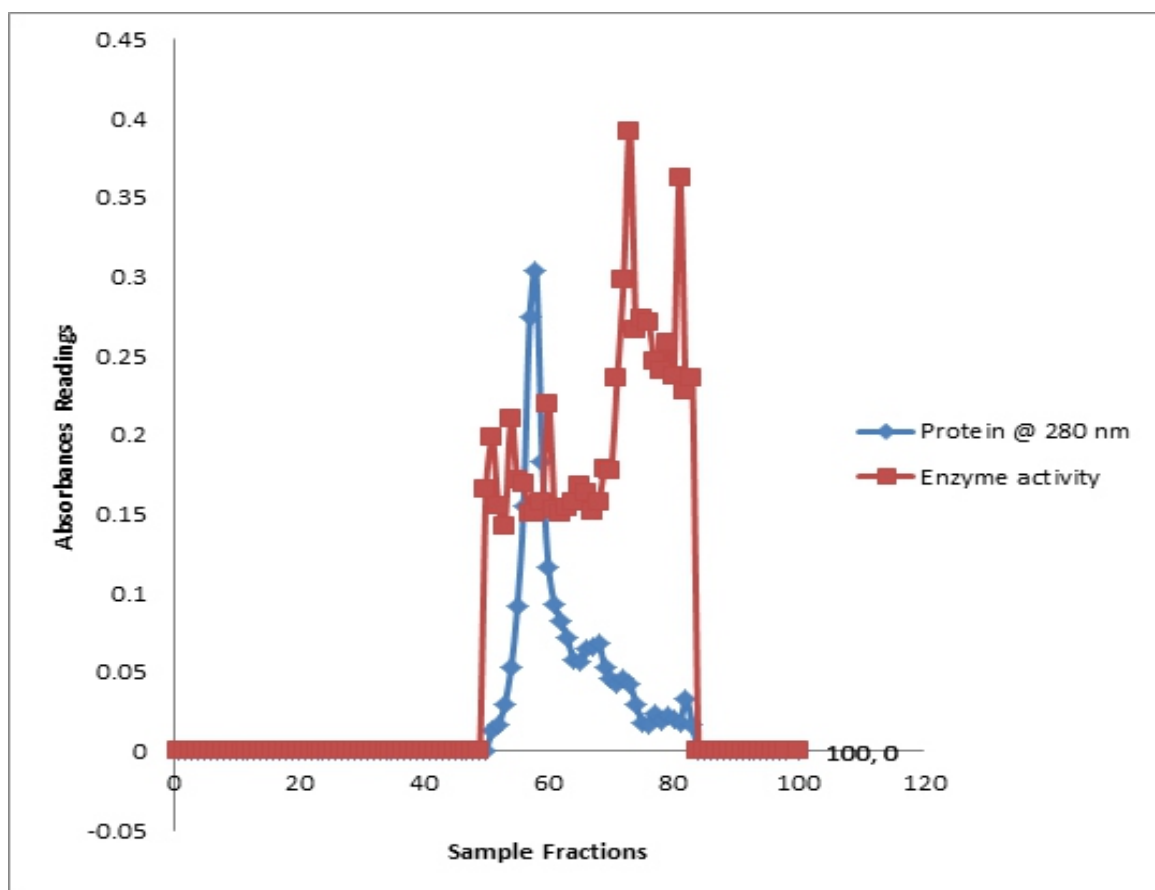


Figure 1: Chromatogram of ion exchange in *L*-asparaginase purification.

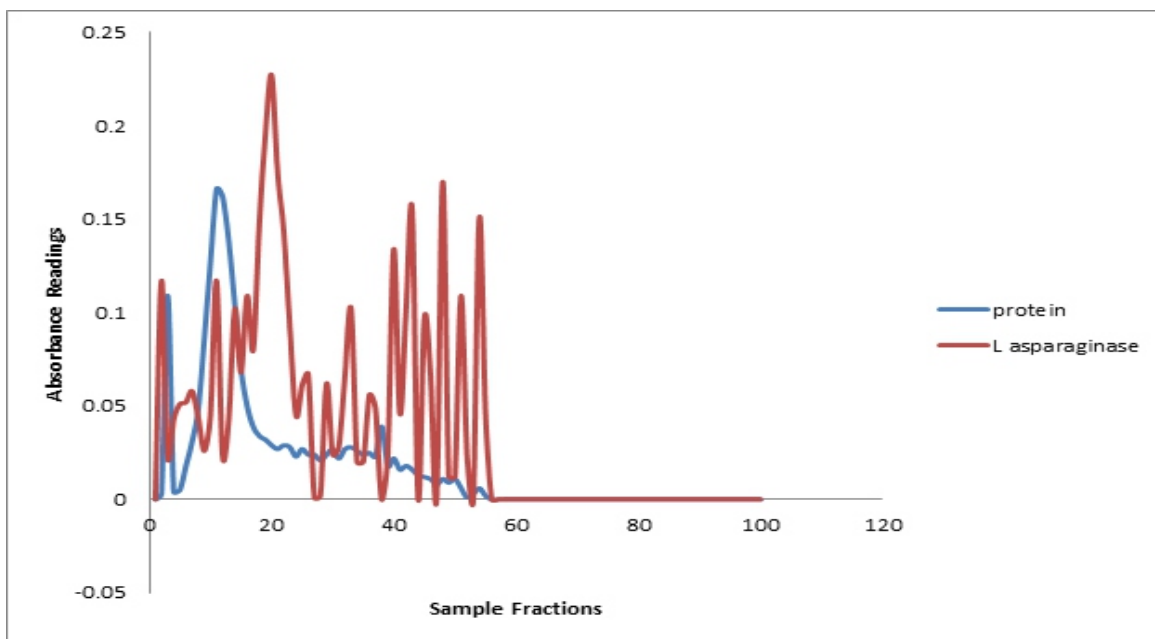


Figure 2: Chromatogram of Gel filtration in *L*-asparaginase purification. Significant protein concentrations correlated with fractions with highest *L*- asparaginase activity.

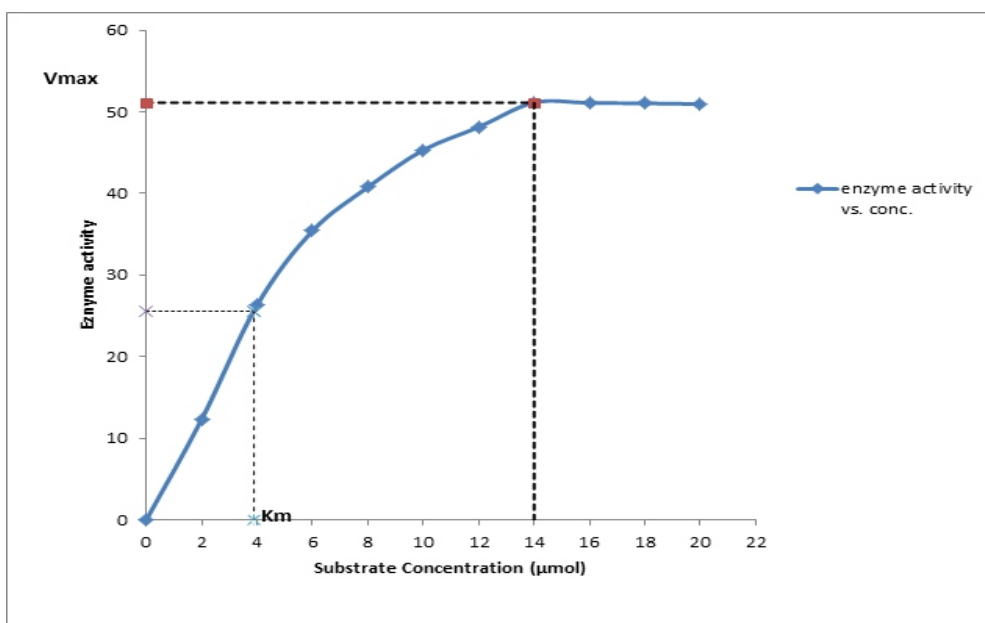


Fig. 3a: Effect of substrate concentration on *L*- asparaginase activity

Note:

Vmax ----- maximum velocity

Km ----- Michaelis – menten's kinetics

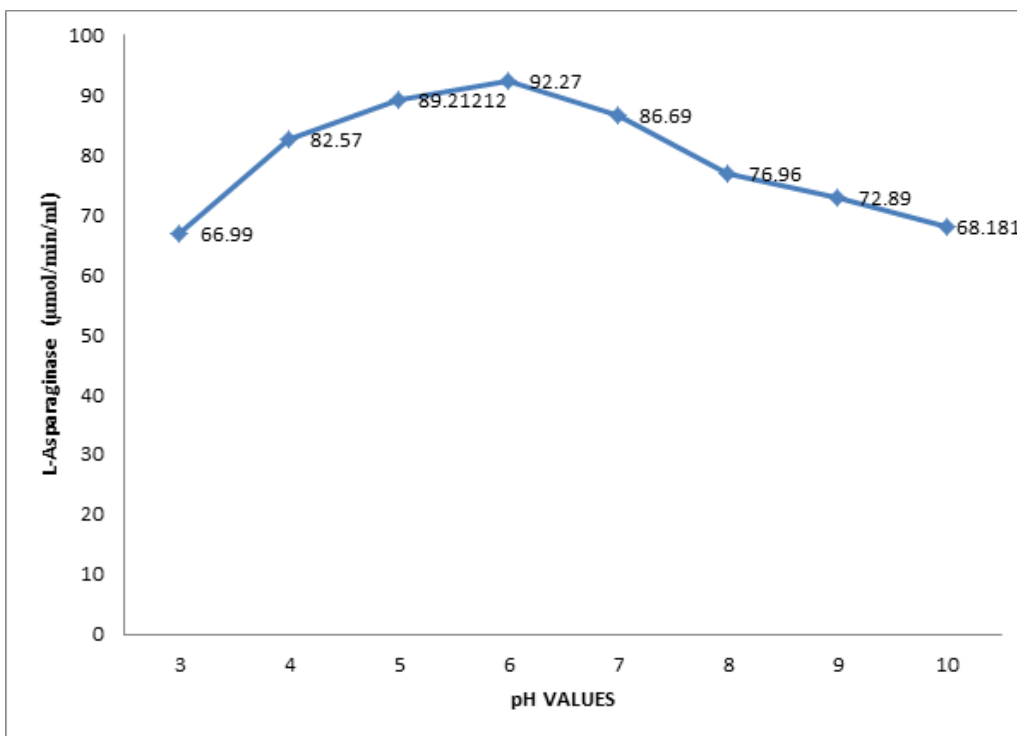


Fig. 3b: Effect of pH concentration on L-asparaginase activity

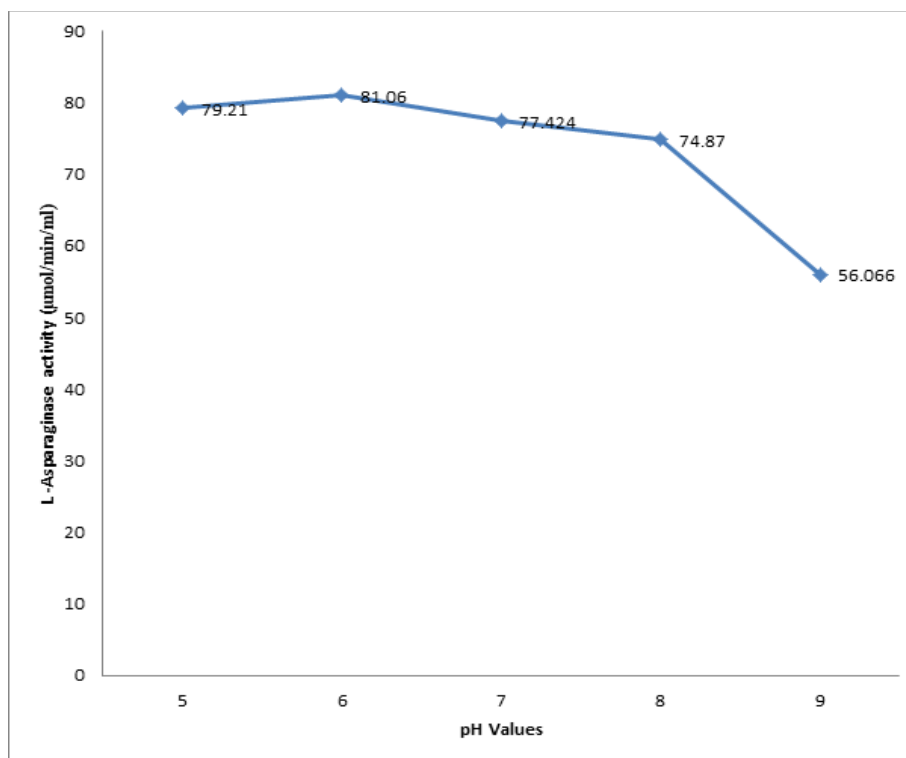


Fig. 3c: Effect of pH stability on L-asparaginase activity

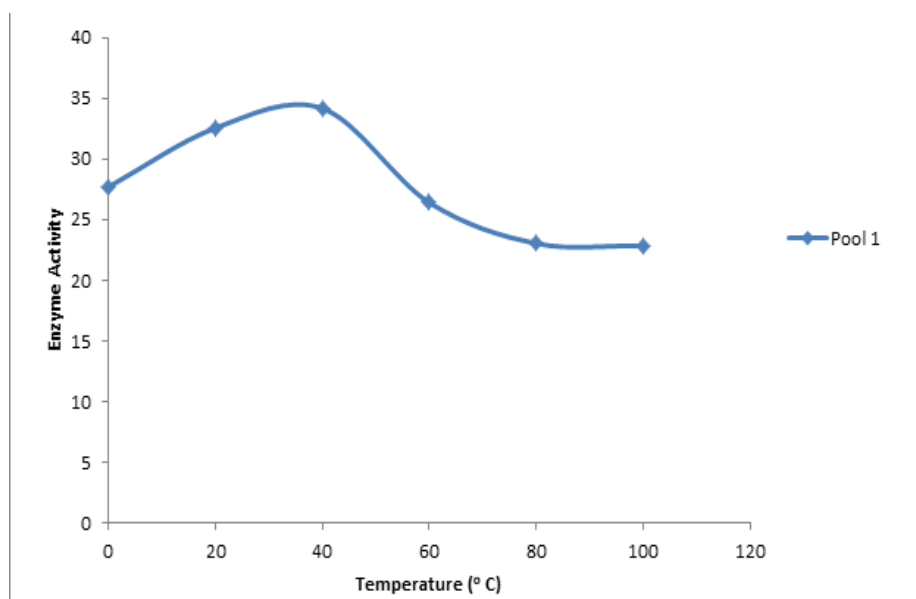


Fig 3d: Effect of temperature on *L*-asparaginase activity.

Note; Figure 3a explains the effect of substrate concentration on *L*-asparaginase activity with maximum velocity at 51.136 $\mu\text{mol}/\text{min}/\text{ml}$ and K_m value of 3.812 μmol .

3b. explains the effects of pH on purified enzyme activity. The highest enzyme activity was at pH 6.0 having a value of 92.27 $\mu\text{mol}/\text{min}/\text{ml}$ of *L*-asparaginase activity and the lowest value at pH 3.0 having an activity value of 66.99 $\mu\text{mol}/\text{min}/\text{ml}$.

3c. explains the effects of pH stability on purified *L*-asparaginase activity. The purified enzyme activity was fairly stable at pH 5.0-8.0 and its stability reduced from 74.87 to 56.066 $\mu\text{mol}/\text{min}/\text{ml}$ at pH 9.

3d. explains the effects of temperature on purified *L*-asparaginase activity. Maximum activity of 34.1515 $\mu\text{mol}/\text{min}/\text{ml}$ was at 40°C.

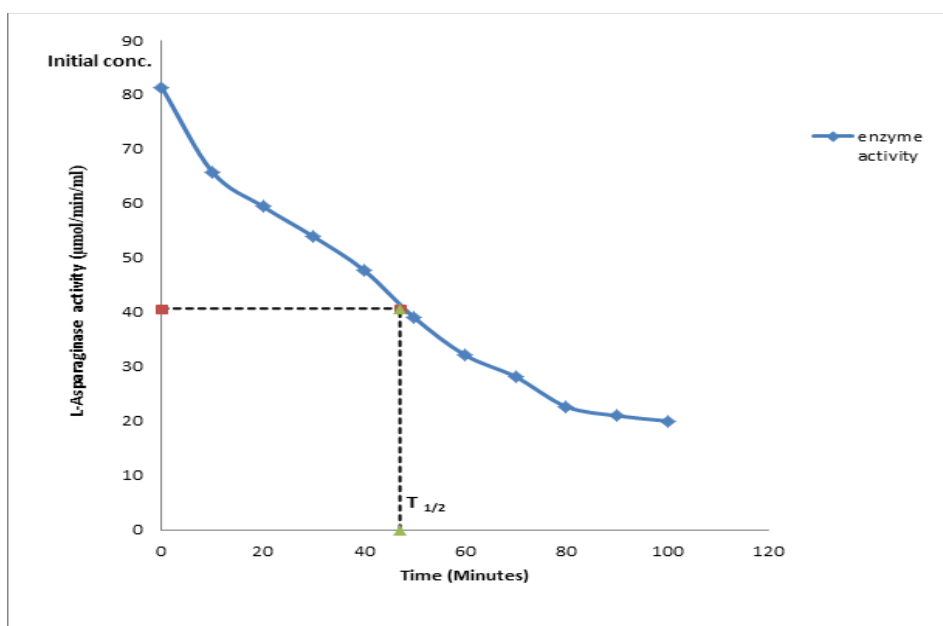


Fig. 2e: Effect of Incubation on purified enzyme activity.

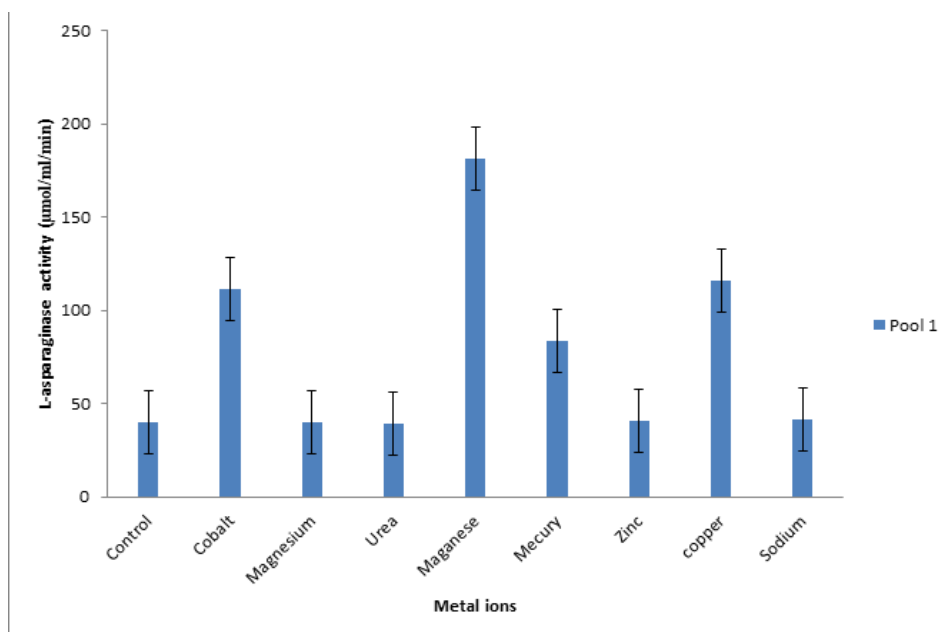


Fig. 3f: Effect of Metal ions and urea on L-asparaginase activity.

DISCUSSION

Enzyme purification has been extensively studied to develop simple rapid and single step process to achieve enzymes with the highest grade of purity. Efforts have been made to attain endotoxin free L-asparaginase with low cost and enhanced efficiency, but to date, the commercially available ones hold a maximum of 60 % of purity^[10]. In this study, partial purification of L-asparaginase was done by using 60 % ammonium sulphate precipitation this was used because it has the potential to precipitate about 99.9 % of total protein present in a medium, this was in accordance with the work carried out by Patro^[11] unlike sodium sulphate. Ion exchange chromatography was used to separate this enzyme based on their charge positive (+ve) and negative charge (-ve). L-asparaginase has a +ve side chain. The positive charged group would attach to the negative charged ions. The proteins that are -ve charged ions would not attract to the beads; hence they travel faster and are collected. The positively charged proteins would attract or bind to the negatively charged

ions and travel very slowly^[13]. To improve the travel rates salt solutions such as NaCl is added. The NaCl ions dissociate into the solutions to form Na^+ and Cl^- . The positively charged Na^+ would interact with the negatively charged beads.

Gel filtration was used for final purification step using gel beads. Enzymes produced from marine organisms including algae, bacteria, fungi and sponges exhibit unique physiological properties such as hyper-thermal stability, barophilicity, salt and pH tolerance, adaptability to extreme cold conditions and novel chemical and stereochemical properties^[14]. Moreover, most halophilic bacterial enzymes often found in marine organisms like the marine actinomycetes are considerably thermotolerant and remain stable at room temperature over a long period of time^[14]. In this study, the maximum velocity (V_{\max}) of the purified L-asparaginase was 51.136 $\mu\text{mol}/\text{min}/\text{ml}$ and K_m value of 3.812 μmol .

Michaelis menten constant (Km) explains the time it takes for an enzyme to reach half of its initial velocity. The purified enzyme was also incubated at different intervals a half-life of 50 minutes at 60 °C was observed. Half-life explains the time it takes for a substance to reduce to half of its initial concentration. In this study, an initial concentration of 81.212 µmol/min/ml was discovered. The result from this experiment followed a second order reaction, in which the half-life increases with decreasing concentration.

Meanwhile for the purified enzyme, maximum activity of 92.2727 µmol/min/ml at pH 6.0 and a minimum activity of 66.939 µmol/min/ml were recorded at a pH 3.0. pH stability result shows that the enzyme was fairly stable from pH 5.0 to 8.0. A significant drop of stability was recorded at pH 9.0. Effect of temperature was also used to characterize the purified L-asparaginase and it was discovered that L-asparaginase had maximum activity of 34.1515 µmol/min/ml was recorded at 40 °C and a minimum activity of 22.8485 µmol/min/ml was recorded at 100 °C. However, in the study carried out by Luhana^[6] maximum enzyme activity of 30.8 µmol/min/ml was recorded at 37 °C, so 37 °C was considered as the optimum temperature for enzyme isolated from *Aspargillus niger* AKMB and at 4 °C and 50 °C, the enzyme had lost its activity.

The inhibition mechanism of metal ion against carbonic anhydrases is probably due to the interactions with histidine residues present at the entrance of the active site cavity of these enzymes, which thereafter prevents the proton shuttling effects in which these amino acid participate and which are essential for the catalytic site. Also, metal ions such as Ag, Hg and Pb denature proteins by reacting with the sulphhydryl groups to form stable, metal –sulfur bonds. This prevents formation of needed disulfide bonds. Metals such as sodium (Na⁺), potassium (K⁺), iron (II) and iron (III), Zinc (Zn²⁺), calcium (Ca²⁺), Copper (Cu²⁺) Mercury (Hg⁺) and Urea, did not have

positive effects on purified L-asparaginase activity^[6]. Categorically, Manganese had the highest activity of 181.576 µmol/min/ml and mercury (Hg²⁺) had the least significant increase in activity of 83.363 µmol/min/ml) in this study.

CONCLUSION

The need to properly purify and characterize any protein cannot be overemphasized. Purified L-asparaginase from *Streptomyces avermitilis* showed a Maximum velocity of 51.136 µmol/min/ml and Km value was about 3.812 µmol. The purified enzyme had half - life of 50 minutes at 60 °C and was inhibited by urea. Metals such as Mn²⁺, Co²⁺, and Hg⁺, had significant increase on purified L-asparaginase activity. Conclusively, this purified enzyme showed viability under various conditions in the study carried out hence it can be applied in food industry in reducing acrylamide content or used in medicine in treatment of acute lymphoblastic leukemia..

Conflict of Interest

There was no conflict of interest in this study

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