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Research Article

Extraction And Purification of L-Asparaginase from *Cucurbita Pepo* Leaves and Nano encapsulation of L- Asparaginase

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ABSTRACT

L-asparaginase (E.C. 3.5.1.1) is an essential anticancer enzyme that plays a pivotal role in inducing cellular starvation in 3T3 cancer cell lines. Plant-derived L-asparaginase offers a safer and more sustainable alternative to microbial sources. With lower immunogenicity, it minimizes adverse immune responses, provides a renewable and eco-friendly enzyme source, and enables cost-effective production, thereby making cancer treatments more accessible. In this study, various plant samples were screened for L-asparaginase activity using the Nesslerization method, with *Cucurbita pepo* leaves consistently showing the highest absorbance values. Consequently, these leaves were selected for L-asparaginase production. The crude extract underwent a three-step purification process, including salt precipitation and dialysis, ion exchange chromatography, and gel filtration chromatography. The purified enzyme exhibited the highest specific activity of 25,850 U/mg with a protein fold increase of 53.080. Enzyme kinetics were evaluated by analyzing the effects of time, pH, temperature, substrate concentration, and metal ions on enzyme activity. The optimal conditions for maximum enzyme activity were determined to be 30 minutes of reaction time, pH 8.0, a temperature of 40°C, and the presence of Ca2+ ions, which acted as an enzyme activator. The Km and Vmax values were 31.54 µM and 63.094 µM, respectively. The molecular weight of the enzyme was determined by SDS-PAGE as 50 kDa. Additionally, L-asparaginase was nanoencapsulated using chitosan nanoparticles via the ionotropic gelation method. MTT assays on 3T3 mouse embryonic fibroblast cancer cell lines yielded IC50 values of 36.033 µg/mL for the partially purified enzyme and 45.714 µg/mL for the nanoencapsulated enzyme.

KEY WORDS: Anticancer enzyme, Nesslerization, nanoparticles, MTT assay, 3T3 cancer cell lines

Introduction

L-asparaginase (E. C. 3.5.1.1) is a vital anticancer enzyme that plays a significant role in inducing cellular starvation in cancer cells. Generally, normal cells remain unaffected by this deprivation, as they can convert the resulting products into essential compounds for their growth. L-asparagine, a non-essential amino acid, is hydrolyzed by L-asparaginase into aspartic acid and ammonia, making it unavailable for cancer cell growth.

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Cancer cells have a higher demand for L-asparagine than normal cells and lack the ability to synthesize it internally. In contrast, normal cells possess the Asparagine synthetase gene on Chromosome 7, allowing them to convert aspartate into asparagine using ATP as an energy source.

Tumor cells, however, do not have this gene, rendering them unable to produce L-asparagine. This deficiency leads to cellular starvation, resulting in apoptosis and the disruption of the cell cycle. L-Asparaginase has been extracted from various sources such as plants, fungi, bacteria,algae and actinomycetes (Abhinav Shrivastava et al., 2015). The production of L- Asparaginase from plants offers advantages such as reduced production costs, lower risk of

immunogenic reactions, and a sustainable, eco-friendly source. Plant-derived enzymes can also be engineered for improved activity and stability, making them a viable alternative to microbial sources. Previously, L-asparaginase has been reported in different plants such as *Pisum sativum*, (Zena Abdullah Khalaf, 2012), *Tamarindus indica*, *Vigna radiata* (Muhammad Anjum Zia, 2020), *Chromolaena odorata* (Yusriadi et al., 2019) which have shown significant antimicrobial activity, cytotoxic effects on cancer cell lines and helped in quenching the acrylamide content in fried foods items. (Archana Vimal & Awanish Kumar, 2017). *Cucurbita pepo* leaves were selected as the plant source for the production of L- Asparaginase in this study. Nanoparticles in enzyme encapsulation offer enhanced stability, protection, and controlled release of enzymes, making them valuable in various applications. They improve enzyme activity and lifespan by shielding against environmental factors like temperature, pH, and proteolysis. In biotechnology, nanoparicles are utilized in a multitude of applications, including drug encapsulation, magnetic drug delivery, gene transport, targeted and controlled drug delivery systems, magnetic separation of biomolecules, bio-labelling with fluorescent quantum dots, and bio-imaging. (Muhammad Irfan Majeed & Haq Nawaz Bhatti, 2020).

Material and Methods

Extraction of Crude enzyme: *Cucurbita pepo* leaves of 20g was weighed, washed under the tap water and then rinsed with distilled water. The plant samples were homogenized using 0.1M of phosphate buffer of pH 7.0 to carry out enzyme assay. The homogenate was centrifuged at 6000 rpm for 10 minutes. The supernatant served as the crude enzyme extract..

Enzyme Assay: Nesslerization is a standard method for detecting the amount of ammonia present in a sample. Different aliquots of ammonium sulphate solution (0.25, 0.5, and 1 mL) were added to different test tubes. The assay was performed by adding 1 mL of 50 mM Tris-HCl buffer at pH 8.6 and 0.1 mL of 189 mM L-asparagine and incubated for 5 min at 37°C. Enzyme extract of 0.1 mL to each test tube. A blank was prepared without adding the enzyme extract. The tubes were incubated for 30 minutes at 37°C. Following incubation, 0.1 mL of 1.5 M trichloroacetic acid (TCA) was added to stop the reaction. Clarified by centrifugation, 4.3 mL of distilled water was added to 0.2 mL of supernatant followed by the addition of 0.5 mL of Nessler's reagent, and the absorbance was measured at 436 nm against the blank.

Purification and Characterization of L-Asparaginase

Salt precipitation and Dialysis: The crude extract was salted out using 70% ammonium sulphate and refrigerated overnight. The supernatant was centrifuged at 8500 rpm for 15 minutes. The resulting pellet was dissolved in 10 mL of 10 mM Tris-HCl and was subjected to dialysis. The dialyzed sample was centrifuged at 6000 rpm for 10 minutes.

Ion exchange Chromatography: The dialyzed sample was further purified using Sintered silica G0 column (25 x 8cm) packed with DEAE anion exchange gel. Seven different elutions, each consisting of 25mM Tris-HCl and different concentrations of NaCl (25mM, 50mM, 75mM, 100mM, 125mM & 150mM) were used to purify the dialyzed sample.

Gel Filtration Chromatography: The Sintered silica G0 column (25 x 8cm) was packed with Sephadex G-75 gel and the elution sample was carefully applied along with 20 mL of 0.1M phosphate buffer at pH 7.0. The eluate was collected in 1 mL fractions, with each fraction being collected in separate 50 Eppendorf tubes and the absorbance was measured at 280 nm.

Total protein was estimated by Lowry's method and enzyme assay was carried out by Nesslerization method to determine both enzyme activity and specific enzyme activity for all the samples.

Molecular Weight Determination by SDS-PAGE: The purified samples along with the crude were subjected to Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determine the molecular weight of the enzyme. Bovine Serum Albumin (BSA) was used as standard marker. Prior to loading, the samples were mixed with SDS loading dye and denatured in a hot water bath at 80°C for 15 minutes. Electrophoresis was carried out at 50V and 100mA. Coomassie Brilliant Blue stain was used to observe the protein bands.

ENZYME KINETICS

Time Kinetics: Seven test tubes were labelled as Blank, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min. Each tube received 1 mL of 50 mM Tris buffer, 0.1 mL of 189

mM L-Asparagine, and 0.1 mL of a crude sample. The tubes were incubated at 37°C for their respective times, with the blank incubated for 30 minutes. After incubation, the reaction was stopped by adding 0.1 mL of TCA. Then, 0.2 mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a graph of incubation time versus absorbance was plotted.

pH Kinetics: A set of 0.1 M buffers (sodium acetate at pH 5.0 and 6.0, potassium phosphate at pH 7.0 and 8.0, glycine at pH 9.0 and 10.0) was prepared. Twelve test tubes were labelled by pH, with separate blanks and tests. Each tube received 1 mL of the corresponding buffer, 0.1 mL of 189 mM L-Asparagine, and 0.1 mL of a crude sample. The tubes were incubated at 37°C for 30 minutes, and the reaction was stopped by adding 0.1 mL of TCA. Then, 0.2 mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a graph of pH versus absorbance was plotted.

Temperature Kinetics: Seven test tubes were labelled as Blank, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Each tube received 1 mL of 50 mM Tris buffer, 0.1 mL of 189 mM L-Asparagine, and 0.1 mL of a crude sample. The tubes were incubated at their respective temperatures for 30 minutes, with the blank at 37°C. After incubation, 0.1 mL of TCA was added to stop the reaction. Then, 0.2 mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a temperature versus absorbance graph was plotted.

Km and Vmax: Seven test tubes were labelled as Blank, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, and 6 mg. Each tube contained 1 mL of 50 mM Tris buffer and 0.1 mL of L-asparagine at corresponding concentrations. The blank tube had 1 mg of L-asparagine. After adding 0.1 mL of a crude sample, the tubes were incubated at 37°C for 30 minutes. The reaction was stopped with 0.1 mL of TCA, and 0.2 mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a graph of substrate concentration versus absorbance was plotted to calculate Km and Vmax.

Metal ions: Seven test tubes were labelled as Blank, $ZnCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, and $CaCl_2$. Each tube contained 1 mL of 50 mM Tris buffer, 0.1 mL of 189 mM L-Asparagine, and 0.1 mL of a crude sample. Metal ion tubes received 0.1 mL of their respective metal solutions. All tubes were incubated at 37°C for 30 minutes, with the blank following standard protocol without metal ions. The reaction was stopped with 0.1 mL of TCA, and 0.2 mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a bar graph of metal ions versus absorbance was plotted.

Effect of Different Concentrations of Ca^{2+} ions: Seven test tubes were labelled as Blank, 100 µL, 200 µL, 300 µL, 400 µL, 500 µL, and 600 µL. Each tube contained 1 mL of 50 mM Tris buffer, 0.1 mL of 189 mM L-Asparagine, and 0.1 mL of a crude sample. The tubes received 0.1 mL of calcium ions at the corresponding volumes and were incubated at 37°C for 30 minutes. The blank followed the same protocol without calcium ions. After incubation, the reaction was stopped with 0.1 mL of TCA. Then, 0. 1mL of supernatant was mixed with 4.3 mL of water and 0.5 mL of Nessler's reagent. Absorbance was measured at 436 nm, and a bar graph of calcium ion concentration versus absorbance was plotted.

Nanoencapsulation of L-asparaginase with Chitosan Alginate nanoparticles: Chitosan nanoparticles were synthesized using ionotropic gelation method. 10 mL of 0.3% sodium alginate solution was stirred on a magnetic stirrer for 30 min, simultaneously followed by the drop wise addition of 2 mL of 0.2% calcium chloride, forming a microgel. Next, 2 mL of 0.05% chitosan was added, with continuous stirring for an hour. After 24 hours, the suspension was centrifuged, re-suspended, and sonicated. For L-asparaginase encapsulation, 200 µL of the gel filtered enzyme was mixed with calcium chloride, then added to sodium alginate, followed by chitosan. The solution was equilibrated, centrifuged, and the supernatant was analysed for free enzyme. Both nanoparticle types were characterized by Zeta sizer to determine the particle size. (Apoorva Singh et al., 2020).

Anti-proliferative Activity of Cancer Cell Lines using MTT Assay: The MTT assay was conducted on 3T3 mouse embryonic fibroblast cells in a 96-well plate to evaluate cell cytotoxicity. Border wells were filled with water to maintain moisture. Wells labelled as blank, control, and with varying sample concentrations (10-50 µL) were added with 100 µL of DMEM. Partially purified and gel filtered nanoencapsulated enzyme samples were added to designated wells, followed by 100 µL of 3T3 cells. After 48 hours of incubation in a 5% CO_2 incubator, 10 µL of

MTT dye was added, and the plate was incubated for another 4 hours. The supernatant was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 575 nm against blank. The percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \text{Control} - \text{Test} / \text{Control}$$

The graph of concentration of plant extract in µL on X-axis and percentage inhibition on Y-axis was plotted. The IC50 (Half Inhibition Concentration) value was calculated based on the intercept and the slope value which were obtained from the graph**.**

Results and discussion

The crude leaf extract of *Cucurbita pepo* showed L-asparaginase activity of 319 U/mL, a protein concentration of 655 µg, and a specific activity of 487 U/mg, confirming the enzyme's presence. The crude enzyme extract was purified through ammonium sulfate precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. Protein content was measured using Lowry's method. Post-salt precipitation, the enzyme extract displayed an activity of 335.8 U/mL, protein concentration of 132.0 µg, and specific activity of 2543.9 U/mg. The dialyzed extract reported an enzyme activity of 386.20 U/mL, protein concentration of 90.0 µg, and specific activity of 4291.1 U/mg. Ion exchange chromatography resulted in an enzyme activity of 216.42 U/mL, protein concentration of 42.0 µg, and specific activity of 5152.0 U/mg. Gel filtration chromatography produced an enzyme activity of 429.11 U/mL, protein concentration of 16.6 µg, and specific activity of 25850 U/mg. The protein fold value after gel filtration reached 53.080.

Table 1: Purification steps of L-Asparaginase

	Enzyme activity (U/mL)	Protein Concentration (µg)	Specific Enzyme activity (U/mg)	Protein Fold
Crude	319	655	487	1
Salt precipitation	335.8	132	2543.9	5.223
Dialysis	386.20	90	4291.1	8.811
Ion exchange Chromatography	216.42	42	5152.8	10.580
Gel filtration Chromatography	429.11	16.6	25850	53.080

Molecular weight determination by SDS-PAGE: SDS-PAGE is crucial for determining the molecular weight of purified enzymes. Proteins are denatured, coated with SDS, and migrate through a gel under an electric field. Smaller proteins travel faster. Comparing the enzyme's migration with standard markers allows accurate molecular weight estimation, confirming enzyme purity and characterizing its properties. The molecular weight of L-Asparaginase through SDS-PAGE was found to be 50 kDa using Pre-stained Protein Ladder, while in *Solanum nigrum* (Om Sathya, 2018) it was found to be 66 kDa (Om Sathya, 2018) and 85 kDa in *Phyllanthus emblica* (Apoorva Singh, 2018).

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Figure 1: Molecular Weight Determination by SDS-PAGE

Time Kinetics: Incubation time influences enzyme kinetics by allowing sufficient interaction between the enzyme and the substrate. Optimal incubation ensures maximum reaction progress, while deviation in the time can lead to substrate depletion, product inhibition thereby affecting the overall efficiency of the enzyme. The optimum incubation time for L-asparaginase was found to be 30 minutes. The same optimum incubation time was reported in *Vigna unguiculata* (Sorial Moharib, 2018).

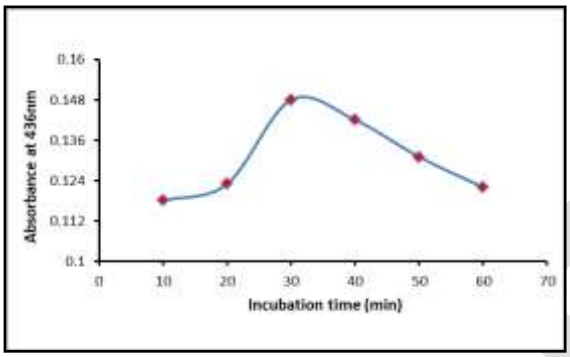

Fig 2: Effect of Incubation time on Enzyme activity

pH Kinetics: pH significantly impacts enzyme kinetics by influencing the enzyme's active site and overall structure. The maximum activity is observed at optimum pH, deviations can reduce the enzyme efficiency or cause denaturation thereby disrupting the enzymatic function. The optimum pH of L-asparaginase was found to be 8.0. The same optimum pH was reported in *Solanum nigrum* (Om Sathya, 2018) and *Spinacia oleracea* (Sarina Khabade, 2017).

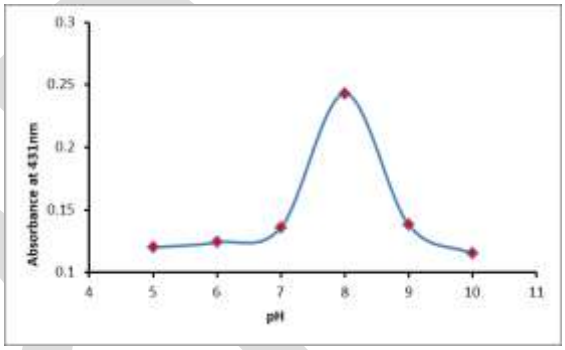

Fig 3: Effect of pH on Enzyme activity

Temperature Kinetics: Temperature influences enzyme kinetics by affecting the rate of enzymatic reactions and maintaining its stability. As temperature increases, reaction rate typically increases due to enhanced molecular collisions, up to an optimum temperature. Beyond this point, the enzyme may denature leading to decrease in the activity. The optimum temperature of L-asparaginase was found to be 40°C. The same optimum temperature was reported in *Solanum nigrum* (Om Sathya, 2018) and Withania somnifera (Vishal P. Oza, 2009).

Km and Vmax: Substrate concentration affects enzyme activity by determining the rate of the reaction. As the substrate concentration increases, the reaction rate also increases until the enzyme becomes saturated. Beyond this point, addition of more substrates does not increase the reaction rate as all the active sites would be occupied. The kinetic parameters, Km and Vmax of the crude enzyme extract were found to be 31.54 µmol and 63.094 µmol, respectively. The similar kinetic parameters, Km and Vmax of the purified enzyme of *Phaseolus vulgaris* were reported to be 6.72mM asparagine and 0.16 µM Ammonia/mL, respectively. (Saleh Mohamed et al., 2015).

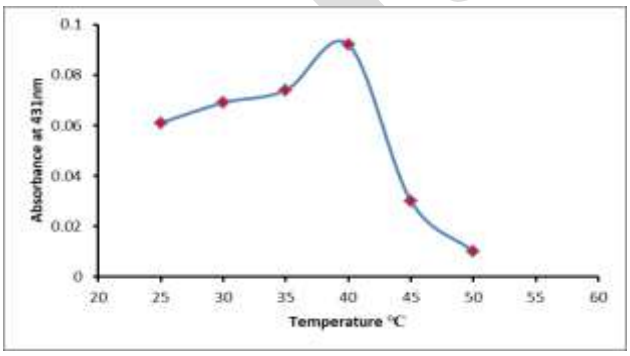

Fig 4: Effect of Temperature on Enzyme activity

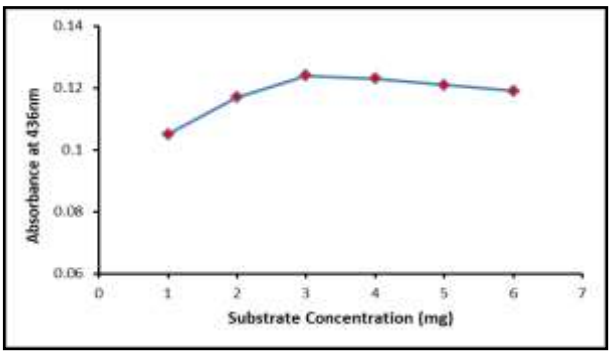

Fig 5: Effect of Substrate concentration on Enzyme activity

Effect of Metal ions: Metal ions can act as activators, enhancing enzyme activity by stabilizing structures, or as inhibitors, disrupting enzyme function by altering active sites or competing with substrates, thus impacting reaction rates. Calcium (Ca2+) ion was found to be the activator of L- asparaginase enzyme, while the other metal ions were found to be the inhibitors of the enzyme. Ca2+ion was found to be the activator of L-asparaginase enzyme, while the other metal ions were found to be the inhibitors of the enzyme. Ca2+ ions were reported as activators in *Phaseolus vulgaris* (Saleh Mohamed et al., 2015), while Fe2+and Zn2+ ions were reported as inhibitors in *Capsicum annum* (Bhavana et al., 2014).

Effect of Ca2+ ions on Enzyme activity: Calcium ions can influence enzyme kinetics by acting as cofactors that stabilize enzyme structure, enhance activity, or facilitate substrate binding, thereby affecting reaction rates and overall enzymatic efficiency. As the concentration of Ca2+ ions increases, the enzyme activity is also increased significantly. Ca2+ ions were reported as activators in *Phaseolus vulgaris* (Saleh Mohamed et al., 2015)

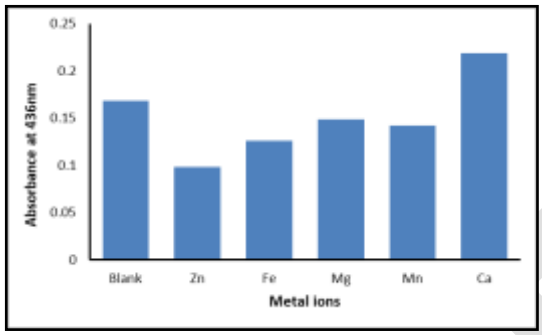

Fig 6: Effect of Metal ions on Enzyme activity

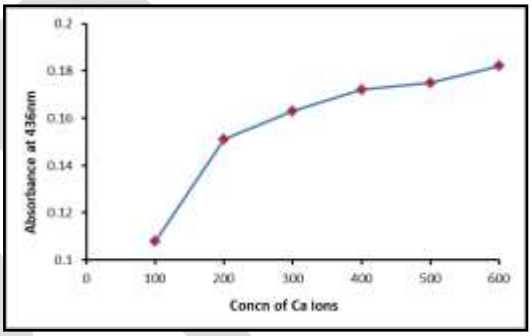

Fig 7: Effect of Ca^{2+} ions Enzyme activity

Nanoencapsulation of L-asparaginase with Chitosan Alginate nanoparticles: The gel filtered L-asparaginase sample was nanoencapsulated using Chitosan alginate nanoparticles. The size distribution report by Intensity for the sample was conducted using Malvern Zeta sizer Ver. 6.20. Polydispersity index of the sample was found to be 0.812. The peaks in the graph represent the size distribution of particles in the sample as measured by their intensity of scattered light.

Size Distribution:
Peak 1:

- Size: 64.20 d.nm
- Percentage Intensity: 10.4%
- Width: 5.800 d.nm

This smaller peak corresponds to a minor population of particles, representing 10.4% of the total intensity. It indicates the presence of smaller particles, approximately 64.2 nm in diameter.

- Peak 2:
- Size: 500.9 d.nm
- Percentage Intensity: 89.6%
- Width: 68.61 d.nm
- This peak is the largest and corresponds to the majority of particles in the sample. Since it has the highest intensity (89.6%), it indicates that most particles are around 500.9 nm in size.
- The similar nanoencapsulation was employed to enhance the stability of L-asparaginase enzyme using sodium and magnesium nanoparticles. (Om Sathya., 2018)

Anti-proliferative activity of cancer cell lines using MTT assay: The MTT assay plays a crucial role in evaluating the cytotoxicity of potential cancer treatments by measuring how effectively a compound kills or inhibits the growth of cancer cells. By assessing the reduction in cell viability after treatment, it is used to determine the effectiveness of chemotherapeutic agents or other anticancer compounds.

The IC50 (Half Inhibition Concentration) value was calculated based on the figure 9. The intercept and the slope value were obtained from the graph. The IC50 value of partially purified enzyme extract was found to be 36.033 µg/mL.

The IC50 (Half Inhibition Concentration) value was calculated based on the figure 10. The intercept and the slope value was obtained from the graph. The IC50 value of nanoencapsulated enzyme extract was found to be 45.7143 µg/mL.

The similar anticancer property was assayed using MTT and was reported in L- Asparaginase extracted from *Solanum lycoperiscum*. The crude sample of L-asparaginase showed anticancer activity against K-562 cell line with an IC50 value of 43.94µg/mL. (Sarina Khabade et al., 2024)

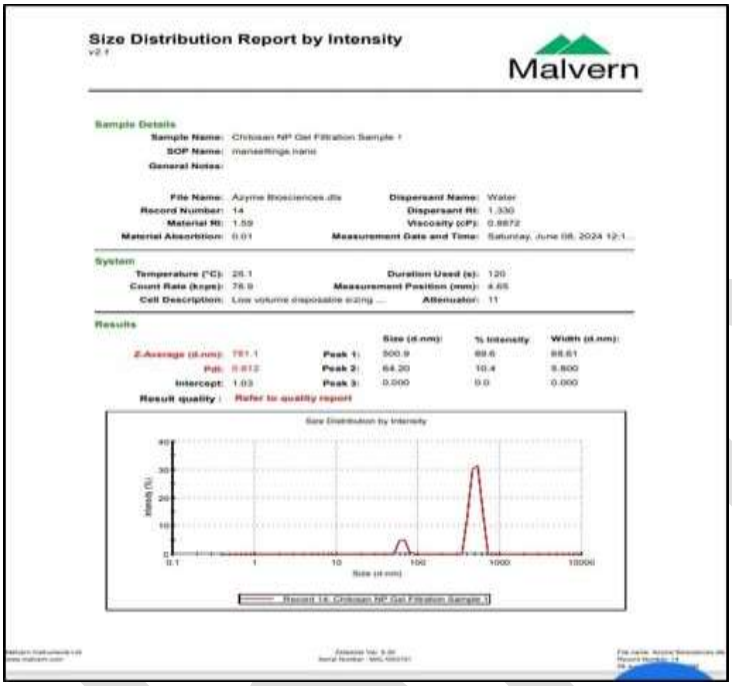

Figure 8: Particle Size Analysis of Nanoencapsulated L-asparaginase

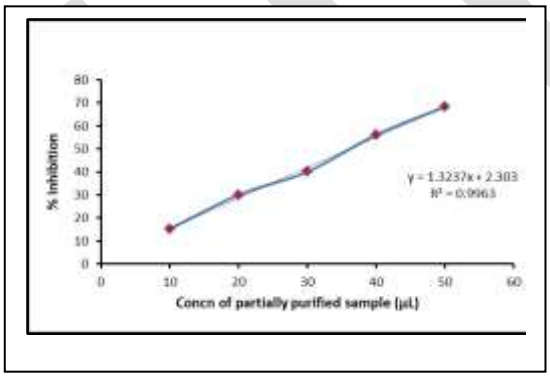

Figure 9: Graph showing the Percentage inhibition of 3T3 cell lines using different concentrations of partially purified L-asparaginase extract

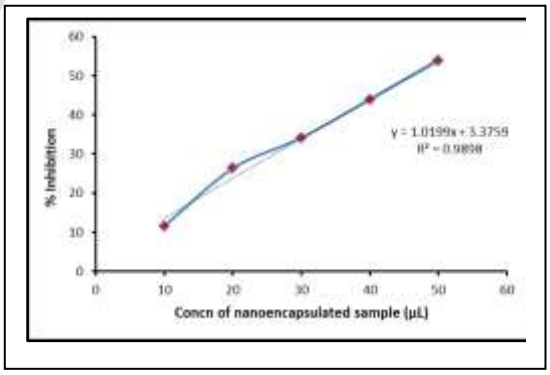

Figure 10: Graph showing the Percentage inhibition of 3T3 cell lines using different concentrations of Nanoencapsulated L-asparaginase extract

Conclusion

Cucurbita pepo exhibited the highest absorbance and enzyme activity, leading to its selection for L-asparaginase production. The crude leaf extract of *Cucurbita pepo* showed concentration of 655 µg, and a specific activity of 487 U/mg, confirming the enzyme's presence. The crude enzyme extract was purified through ammonium sulfate precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. Protein content was

measured using Lowry's method. Post-salt precipitation, the enzyme extract displayed an activity of 335.8 U/mL, protein concentration of 132.0 µg, and specific activity of 2543.9 U/mg. The dialyzed extract had an enzyme activity of 386.20 U/mL, protein concentration of 90.0 µg, and specific activity of 4291.1 U/mg. Ion exchange chromatography resulted in an enzyme activity of 216.42 U/mL, protein concentration of 42.0 µg, and specific activity of 5152.0 U/mg. Gel filtration chromatography produced an enzyme activity of 429.11 U/mL, protein concentration of 16.6 µg, and specific activity of 25850 U/mg. The molecular weight of the protein was determined by SDS-PAGE and found to be 50 kDa. The L-asparaginase enzyme extracted from the leaves of *Cucurbita pepo* demonstrated optimal activity under the following conditions: 30-minute incubation, pH 8.0, temperature of 40°C, and the presence of Ca^{2+} ions as an activator. The kinetic parameters, Km and Vmax of the crude enzyme extract were found to be 31.54 µmol and 63.094 µmol, respectively. In this study, efforts were made to enhance the stability of the L- asparaginase enzyme by creating nanoencapsulated L-asparaginase using chitosan-alginate nanoparticles. The size of the nanoencapsulated enzyme was measured with a Zetasizer, revealing a diameter of 64.20d.nm. The nanoencapsulated L-asparaginase demonstrated effectiveness against mouse embryonic fibroblast cancer cells (3T3 cell lines). To evaluate the cytotoxic activity of the enzyme, an MTT assay was conducted using 3T3 mouse embryonic fibroblast cancer cell lines. Both the partially purified and nanoencapsulated L- asparaginase enzymes extracted from the leaves of *Cucurbita pepo* exhibited strong anticancer properties. The IC50 values were determined to be 36.033 µg/mL for the partially purified enzyme and 45.714 µg/mL for the nanoencapsulated enzyme.

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