Purification of anti cancerous enzyme L-glutaminase from marine Streptomyces parvus HSBT0318

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Abstract

The present study reports purification of extracellular glutaminase enzyme from streptomyces parvus .
Screening was performed from twenty Actinomycetesisolates from soil; one isolate (Isolate HSBT0318) was finally selected based on the activity of glutaminase(36.48 U/ml).The isolate was identified as *Streptomyces* sp. The L-glutaminase produced from *Streptomyces* parvus was purified by ammonium sulphate precipitation, dialysis method and ionexchange chromatography. After the purification of the enzyme by ion exchange chromatography, it has been purified 46-fold from cell-free extract and yield was 3.25%. Characterization of extracellular L-glutaminase showed optimal activity at temperature of 30°C, pH 7, at 3% NaCl and for 0.04M substrate and the Km value was calculated to be 2.8mM and Vmax was 7.57 U/ml. The molecular weight of enzyme as determined by sodium dodecyl sulphate polyacrylamide electrophoresis(SDS-PAGE) was found to be 45 kDa.

Key words: L-Glutaminase, Actinomycetes, Marine sediments, Streptomyces.sp, ion exchangechromatography, SDS-PAGE.

Introduction

Intensive work has been carried out to find sources of glutaminases and to study their properties against tumors. L-Glutaminase is an amido-hydrolase enzyme that cleaves glutamine into glutamic acid and ammonia. Glutamine, a non-essential amino acid and the primary substrate for L-glutaminase, is present in circulating blood and also serves as a fuel for cell growth and nucleic acid synthesis (Hartman 1971; Wade 1980). The consumption of glutamine by cancer cells is faster than that of other amino acids, and its uptake rate is directly proportional to its supply (Souba 1983). Though cancer cells consume more amounts of glutamine, they are incapable of producing their own glutamine de novo (Wise et al. 2010), while normal cells can do so. Hence, a strategy that reduces blood glutamine levels using glutaminase would control the growth of cancer cells under hypoxic conditions. Besides its applications in cancer therapy, Lglutaminase is also a much sought-after enzyme in the food industries as a flavour-enhancing agent(Nandakumar et al. 2003), for the production of acrylamide-free potato food (Pedreschi et al. 2008), and as a biosensor that detects glutamine (Padma et al. 2010). Previous studies have shown that marine environments could be a source of many enzymes that display useful characteristics, including salt tolerance and stability under various conditions (Chandrasekaran et al. 1997). A novel fermentation medium is of critical importance because of its significant influence on product yields; thus, designing an appropriate medium for a particular product is gaining importance as this would also contribute to maximum yields of the product. The enzyme was purified to homogeneity, entrapped in PEG and immobilized on PHB nanoparticles.

Material and methods

Optimal conditions required for maximal growth and L-glutaminase production by the selected strains were determined by subjecting them to various incubation temperatures, different levels of p^H, substrate concentrations, NaCl concentrations, additional carbon and nitrogen sources, glucose concentrations, inoculum concentrations in the growth media and different incubation periods. Minimal glutamine

broth was used for these studies. The prepared media were dispensed in 100 ml aliquots of 250 ml Erlenmeyer conical flasks, autoclaved and used for optimization studies. 1% Concentration of inoculum was used. The growth of bacteria in the minimal glutamine broth was followed by estimating the turbidity of the broth by taking the absorbance at 660 nm in a UV-Visible Spectrophotometer (Hitachi Model 200).

Enzyme production in the media was estimated in terms of enzyme activity except for the incubation temperature as 28°C and incubation time as l5 minutes instead of 30 minutes, since it was observed that maximal amounts of enzyme units are obtained under these conditions. pH of the buffer varied from 6 to 8 according to the optimum pH of enzyme from each organism.

Inoculum preparation

Initially a loopful of 24 hours old agar slope culture was transferred to 10 ml of NBG (Nutrient Broth added with Glutamine) and grown for 24 hours at room temperature(28 ± 2°C). One ml of the cultured broth was then aseptically transferred into another 50 ml of NBG media and incubated for 24 hours in a rotary shaker (150rpm) at room temperature (28 ± 2°C).Cells were harvested by centrifugation at 5000 rpm for 20 min.The harvested cells were made upto 10 ml volume using physiological saline (0.85% NaCl) after repeated washing with the same. The prepared cell suspension was used as inoculum at 1% level for further inoculation of 50 ml MGB. All the flasks were uniformly inoculated and incubated on rotary shakers (150 rpm) for a period of 24 hours at room temperature (28 ± 2°C).

Submerged fermentation

1ml of inoculum was added to 100ml of the production medium in 250ml Erlenmeyer flasks and incubated in orbital shaker at 28^0C for 108 hrs. The enzyme production medium (EPM) was designed based on the data obtained from the studies conducted for optimization of growth conditions for maximal enzyme production in MSG broth. It was supplemented different carbon and nitrogen sources to study their effect on growth and L-Glutaminase production.

Effect of initial p^H

To determine the effect of pH on growth and production of enzyme L-Glutaminase, the initial pH of the medium was adjusted to different pH values, i.e.: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0.

Initial pH for growth and enzyme production was determined by subjecting the organisms to various p^H levels adjusted in the culture broth (MGB) using 1 N NaOH or 1 N HCl. After inoculation and incubation for 24 hours at room temperature (28 ± 2°C), the culture broths were centrifuged and growth and enzyme production were determined.

Effect of Incubation Temperature

Optimal temperature for maximal growth and enzyme production was estimated by incubating the MG broth inoculated with the test strains at ‘various temperatures (15, 25, 30, 35, 45 and 55°C) for a total period of 108 hours. Growth and enzyme production were determined.

Optimization of Substrate concentration

Optimal substrate concentration that favours growth and enzyme production of the strains was checked by growing them in MSG broth supplemented with different glutamine concentrations (0.25, 0.5, l, 2, 3%). After 24 hours of incubation at room temperature (28± 2°C) the growth and enzyme production were estimated .

Optimization of NaCl concentration

Optimal NaCl concentration that promotes maximal growth and enzyme production of the organisms was determined by subjecting them to different NaCl concentrations (0, l, 3, 5, 7 and 10%) adjusted in the MG broth. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were analyzed.

Effect of Carbon sources

Effect of various carbon sources on growth and production was studied by incorporating them at 1 % (w/v) level into the Pridham and Gottlieb's inorganic salts medium. To optimize the concentration of glucose for maximum growth and

antibiotic production, it was incorporated into the production medium at different concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 g/L After 24 hours of incubation at room temperature (28 ± 2°C), growth and enzyme production were estimated.

Effect of Nitrogen sources

The influence of various nitrogen sources on growth and L-Glutaminase production was studied by adding inorganic nitrogen sources and organic nitrogen sources at 0.2% (w/v) level into the Pridham and Gottlieb's inorganic salts medium. Glucose at 1% (w/v) level was employed as carbon source.

The concentrations of ammonium nitrate used to determine the optimum concentration for growth and L-Glutaminase production are (g/L) 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5. After 24 hours of incubation at room temperature growth and enzyme production were estimated.

Effect of Glucose concentration

Since glucose was found to enhance enzyme production during the studies, optimal requirement of glucose level in the culture medium was estimated by incorporating different concentrations of glucose (0.5, l, 2, 3%) along with 1% glutamine in the MGB. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were estimated.

Effect of Inoculum size

Optimal inoculum size that yields maximal growth and enzyme production was determined in MSG broth at their optimal growth conditions determined by inoculating the broths with various levels of the prepared medium(1-7%). After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production in the media were estimated.

Effect of Incubation period

Optimal incubation time that leads to maximal growth and enzyme production of the strain was estimated by incubating culture flasks for various incubation periods upto a maximum of 108 hours and analysing the samples at regular intervals of 24 hours. Growth and enzyme production in the broths were estimated.

Effect of different concentrations of K_2HPO_4

To optimize the concentration of K_2HPO_4 enzyme L-Glutaminase production, it was incorporated into the production medium at different concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6 and 4.0 g/L.

Effect of different concentrations of $MgSO_4$

To determine the optimum concentration of $MgSO_4$ for L-Glutaminase production, $MgSO_4.7H_2O$ were incorporated into the Pridham and Gottlieb's inorganic salts medium at 0.25,0.5,0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 g/L.

Effect of Agitation

To determine the effect of agitation on the growth and L-Glutaminase production, the fermentation was carried out at different agitation speeds, i.e., 30, 60, 90, 120, 150, 180, 210 and 240 rpm

Growth Curve of enzyme L-Glutaminase with optimized parameters

The growth of the organism was measured as dry weight of the mycelium. The contents of the culture flask were filtered through a previously weighed dry Whatmann No.1 filter paper, washed twice with distilled water and then the filter paper along with the mycelia mass was dried in a hot air oven at 80˚C for 18-24hrs and then the filter paper was weighed.

The prepared media were dispersed in 50 ml aliquots in 250 ml conical flasks, autoclaved and inoculated with 0.5 ml of the prepared inoculum and incubated at room temperature (28 ± 2°C). Samples were drawn at regular intervals and growth was determined by measuring the turbidity at 660 nm in a UV—Visible Spectrophotometer. From the results obtained growth curve was constructed.

Purification of L-Glutaminase

Enzyme purification was carried out following the methods suggested by Hartman (1968) and Roberts (1976). L-Glutaminase produced by *Streptomyces parvus* under submerged fermentation was purified employing ammonium sulfate precipitation, followed by dialysis and gel chromatography on sephadex column as detailed below. All the operations were done at 4 °C unless otherwise specified.

$(NH_4)_2S0_4$ fractionation

Solid ammonium sulphate was slowly added to the crude enzyme filtrate with gentle stirring to bring 40% saturation (fraction I). The mixture was allowed to stand overnight at 4º C. It was centrifuged at 10,000 rpm at 4°C for 20 minutes to remove the precipitate while the resulting supernatant was subjected to the addition of ammonium sulphate until reached to the concentration 50% saturation (fraction II), then it was allowed to the same previous conditions. The resulting supernatant was further subjected to ammonium sulphate precipitation to bring 80% saturation (Fraction III) in a sequential manner. The enzyme precipitate obtained from each saturation was dissolved in a minimal volume of 0.01M phosphate buffer (pH 8) and dialyzed against 0.01M phosphate buffer (pH 8) for 48 -72 hours at 4°C and the buffer were changed occasionally (Davidson L.,et al,. 1977)

Dialysis

The precipitate obtained after (NH4)2SO4 fractionation was dissolved in phosphate buffer (0.2 M) (pH 6 or 8) and dialyzed against the same buffer extensively at 4°C for 24 hours. Enzyme activity and protein content of the dialyzate were determined.

Gel chromatography using Sephadex G-100 column

Finely powdered ammonium sulphate was added with constant stirring to the supernatant of fermented broth obtained after centrifugation and was incubated overnight at 4°C. Maximum L-glutaminase activity was observed with the fraction precipitated at 60–80 % saturation. The Sample after centrifugation was dialyzed against the phosphate buffer for 24 h. Further dialyzed sample was subjected to gel chromatography on sephadex G-100 column. All the fractions were assayed for L-glutaminase activity. L-Glutaminase activity, protein content and specific activity were determined as described earlier and expressed as U/ml, mg/ml and U/mg protein respectively.

Determination of purity by Reverse Phase High Performance Liquid Chromatography

The homogeneity of the L-Glutaminase sample from the active fractions from sephadex G-100 column was determined by RP-HPLC.

Determination of purity and molecular weight by SDS-PAGE

The active fractions were used for testing the purity by polyacrylamide gel electrophoresis. The chromatographic fractions were electrophoretically analyzed on SDS-PAGE.

RESULTS AND DISCUSSION

Growth phase and L-Glutaminase production.

L-Glutaminase production and growth of *Streptomyces parvus* HSBT0318 as a function of time is shown in **Figure 1**. The production of enzyme could be observed from the early exponential growth phase of the strain. It is clear that the maximum specific activity 2.48 U/mg protein was obtained during mid stationary phase i.e., after 108 h of incubation. Results of present study indicated that production of L-Glutaminase was dependent on the bacterial cell growth.

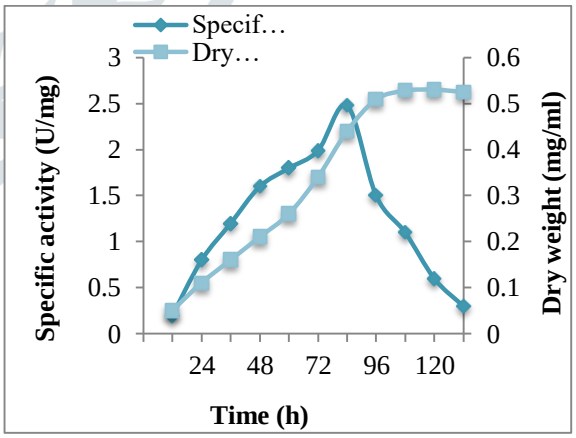

Figure 1: Growth phase and L-Glutaminase

Effect of carbon source

The results demonstrate that glucose was the best carbon source for L-Glutaminase production (**5.01** U/mg protein).

Effect of glucose concentration

The effect of different concentrations of glucose on growth and L-Glutaminase production was studied and

the results are presented in **Figure 2**. At 10 g/lt concentration of glucose, the maximal specific activity (13.5 U/mg protein) and growth (4.5 mg/ml) was observed.

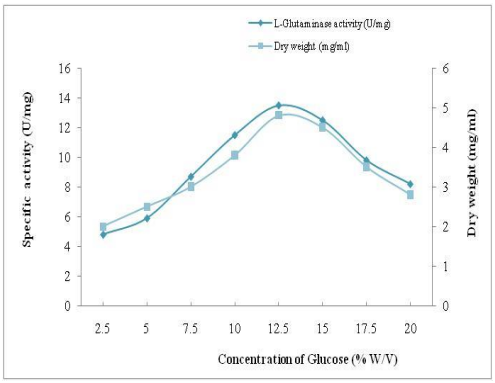

Figure 2: Effect of glucose concentration

Effect of nitrogen source

Among both the inorganic and organic nitrogen sources, Glutamine exhibited the highest level of cell growth (5.5 mg/ml) and L-Glutaminase activity (15.10 U/mg protein).The effect of different concentrations of L-Glutamine on growth and L-Glutaminase production was investigated and the results are presented in **Figure 3**.

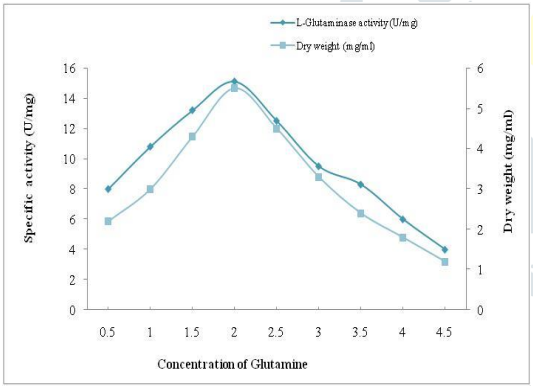

Figure 3: Effect of glutamine concentration

Effect of K_2HPO_4

The effect of different concentrations of K_2HPO_4 on growth and L-Glutaminase production was studied and the results were presented in **Figure 4**. The maximum L-Glutaminase was obtained at 14.01 U/mg activity of K_2HPO_4 with a biomass of 8.3 mg/ml.

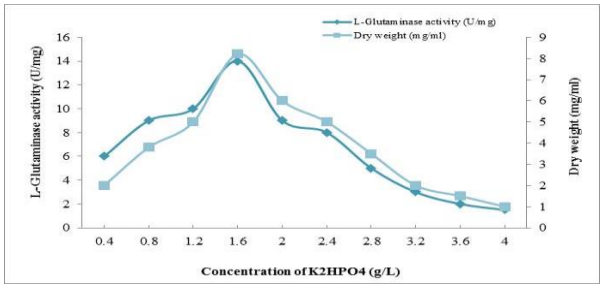

Figure 4: Effect of K_2HPO_4 concentration

Effect of $MgSO_4$

The effect of different concentrations of $MgSO_4$ on growth and L-Glutaminase yield was investigated and the results (**Figure 5**) revealed that, incorporation of $MgSO_4$ in the medium at 1.0 g/lt concentration exhibited the maximum L-Glutaminase activity (15.23 U/mg protein) and biomass (9.86 mg/ml) production.

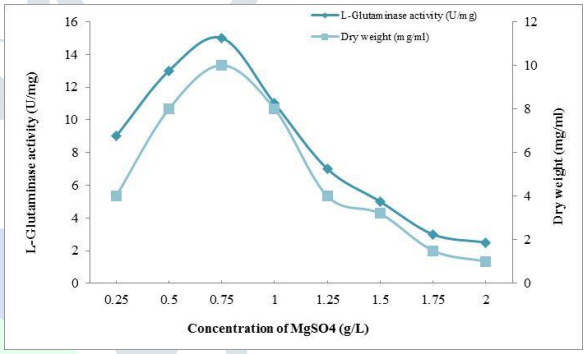

Figure 5: Effect of $MgSO_4$ concentrations

Effect of incubation temperature

In the present study growth and L-Glutaminase yield by strain HSBT0318 was detected at various temperatures (**Figure 6**) within the range of 10–90°C, with 30°C being the optimum temperature for L-Glutaminase production (18..35 U/mg protein) and growth (14.03 mg/ml).

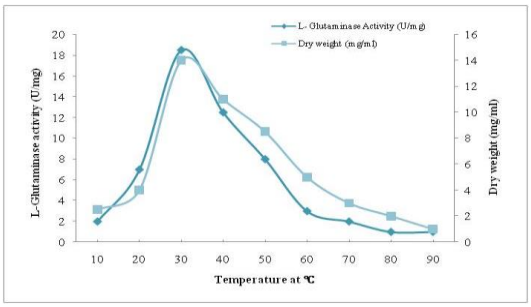

Figure 6: Effect of incubation temperature

Effect of initial pH

In this study initial pH of 7.0 resulted in maxium L-Glutaminase production (22.13 U/mg protein) and cell growth (15. 92 mg/ml) (**Figure 7)**. The maximal enzyme output was noticed at pH 6.0 (2777.7 U/ml) with biomass (0.0117 g/l), either excess or lowering in the pH of the medium led to reduction of enzyme production.

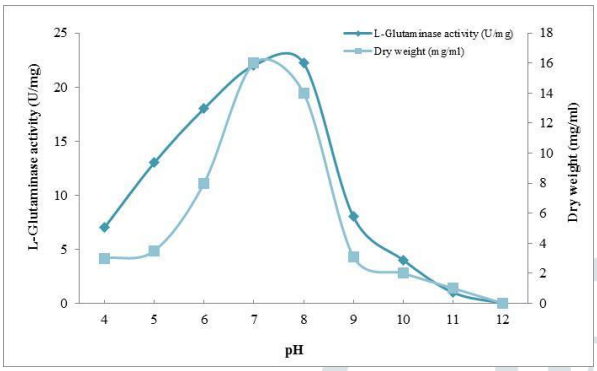

Figure 7: Effect of pH

Effect of incubation period

The effects of incubation periods on L-Glutaminase production and growth was studied. The results (**Figure 8**) indicated the L-Glutaminase activity of 11.03U/mg at 48 hrs of incubation and increased upto 96 hrs reaching a maxmium (23.98 U/mg protein) and then declined on further incubation. The highest biomass production (17.93 mg/ml) was also obtained after 96 hrs of incubation.

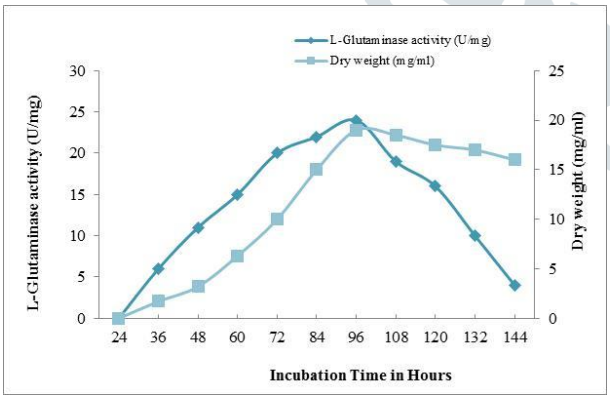

Figure 8: Effect of incubation period

Effect of inoculum size

The results (**Figure 9**) revealed that, L-Glutaminase yield (25.23U/mg protein) and actinobacterial growth (18.35 mg/ml) were optimum when 6.0% (v/v) of inoculum was used. Higher inoculum size at 8.0% (v/v) and 10.0% (v/v) decreased the , L-Glutaminase production.

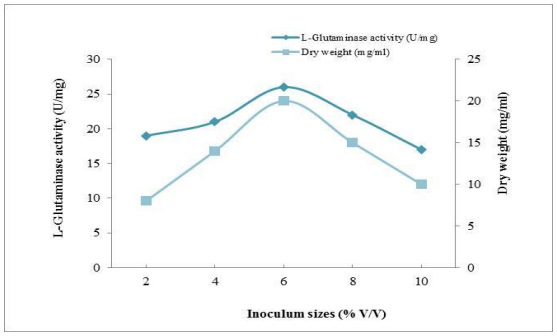

Figure 9: Effect of inoculum size

Effect of agitation

The oxygen demand of a fermentation process is generally met by agitation and aeration. The results represented in **Figure 10** revealed that enzyme production increases with increasing speed. The maximum L-Glutaminase activity (28.02 U/mg protein) and growth (22.35 mg/ml) were observed at 120 rpm.

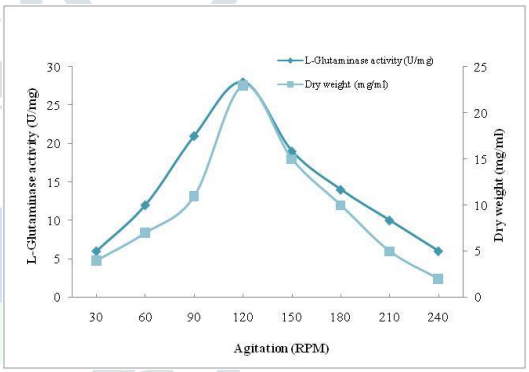

Figure 10: Effect of agitation

Production of L-glutaminase with optimized parameters

The fermentation was conducted by maintaining all the parameters at the optimum levels and the results obtained are presented in **Figure 11**. A 6% (v/v) of 48 hrs aged inoculums was transferred to the modified production medium and incubated at 30°C for 96 h.

It was evident from the result that the L-Glutaminase activity employing optimized fermentation conditions was (32.12 U/mg) 9.4 fold higher than the specific activity using initial fermentation conditions (5.01 U/mg).

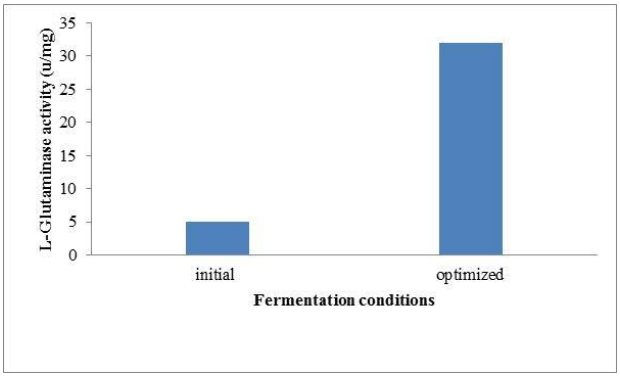

Figure 11: L-Glutamine production by the isolate *Streptomyces parvus* employing initial and optimized fermentation conditions.

Purification of L- Glutaminase

Applying standard protein purification procedures, such as ammonium sulfate fractionation followed by dialysis and gel filtration L- Glutaminase was purified. Different amounts of the enzyme were precipitated from the supernatant by addition of different quantities of ammonium sulfate up to 100% saturation. However the precipitation of the enzyme was maximum between 60 to 80% saturation. The purification elution profile (**Figure 12**) from sephadex G - 100 column exhibited a major peak with L- Glutaminase activity. The L- Glutaminase activity and ultra violet absorbance at 280 nm of the fractions under the peak were superimposed. The enzymatically active fractions from sephadex G-100 column (fraction numbers 20 to 30) were pooled and dialyzed against distilled water for 18 hrs at 4 °C with the change of dialysate four times. Then it was concentrated by placing the dialysis sac containing the protein in sucrose crystals for four hours at 4 °C and lyophilized (LARK).

Employing a purification protocol, involving ammonium sulfate precipitation and sephadex G-100 gel filtration chromatography, L- Glutaminase produced by *S. parvus* was purified to homogeneity with 40% yield and 45 fold purification. These results were in the agreement with that reviewed by Mohana Priya *et al.* (2011).

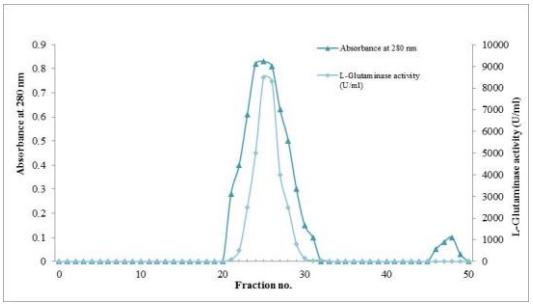

Figure 12: **Elution profile of *Streptomyces parvus* L-Glutaminase through Sephadex G-100 column**

Homogeneity of the L- Glutaminase

The homogeneity of the L- Glutaminase sample from the active fractions of sephadex G-100 column was determined by RP-HPLC and the elution profile is shown in **Figure 13**. The enzyme was eluted as a single symmetrical peak with the retention time of 3.394 mins from RP-HPLC column.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE performed under reducing conditions yielded a single band, thus confirming the monomeric nature of the enzyme (**Figure 14**). The molecular mass of the L- Glutaminase, estimated by comparison with the electrophoretic mobility of marker proteins, indicated that the *Streptomyces parvus* L-Glutaminase has an apparent molecular mass of 45 KDa.

Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

All the fractions which had significant L-Glutaminase activity, obtained after gel chromatography were pooled and lyophilized. They were subjected to Native polyacrylamide gel electrophoresis and the enzyme migrated as a single band in Native-PAGE (**Figure 15**), indicating its homogeneity.

Reverse Phase High Performance Liquid Chromatography

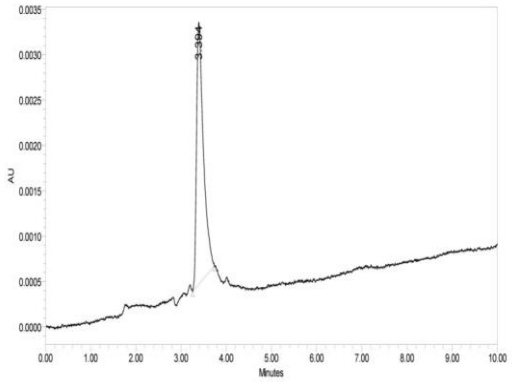

Figure 13: RP-HPLC Elution profile of *Streptomyces parvus*

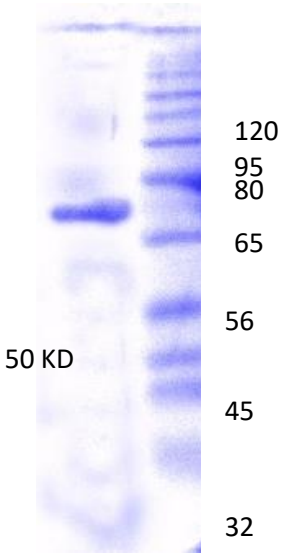

Figure 14: SDS-PAGE of purified sample

CONCLUSION

Since the discovery of its anti-tumour properties, L-glutaminases have been in prime focus and microbial sources of the enzyme are sought.

The purification and characterization of L-Glutaminase from *Streptomyces parvus* HSBT0318 deals with the studies on evolution of the L-Glutaminase by its biochemical characterization. The extracellular L-Glutaminase was purified 40- fold with 34% yield and a specific activity of 32.12.U /mg protein. The enzyme was eluted as a single symmetrical peak with the retention time of 3.394 mins from RP-HPLC column, exhibiting the homogeneity of the purified L-Glutaminase. The molecular weight of L-Glutaminase determined by SDS-PAGE was found to be 45 KDa.

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