Journal of Advanced Zoology

ISSN: 0253-7214

Volume ***44*** *Issue* ***S-2 Year 2023*** *Page* ***197:215***

PURIFICATION, CHARACTERIZATION STUDIES OF L-GLUTAMINASE FROM *ASPERGILLUS FLAVUS* STRAIN S4

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Article History

Received: 11 Aug 2023

Revised: 21 Sept 2023

Accepted: 01 Oct 2023

Abstract

L-glutaminase [EC.3.5.1.2] is an amidohydrolase that catalyzes the hydrolytic deamination of L-glutamine, resulting in the production of L-glutamic acid and ammonia. The L-glutaminase has received a significant attention due to its potential as an anticancer agent. In the present study, soil sample were collected from the Visakhapatnam District, Andhra Pradesh. The aim of this work was to purify L-glutaminase from Aspergillus flavus strain S4. Saturation at 70% ammonium sulphate concentration showed the maximum specific activity (38.22 U/mg protein) with 2.50-fold purification. The purity of the L-glutaminase after DEAE cellulose was increased by 4.56-fold when compared to the crude enzyme. The specific activity and purification fold of the enzyme after DEAE cellulose ion exchange was (69.61 U/mg protein) with a recovery of 37.61%. Molecular weight of L-glutaminase was determined from the standard graph and was found to be 23 kDa. Protein sequencing of the purified enzyme was carried out using MALDI-TOF and the obtained sequence is 204 amino acids in length (21.96 kDa). the results obtained in the present investigation demonstrated that the new fungal strain *Aspergillus flavus* strain S4 was isolated and identified. The L-glutaminase obtained from this strain showed unique properties like optimum activity at neutral pH (7.0), optimum temperature at 30^{o}C with great pharmaceutical applications. Low K_M exhibited by this L-glutaminase indicates that it is advantageous as it has greater affinity with the substrate and provides a scope for its use in pharmaceutical sector.

Key Words: *Aspergillus flavus*; L-glutaminase; characterization; purification.

Introduction

L-glutaminase purification is crucial for improving our comprehension of the enzyme's mechanism of action. The many methods used to purify enzymes follow similar patterns to those used to purify proteins. Enzymes are purified using a generalised strategy that includes initial protein recovery, concentration/primary purification, and eventually high-end resolution utilising chromatographic techniques, despite the fact that the origins of enzymes vary widely (Kalyani *et al.*, 2016). In order to check the activity of all fractions and the overall protein content, an enzyme assay is carried out at each stage of purification. Any specific protein's purification process is determined by a number of variables, including the source material used, the target protein's location (extracellular or intracellular), amount of expression, physiochemical properties of the protein, and the intended use of the protein. L-glutaminase purification does not, however, follow any fixed guidelines (Gupta *et al.*, 2002). Additionally, L-glutaminase can be purified using a variety of chromatographic techniques. The procedure becomes more laborious when there are too many steps, thus using the fewest steps possible to purify concentrated enzyme can be a good option.

Recovery of the enzyme from the source is the first stage in any purification technique; the difficulty of this step depends on whether the enzyme is intracellular or extracellular. The majority of microbial enzymes are extracellular and are released into the fermentation medium; in these circumstances, entire cells are often separated from the medium by centrifugation or, in rare situations, by filtration. While proper cell harvesting and disruption strategies are used in the case of intracellular microbial enzymes (Walsh, 2004). The removal of water becomes important because the enzyme of interest is typically present in the cell-free supernatant in extremely diluted amounts. The volume is made manageable for the subsequent purification processes by the concentration procedure.

Precipitation-based concentration is one of the oldest known techniques. Agents including neutral salts, organic solvents, high molecular mass polymers, as well as the right pH modifications, can all help to promote protein precipitation (Kalyani *et al.*, 2021). The typical agents used for precipitation include neutral salts (ammonium sulphate) and organic solvents that reduce the solubility of the required proteins in an aqueous solution (Kumar and Takagi, 1999). Ethanol and acetone are two organic solvents that are widely used to encourage precipitation.

A combination of one or more chromatographic methods, including affinity chromatography (AC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and gel filtration chromatography, is used to further purify the enzyme.

For this reason, the present study was focused on purification and characterization of L-glutaminase from *Aspergillus flavus* strain S4. Further, the enzyme L-glutaminase was analyzed for its *in vitro* anticancer activity.

Materials and methods

Estimation of L-glutaminase activity

One unit of glutaminase was defined as amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme activity was expressed as units/ml (U/ml). The enzyme activity was calculated by using Imada *et al.*, (1973) method.

Specific activity of L-glutaminase

Specific activity of L-glutaminase was expressed in terms of L-glutaminase activity Units per milligram protein.

$$\text{Specific activity} = \frac{\text{Enzyme activity Units (U)}}{\text{mg Protein}}$$

Purification of L-glutaminase produced from *Aspergillus flavus* strain S4

In order to use biological catalysts in significant commercial applications like food and pharmaceuticals, a purification process was carried out to ascertain their structure and function. Essentially, the goal of purification is to increase a particular activity. The proportion of enzyme activity to protein concentration in the enzyme assay is known as the specific activity. The integrity of an enzyme's original protein structure determines its catalytic activity. Depending on a protein's solubility, size, charge, and binding affinity, it can be purified. The enzyme was tested and the protein concentration was calculated at each stage of the purification.

Ammonium sulphate precipitation/Salting out

The enzyme produced from *Aspergillus flavus* under optimized conditions was centrifuged at 5,000 rpm for 20 min. The collected supernatant was subjected to (30, 50, 70 and 100%) ammonium sulfate precipitation at 4^{o}C. During ammonium sulfate precipitation, the salt has to be added in small portions with constant stirring to prevent increase of high salt local concentration. This mixture was incubated for overnight at 4^{o}C. The precipitate was centrifuged at 5,000 rpm for 20 min in refrigerated centrifuge (REMI, C-24, Mumbai). The pellet obtained was dissolved in phosphate buffer at pH 7.6. The enzyme activity and specific activity were determined.

Dialysis

Dialysis is used to remove the salts. Selection of specific molecular weight cut off dialysis membrane plays an important role in dialysis. The dialysis membrane was primarily washed with the buffer. One end of the membrane was tagged, later the dialysate was added and another end of the membrane was also tagged with nylon thread gently. Then membrane bag was dialyzed against the same buffer in a glass beaker, the contents were gently stirred with magnetic stirrer. The dialysis process was carried out at 4^{o}C for 12 h and the buffer was changed at every 2 h for desalting. The buffer was tested for sulphate ions by Barium chloride ($BaCl_2$) test. The positive test gives the white turbidity of Barium sulphate ($BaSO_4$) indicates the presence of sulphate ions. The sample obtained from dialysis was stored at 4^{o}C for further use.

Ion exchange chromatography

DEAE-cellulose was acquired from Sigma Chemical Company in St. Louis, Missouri, and activated in accordance with the directions provided by the manufacturer. The resin was arranged in a 30-by-2-cm column. Air bubbles were carefully avoided being trapped. Before each run, all of the buffers were filtered and degassed. Phosphate buffer (pH 7.6) was pre-equilibrated in the column. The equilibration buffer was used to elute the unattached proteins. The bound proteins were released using a linear gradient of 0.1 to 1.0 M NaCl, and fractions of 5 ml were collected at a flow rate of 60 ml/h. The proteins' absorbance at 280 nm was measured, and glutamine was used as a substrate to measure the enzyme activity. The active fractions were combined, concentrated, dialyzed at 4 degrees Celsius against distilled water, and lyophilized.

Gel filtration on sephadex G-100

The sample from the preceding stage was dissolved in phosphate buffer (pH 7.6), placed onto a Sephadex G-100 column (30 1.5 cm), pre-equilibrated with phosphate buffer (pH 7.6), and eluted with phosphate buffer. At a flow rate of 12 ml/h, two millilitres (2 ml) fractions were collected, and the protein concentration was checked using an enzyme test and a 280 nm absorbance measurement. The active fractions were combined, dialyzed at 4 oC against the buffer, and lyophilized.

The purity of the sample at each step was checked by polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of native protein (enzyme) was done by the method of Laemmli (1970) in 10% polyacrylamide gel with Tris/Glycine buffer pH 8.3.

Coomassie Brilliant blue staining

Visualization of proteins by Coomassie brilliant blue was performed and the gel was stained with staining solution (0.25 g Coomassie blue, 40 ml methanol, 10 ml glacial acetic acid and 50 ml double distilled water) for overnight. The gel was destained with destaining solution (40 ml methanol, 10 ml glacial acetic acid and 50 ml double distilled water). Destaining solution was changed for 3-5 times for clear visualization of blue colour protein bands.

Determination of molecular weight of the enzyme

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

According to Laemmli's (1970) method, one-dimensional SDS-PAGE was used to determine the glutaminase's molecular mass in 5% stacking gel (pH 6.8) and 12% resolving gel (pH 8.8). Under decreasing circumstances, sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out (sample buffer: 0.5 M Tris-HCl buffer, pH 6.8, 4% SDS, 10% glycerol, and 10% 2-mercaptoethanol). For the purpose of calculating molecular weight, a wide variety of typical recombinant protein markers (Puregene protein markers) were applied. A steady current of 12 mA was used throughout electrophoresis. The sample was loaded into the wells and subjected to electrophoresis until the dye front reached the bottom

of the gel (protein sample in sample buffer was held at 100oC for 2 min). After electrophoresis, the gel was carefully removed, dyed with 0.25 percent Coomassie Brilliant Blue R-250, and then thoroughly cleaned with a 50:10:40 mixture of methanol and acetic acid until the band was completely clear. The standard proteins' migration distance was plotted against their logarithmic molecular weight. From the conventional graph, the molecular weight of L-glutaminase was ascertained.

Molecular weight by gel filtration on Sephadex G-200

The molecular weight of the L-glutaminase was determined using molecular sieve chromatography on Sephadex G-200 according to Andrews' method from 1964. Sephadex was packed in a 1.65 94 cm column with a bed capacity of 185 ml after being inflated in 0.1 M sodium phosphate buffer with a pH 7.6. The same buffer was used to develop and equilibrate the column. Use of Blue Dextran was used to calculate the void volume (Vo). 1 ml-sized fractions were gathered. The calibration curve was created by plotting the elution volume (Ve) versus log molecular weight for the molecular weight standards phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa), chymotrypsinogenA (25 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14 kDa). The calibration curve was used to quantify the L-glutaminase's molecular weight.

Characterization of purified enzyme

Relative activity

It is the percentage enzyme activity of the sample with respect to the sample for which maximum activity is obtained.

$$\text{Relative activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Enzyme activity of control (untreated sample)(U/ml)}}$$

Effect of substrate concentration on L-glutaminase

Effect of different substrate concentrations on purified L-glutaminase enzyme activity was performed by taking the glutamine at various concentrations 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 (mg/ml) dissolved in phosphate buffer (pH 7.6). This mixture was incubated at 30^{o}C for 20 min. The L-glutaminase enzyme assay was performed by following Imada *et al*., (1973) method.

Effect of pH on enzyme activity

The effect of pH on L-glutaminase activity was evaluated over a wide pH range 2.0-10.0, using different buffers such as KCl-HCl (pH 2.0), Citrate (pH 3.0 to 6.0), phosphate (pH 7.6), Tris-HCl (pH 8.0 to 9.0), Glycine-NaOH (pH 10.0) in the reaction mixture. The activity of the enzyme was assayed.

Effect of temperature on the enzyme activity

By performing the assay at various temperatures ranging from 20 to 70 oC and incubating the enzyme (100 g/ml) in phosphate buffer, pH 7.6, in a water bath at various temperatures for 10 min, the influence of temperature on the enzyme activity was determined.

Following heat treatment, the solutions were promptly chilled in ice before being divided into the necessary aliquots for measuring L-glutaminase activity using glutamine as the substrate.

Effect of different metal ions on enzyme activity

Enzyme assays were conducted in the reaction mixture as stated above with various metal ions at a final concentration of 1.0 mM to assess the impact of different metal ions on enzyme activity. The activity that was measured when no metal ions were present was control. $MnCl_2$, $CaCl_2$, $FeCl_2$, $BaCl_2$, $NiCl_2$, $ZnCl_2$, and $CoCl_2$ are among the metal ions that were evaluated. The Imada et al., (1973) approach was followed to carry out the enzyme assay. The enzyme's relative activity (%) was calculated.

Determination of kinetic parameters

The kinetic parameters for purified L-glutaminase were calculated by using various concentrations of glutamine with standard assay conditions. The kinetic rate constants, K_M and V_{max}were determined by following the Michaellis- Menten equation.

$$\mathbf{V= V_{max}\,[S]\,/K_M+[S]}$$

Here, V is the velocity of enzymatic reaction and [S] is the concentration of the substrate.The reaction velocity V_i is a function of the substrate concentration [S] for an enzyme. This relationship is called the Michaelis- Menten equation.

V_i: Initial velocity

[S]: Molecular substrate concentration

V_{max}: Maximum velocity

$K_{M:}$ Substrate concentration when V_i is one-half of V_{max} (Michaelis-Menten Constant). The Lineweaver- Burk equation is used to make the Michaelis- Menten equation linear by taking its reciprocal as given below.

$$\mathbf{1/V= K_M/V_{max}.1/[S]+1/V_{max}}$$

Plotting the reciprocals of the same data points yields a “double-reciprocal” or Lineweaver-Burk plot (Hans Lineweaver and Burk, 1934). The intercept on the y-axis gives us: $1/V_{max}$ (Set [S] = 0 (intercept on the y-axis) and $1/V=1/V_{max}$, and the intercept on the x-axis gives us $-1/K_M$ (set 1/V=0 (intercept on the x-axis) and slope for $1/[S]= -1/K_M$.

Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectra (MALDI-TOF MS)

MALDI- TOF mass spectra are used for the analysis of peptide mass fingerprinting and MS/MS ion search.

In-gel tryptic digestion

With a few adjustments, the Lazarev *et al.*, 2009 method of trypsin digestion was used. A 10% SDS-PAGE was used to resolve purified protein (20 g), and CBB R-250 was used to see the results. The protein bands were removed from the gel, cut into 1 mm3 cubes, and treated with 200 l of 50 mM NH4HCO3 in 40% ethanol for an overnight period at room temperature. The liquid was drained and refilled with 150 ml of 50 mM NH4HCO3 once the stain had been removed. After that, 150 l of acetonitrile was added in place of the NH4HCO3 buffer, pH 8.0, and the mixture was incubated for 10 minutes. The gel bits were dried for a short while after this procedure was performed two times. The tube was filled with 20 microliters of freshly prepared trypsin solution (12.5 mg/l) in 50 mM NH4HCO3 buffer. After 10 minutes of rehydration, excess enzyme solution was removed using the gel loading tips. The excess solution was then replaced with an equivalent volume of fresh 25 mM NH4HCO3 buffer, which was then incubated for 12 hours at 36 degrees Celsius. By adding 10 l of 1% trifluroacetic acid (TFA) for MALDI-TOF MS analysis, the digestion was put to an end.

Sample preparation for MALDI-TOF MS

The 20 µl (20 µg) of trypsin digested purified protein solution was used for the MALDI-TOF MS analysis, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) time-of-flight spectrometer, with a pilsed nitrogen laser (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive high-mass mode. A saturated solution of a-cyno-4-hydroxynnamic acid in a 1:1 mixture of acetone and water along with 0.1% trifluoroacetic acid was used for obtaining the mass spectra.

Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean ± standard deviation calculated using Microsoft excel.

Results and Discussion

Purification of L-glutaminase

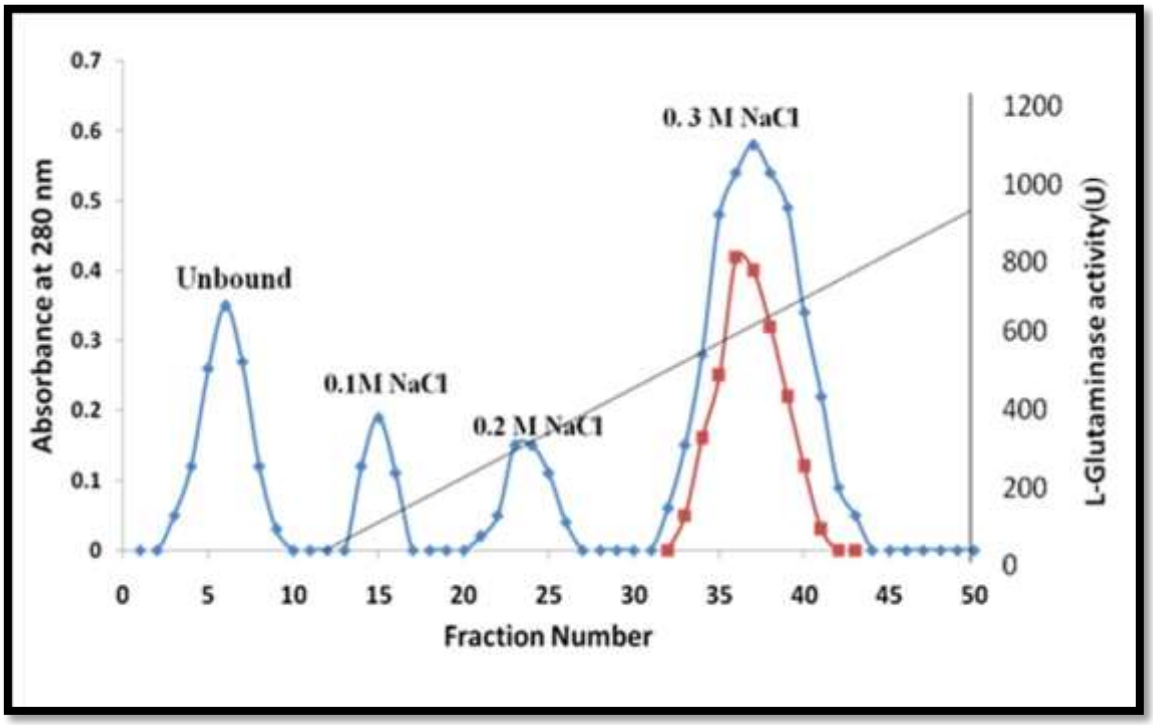
L-glutaminase from *Aspergillus flavus* strain S4 has been purified following conventional methods of protein purification such as ammonium sulphate fractionation, Ion-exchange chromatography and gel filtration.

Ammonium sulphate precipitation

The salt concentration at which a protein precipitates differs from one protein to another. Salting out is useful for concentrating dilute solutions of proteins. Ammonium sulphate precipitation was performed at different concentrations ranging from (30% to 100%) for L-glutaminase recovery from cell free broth. Saturation at 70% ammonium sulphate concentration showed the maximum specific activity (38.22 U/mg protein) with 2.50 fold purification. The precipitate collected from the 70% ammonium sulphate saturation was dissolved in required amount of phosphate buffer at pH 7.6 and dialysis was performed. Since ammonium sulphate has the ability to neutralize charges at the surface of the protein and disrupt the water layer surrounding the protein, it eventually causes a decrease in the solubility of the protein which, in turn leads to the precipitation of the protein by the effect of salt (Volesky and Loung, 1985).

Ion exchange chromatography

The dialyzed enzyme was loaded on DEAE-cellulose column. The stepwise gradient elution pattern of L-glutaminase was shown in Fig.1. For gradient elution, phosphate buffer (pH 7.6) containing NaCl (0.1 to 1.0 M) was used. The pooled distinct peak was obtained showing enzyme activity (11485 U) at 0.30 M concentration of NaCl. The purity of the L-glutaminase after DEAE cellulose was increased by 4.56 fold when compared to the crude enzyme. The specific activity and purification fold of the enzyme after DEAE cellulose ion exchange was (69.61 U/mg protein) with a recovery of 37.61% (Table. 5.1). Reda (2015) has reported L-glutaminase from *Streptomyces canaries* FR KC 460654 with a final specific activity of 132.2 (U/mg protein), 17.9 fold purification and 28% yield recovery after employing ammonium sulphate precipitation followed by DEAE-cellulose column chromatography, gel filtration on sephadex G-100.



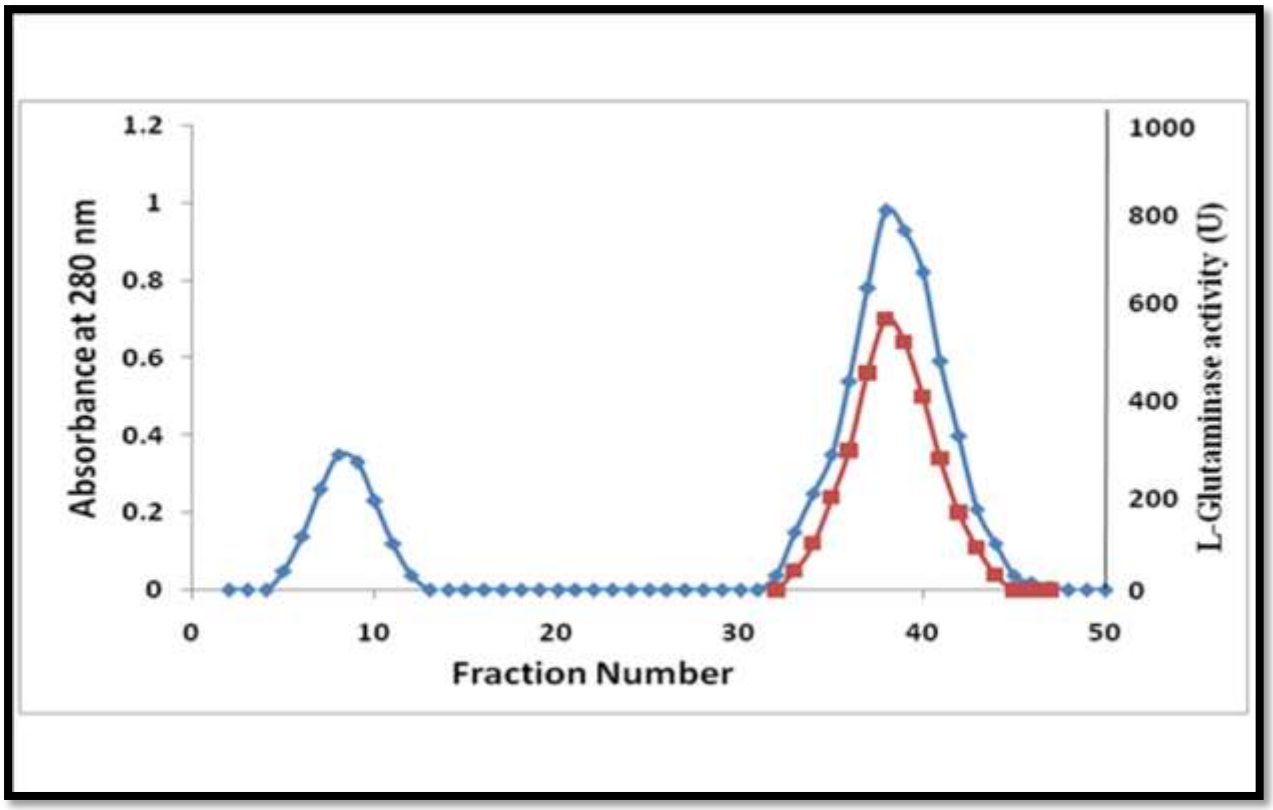
♦----------♦ Protein was monitored by absorbance at 280 nm

■----------■ L-Glutaminase activity

Fig. 1 Elution profile of DEAE cellulose

Gel filtration chromatography

Sephadex G-100 was used in the next step purification. Upon loading the dialyzed sample, sixteen fractions were eluted with phosphate buffer, pH 7.6. The elution profile was depicted in Fig. 2. The active fractions contained enzyme activity (9950 U), with the specific activity (88.84 U/mg protein) and fold purification (5.82) with a recovery of 32.59% were recorded as shown in Table 1.



♦---------♦ Protein was monitored by absorbance at 280 nm

■----------■ L-Glutaminase activity

Fig. 2 Elution profile on Sephadex G-100

Table 1 Summary of purification of L-glutaminase from *Aspergillus flavus* strain S4

Purification steps	Volume of enzyme (ml)	Total enzyme activity (Units)	Total protein (mg)	Specific activity (U/mg protein)	Recovery Or % yield	Fold purification
Crude	100	30530	2000	15.26	100	1
Ammonium sulphate fractionation (70%)	50	15480	405	38.22	50.70	2.50
DEAE cellulose chromatography	30	11485	165	69.61	37.61	4.56
Gel filtration on Sephadex G-100	25	9950	112	88.84	32.59	5.82

As a result of this purification procedure an enzyme with specific activity of 88.84 U/mg protein was obtained. The purification fold was 5.82, whereas the recovery was

32.59%. The yield recovery of the L-glutaminase in this study was decreased at every step of purification.

Homogeneity of L-glutaminase

Polyacrylamide gel electrophoresis (PAGE)

The homogeneity of L-glutaminase was determined by Polyacrylamide gel electrophoresis (PAGE). The gel was loaded with crude enzyme, ammonium sulphate precipitate, DEAE cellulose fraction and Sephadex G-100 fractions of *Aspergillus flavus* strain S4. Polyacrylamide gel electrophoresis (PAGE) of the purified enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of the Sephadex G-100 column. The results were depicted in Fig.3.

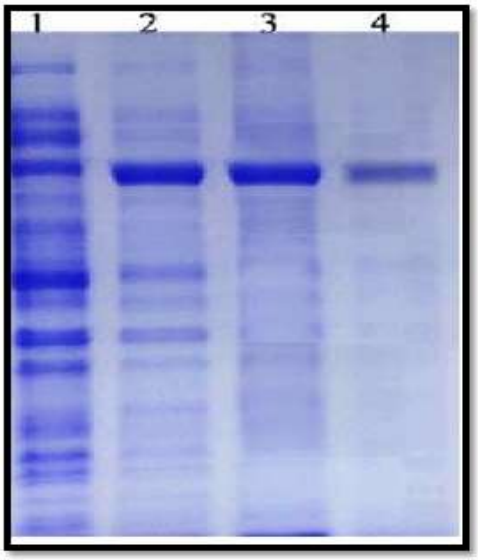


Fig. 3 Native-PAGE

Lane 1: Crude enzyme
Lane 2: Ammonium sulphate fraction
Lane 3: DEAE-Cellulose fraction
Lane 4: Sephadex G-100 fraction

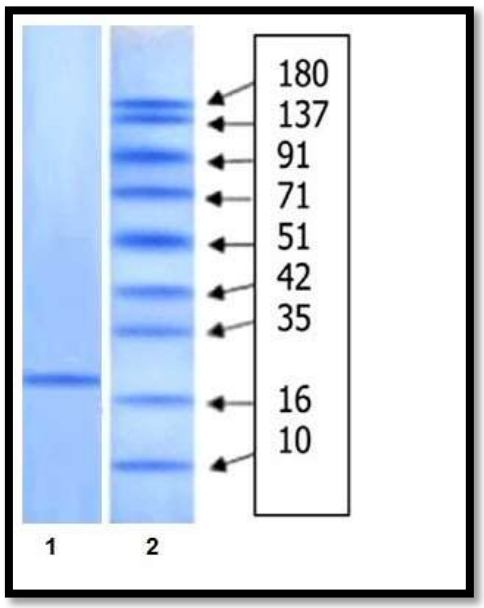
Determination of Molecular weight of L-glutaminase

Molecular weight of the L-glutaminase was determined by using SDS-PAGE and gel filtration chromatography.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

L-glutaminase gave single sharp band on the SDS-PAGE, supporting the monomeric nature of enzyme. Molecular weight of the purified protein was determined by SDS-PAGE by using standard recombinant protein markers (Puregene protein markers) (Fig. 4). The distance migrated by the standard proteins were plotted against their log molecular weight. Molecular weight of L-glutaminase was determined from the standard graph and was found to be 23 kDa (Fig. 5). There has been wide variation (40 and 180 kDa) in the molecular weight of L-glutaminases produced from different sources. L-glutaminase with a higher

molecular weight (148 kDa) was purified from *Pseudomonas aurantiaca* by Imada *et al*., (1973). While 40 kDa molecular weight L-glutaminase was obtained from *Pseudomonas nitroreducens* (Tachiki *et al.*, 1996), the lowest molecular weight recorded for amidase enzyme produced by microorganisms. Most of the L-glutaminases are monomers; however, some of the bacterial glutaminases were reported as dimers and tetramers.



Lane 1: Purified L-glutaminase

Lane 2: Recombinant protein markers

Fig. 4 SDS-PAGE

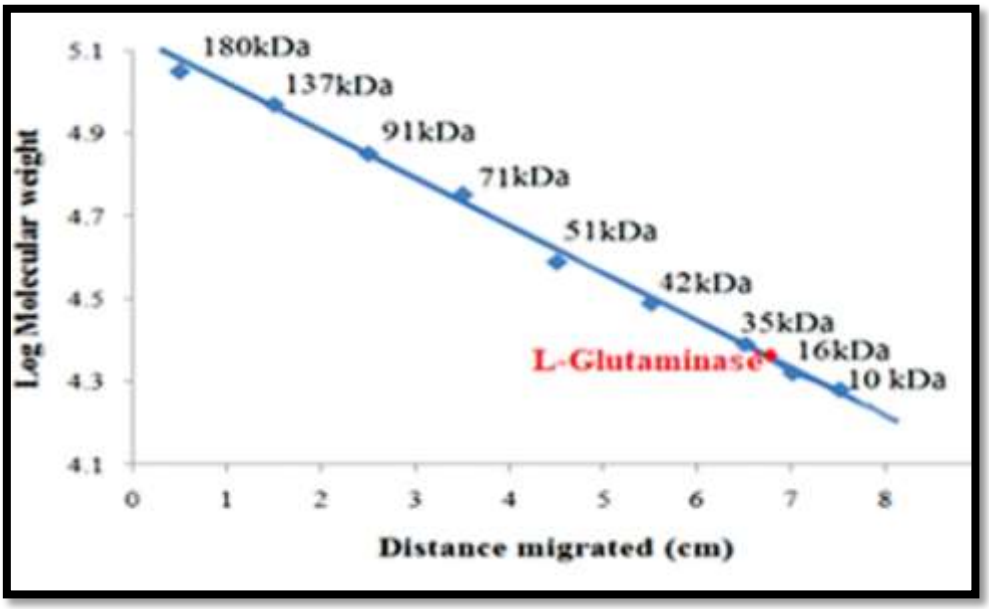


Fig. 5 Molecular weight determination of L-glutaminase by SDS-PAGE

Molecular weight determination of L-glutaminase by Gel filtration on Sephadex G-200

Gel filtration of L-glutaminase on Sephadex G-200 was carried out as described under methods. L-glutaminase was eluted out as a single protein with a corresponding activity peak (Fig 6). The molecular weight of L-glutaminase as calculated from the plot (Ve /Vo versus log molecular weight) was found to be 22.8 kDa (Fig 7).

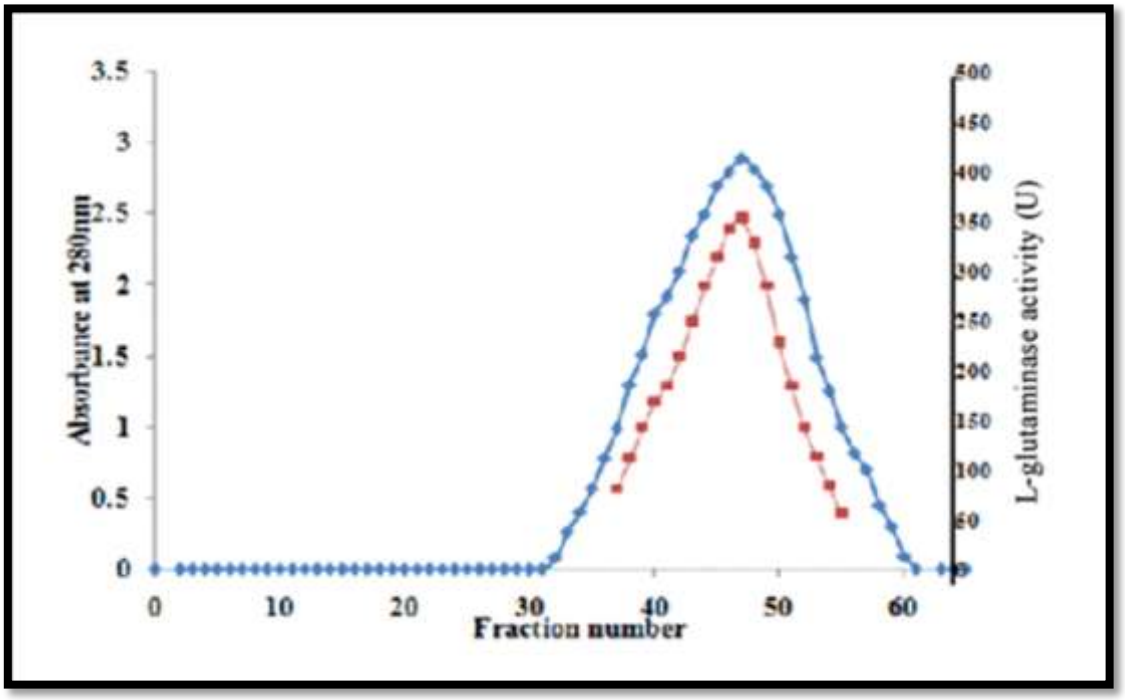


Fig. 6 Elution pattern of L-glutaminase on Sephadex G-200 column

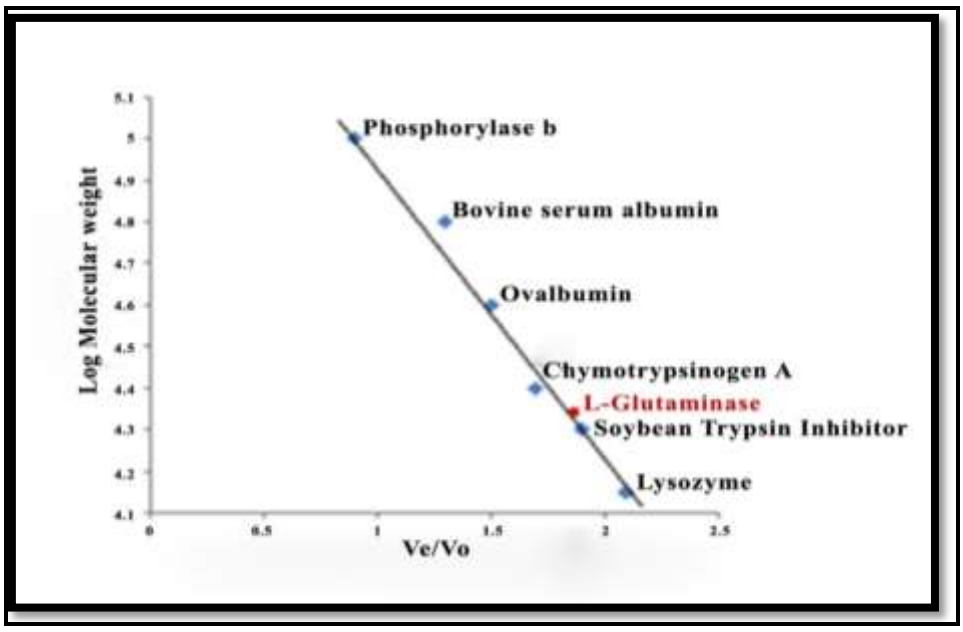


Fig. 7 Molecular weight determination of L-glutaminase by gel filtration on Sephadex G- 200 column

Characterization of purified L-glutaminase

Effect of glutamine concentration on purified L-glutaminase activity

The effect of different glutamine concentrations (1-5%) on purified L-glutaminase was studied. Maximum (382±12 U/ml) and minimum (150±23 U/ml) enzyme activities were observed with 4% and 1% of glutamine concentrations respectively. As the substrate concentration increased above 4%, L-glutaminase activity dropped sharply which may be due to the substrate inhibitory effect. Increase in the enzyme activity was observed from 1% to 4% of glutamine concentration. The results were depicted in Fig. 8.

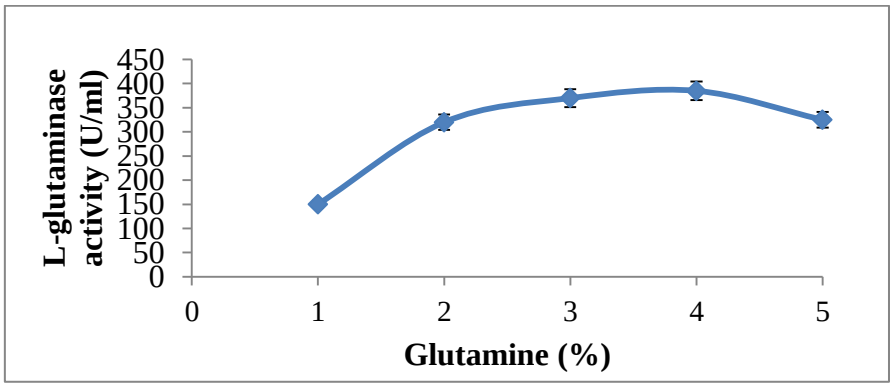


Fig. 8 Effect of glutamine concentration on Purified L-glutaminase
The bars indicate the standard deviation of three replicates analyzed.

Effect of pH on purified enzyme

Effect of different pH on purified L-glutaminase activity was studied. Maximum (378.5±24 U/ml) enzyme activity was observed at pH 7.0 and minimum (79.25±32 U/ml) enzyme activity was observed at pH 2.0. Increase in pH above 7.0 results in decrease in L-glutaminase activity. It may be due to lack of stability towards increase in pH. The enzyme activity was gradually increased from pH 3 to pH 7.0. The results were depicted in Fig. 9.

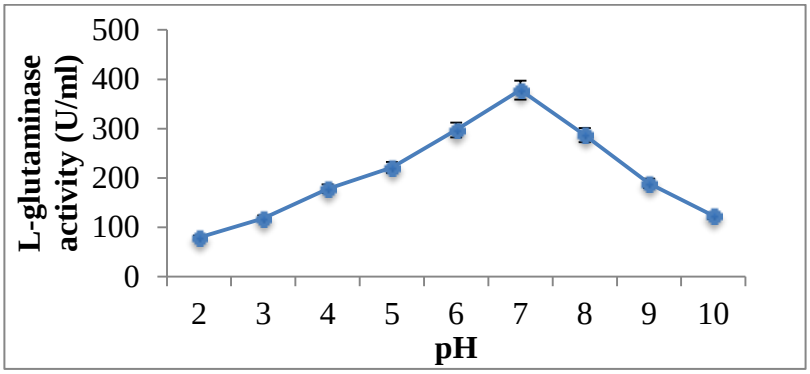


Fig.9 Effect of pH on purified enzyme activity
The bars indicate the standard deviation of three replicates analyzed.

Effect of temperature on purified enzyme

The effect of different temperatures on purified L-glutaminase was studied. The maximum activity (410.65±3.2 U/ml) at 30^{o}C and minimum activity (267.24±6.0 U/ml) at 70^{o}C was observed. Increase in enzyme activity was observed from 20^{o}C to 30^{o}C further increase in temperature decreased the enzyme activity which may be attributed to the denaturation of the enzyme due to the structural and conformational changes of the protein

molecule. The results are depicted in Fig.10. It is evident from the literature that a wide range of temperature optimum was exhibited by L-glutaminase purified from different bacterial sources and the optimum temperature was above 40°C. The temperature optimum of glutaminase has been reported as 37°C for *Bacillus pasturii* (Klien *et al*., 2002), 35°C for *Bacillus cereus* (Singh *et al.*, 2013), 40°C for *Streptomyces canarius* (Reda, 2015), 50°C of *Bacillus subtilis* RSP-GLU.

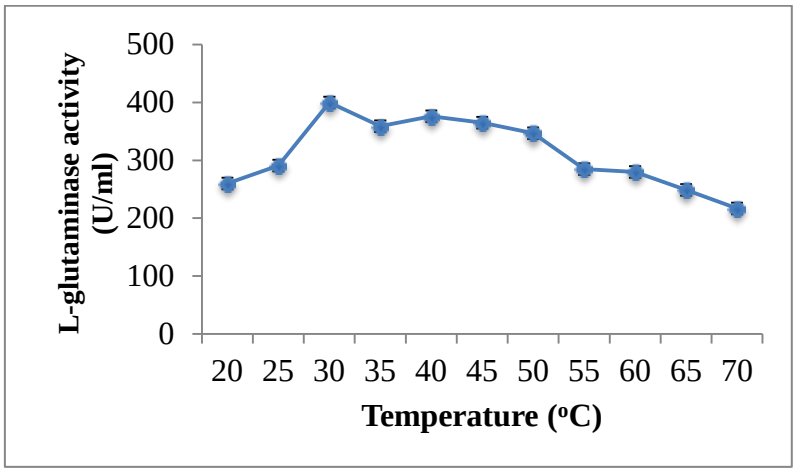


Fig.10 Effect of different temperatures on purified enzyme activity
The bars indicate the standard deviation of three replicates analyzed.

Effect of metal ions on purified L-glutaminase

Effect of metal ions on the activity of L-glutaminase was studied. The impact of various metal ions on catalytic potency of isolated L-glutaminase was evaluated by pre-incubation of the enzyme with each metal ion (0.1 to 1 mM) for 30 min without substrate. Maximum relative activity was observed with Manganese chloride (111.8%) followed by Cobalt chloride (109.2%), Calcium chloride (107.4%) and the activity was decreased on treatment with Barium chloride, (63.1%), Ferrous chloride, (59.2%) Nickel chloride (52.6%) and Zinc chloride (43.4%) at a concentration of 1 mM when compared to enzyme activity of untreated L-glutaminase (100%). The results were depicted in Fig. 11. From the results, it was evident that metal ions such as Manganese chloride, Cobalt chloride, Calcium chloride were found to increase L-glutaminase activity and Nickel chloride, Zinc chloride, Ferrous chloride, Barium chloride were shown to exhibit inhibitory effect on enzyme activity. The metal ions tested at 0.1 mM and 0.5 mM concentrations did not show any effect on enzyme activity.

Nagwa *et al*., (2012) reported L-glutaminase activity from *Streptomyces avermitilis*. The enzyme activity was enhanced by $MgSO_4$ and NaCl with 19.3 and 19.8 (U/ml) activities and 104.9 and 107.6% residual activity respectively. On the other hand, $CuSO_4$, $ZnSO_4$ and EDTA decreased the activity. These results are in accordance to those reported by Lu *et al*., (1996) who indicated that the activity of glutaminase from *Actinomucor taiwanensis* was enhanced by addition of $MgSO_4$ and NaCl.

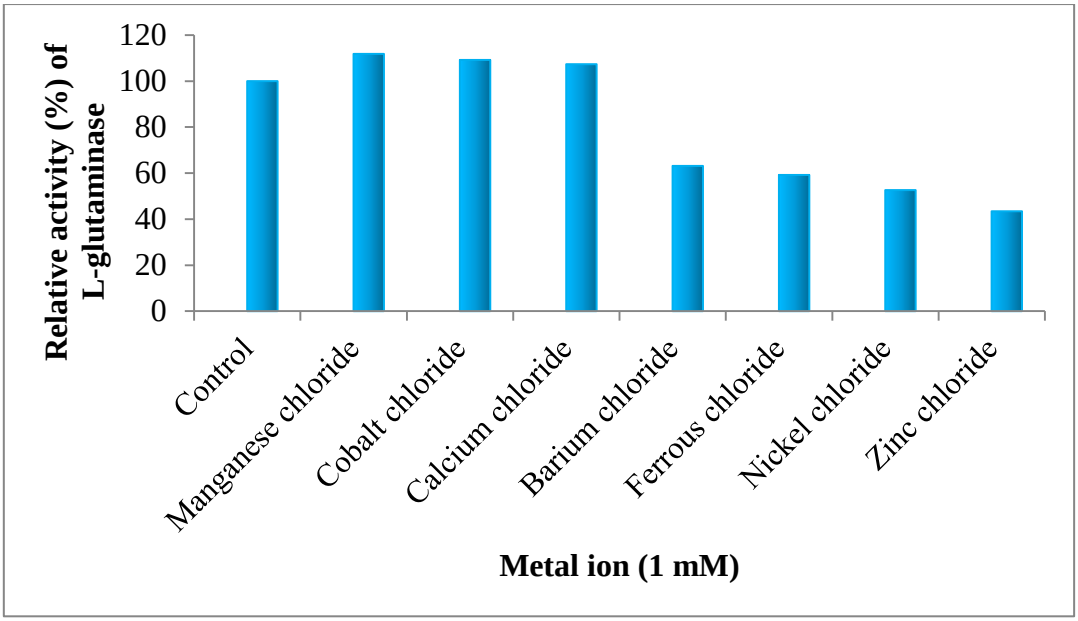


Fig.11 Effect of metal ions on purified enzyme activity

Kinetic studies of L-glutaminase

Effect of glutamine concentration on glutaminase activity was determined by incubating the L-glutaminase from *A. flavus* strain S4 with 1-5% of glutamine at temperature 30^{o}C and pH 7.0. It was observed that the L-glutaminase activity was increased with increase in substrate concentration and maximum L-glutaminase activity was observed at 4.0% glutamine, beyond which there was a moderate inhibition in activity was recorded (Fig.12).

Substrate -Velocity curve

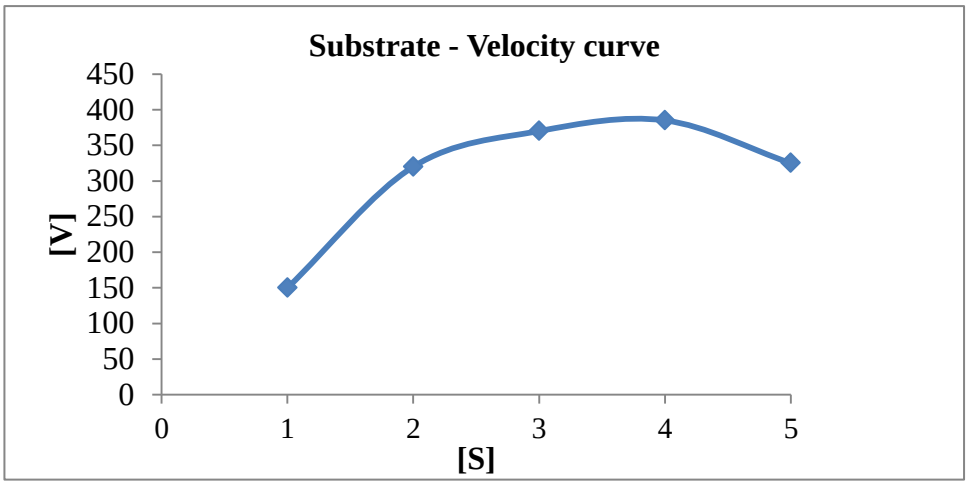


Fig.12 Hyperbolic curve (substrate-velocity curve) of enzyme catalyzed reaction

The hyperbolic curve failed to provide a precise number for K_M and Vmax. L-glutaminase activity was therefore displayed as a linear Lineweaver-Burk plot (Fig.13), and the K_M and Vmax values were found to be 5.28 mM and 454.5 U/ml, respectively. An

increased binding affinity of this L-glutaminase to substrate is indicated by a lower value of K_M.

Lineweaver-Burk plot

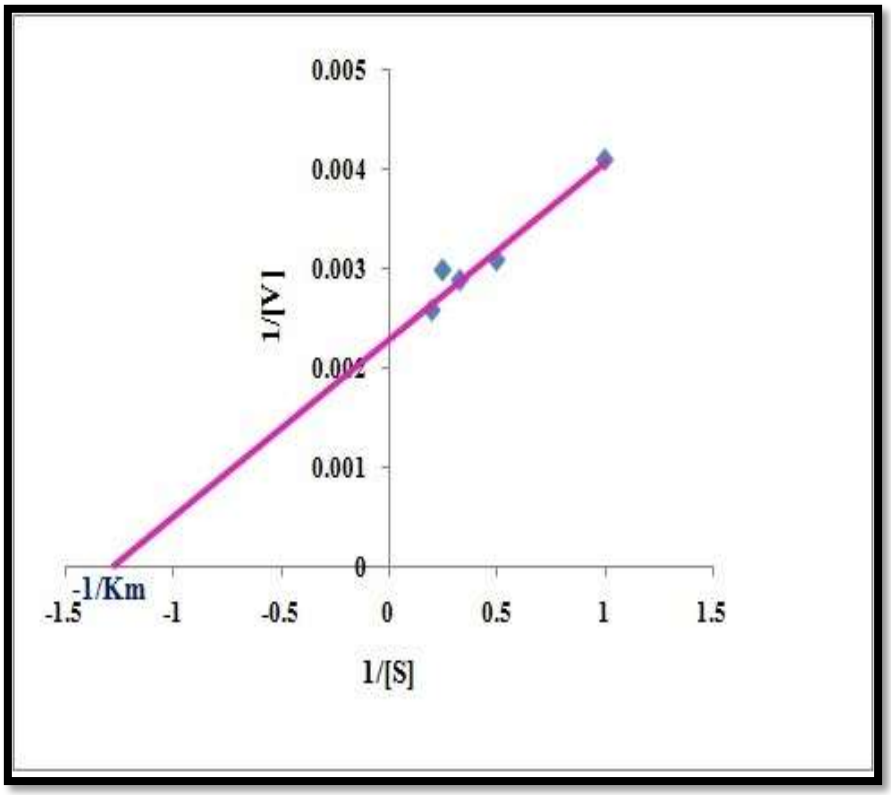


Fig.13 Lineweaver-Burk plot for L-glutaminase showing Michaelis-Menton Kinetics

The KM and Vmax for the L-glutaminase from Bacillus sp. were 66.83 mM and 0.755 mM/min, respectively, according to the investigations by Sinha and Nigam (2016). Additionally, it was discovered that the L-glutaminase displayed its greatest activity at 37 °C with a 3 hr half life. L- glutaminase isolated from *Debaryomyces* sp. CECT 11815 had a higher KM value (4.5 mM) than previously reported by Dura et al., in 2002. L-glutaminase activity purified from Lactobacillus rhamnosus exhibited typical Michaelis-Menten behaviour, according to Weingand-Ziade *et al.*, 2003, with an affinity constant, KM, of 4.8 mM for L-glutamine.

***In silico* analysis of L-glutaminase**

Peptide Mass Fingerprinting (PMF)

By using Matrix Assisted Laser Desorption Ionization/Mass Spectroscopy (MALDI/MS) analysis, the isolated protein was further characterised. A very effective tool for protein analysis is the combination of electrophoretic separation with mass spectrometric analysis. While utilising less protein and less work, peptide mass fingerprints can be just as discriminating as linear peptide sequences. This frequently enables a sample protein to be quickly identified before being subjected to protein sequence analysis (Pappin *et al.*, 1993).

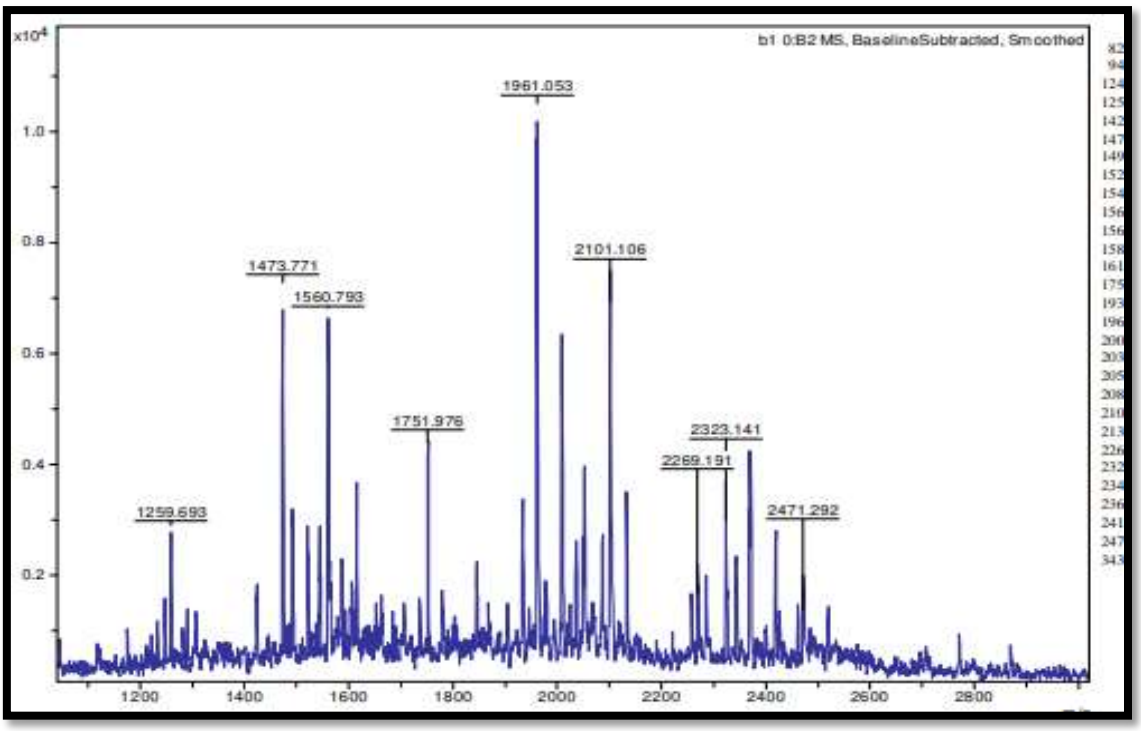


Fig.14 MALDI Spectra

The MALDI spectrum of the L-glutaminase was shown in the Fig. 5.14. Matrix-assisted laser desorption ionization (MALDI) mass spectra recorded in the mass range of 1000-40000 Da. Peptide masses obtained were determined using the Mascot program for protein identification. For search parameter mass values were monoisotopic and peptide mass tolerance was +/- 1.2 Da.

The molecular weight of L-glutaminase deduced from MALDI-TOF analysis was found to be 21.96 kDa which was similar to that obtained by SDS-PAGE (23.0 kDa) and gel filtration chromatography (22.8 kDa). Similar reports were also observed by Hattori *et al*., (2005). The total number of amino acids of L-glutaminase were 204 (Fig. 14) and the isoelectric point of the enzyme was found to be 5.6.

Conclusion

Crude enzyme extract from *Aspergillus flavus* strain S4 was subjected to purification by conventional chromatographic techniques. Ammonium sulphate saturation at 70% showed increased specific activity followed by ion exchange chromatography (0.1 to 1.0 M Nacl elution) and gel filtration chromatography (Sephadex G-100). Purity of the enzyme at each step was checked by performing native PAGE. As a result of this procedure, a protein with specific activity of 88.84 U/mg protein was obtained. The purification fold was 5.82, where as the recovery was 32.59%. Molecular weight of the purified protein was determined by reducing SDS-PAGE (23 kDa) and gel filtration chromatography (Sephadex G-200, 22.8 kDa). Protein sequencing of the purified enzyme was carried out using MALDI-TOF and the obtained sequence is 204 amino acids in length (21.96 kDa). the results obtained in the present investigation demonstrated that the new fungal strain *Aspergillus flavus* strain S4 was isolated and identified. The L-glutaminase obtained from this strain showed unique properties

like optimum activity at neutral pH (7.0), optimum temperature at 30^{o}C with great pharmaceutical applications. Low K_M exhibited by this L-glutaminase indicates that it is advantageous as it has greater affinity with the substrate and provides a scope for its use in pharmaceutical sector.

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