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Submerged fermentation, purification, and characterization of L-asparaginase from *Streptomyces* sp. isolated from soil

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ABSTRACT

Actinomycetes are most valuable microbes because they are reported to produce different kinds of novel antibiotics and bioactive compounds. L-asparaginase enzyme is a chemotherapeutic agent used in the treatment of cancer. The present study reports production and characterization of purified L-asparaginase from an actinomycetes isolate. The isolate (A-164) was found to be a potent producer of L-asparaginase with an enzyme activity of 26.67 international unit (IU)/ml. Biochemical characterization of the isolate indicated that it belongs to genus *Streptomyces*. Laboratory scale enzyme production studies were undertaken using the standard medium. Further, the enzyme was purified by salt precipitation, anion exchange chromatography, and size exclusion chromatography. The purified enzyme exhibited the specific activity of 390 IU/mg with 60-fold purification and yield of 42% with a molecular weight of 34 KDa. The purified enzyme demonstrated the highest activity at pH 6.0 and temperature 35°C. The enzyme retained its activity till temperature of 50°C, indicating that it is a thermostable enzyme. A study on the effect of various additives on enzyme activity was also undertaken. The K_m and V_{max} values for the purified L-asparaginase were 0.065 mM and 20.80 IU/ml, respectively. The enzyme can further be used for health care and industrial applications.

1. INTRODUCTION

Actinomycetes are omnipresent and are most valued organisms. They are branching bacteria with the fungal type of morphology. They are Gram-positive bacteria with DNA rich in G+C content (57–75%), frequently filamentous and sporulating. Most of the actinomycetes are saprophytic bacteria and thrive in a wide range of habitat. Actinomycetes are major source of important bioactive compounds having high commercial value. They are reported to produce a wide range of bioactive metabolites which include antibiotics, antitumor agents, immunosuppressive agents, and enzymes [1]. *Streptomyces* is the largest antibiotic-producing genus, producing both antibacterial agents (Erythromycin, Neomycin, Streptomycin, Tetracycline, Vancomycin, etc.) and antifungal agents (Nystatin, Amphotericin B, Natamycin, etc.). Other genera which are frequent producers of antibiotics include *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora,* and *Streptosporangium* [2]. An actinomycetes metabolite, Adriamycin avert DNA replication process and hence is used in the treatment of leukemia. Another metabolite, rapamycin has immune suppression property and is used in organ transplantation [3]. Actinomycetes are also known for their capacity to produce various enzymes such as amylase, protease, lipase, cellulase, pectinase, xylanase, laccase, asparaginase, and glutaminase [4].

Microbial L-asparaginases (L-asparagine amidohydrolase, E.C.3.5.1.1) are generally used as therapeutic agents in the treatment of human cancers [5]. L-asparaginase enzyme catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia [6]. L-asparagine is required by both normal and tumor cells. Under natural conditions, L-asparagine is synthesized by enzyme L-asparagine synthetase using substrates aspartic acid and glutamine. L-asparagine can also be absorbed from external sources. Cancerous cells lack the enzyme L-asparagine synthetase and hence are unable to synthesize their own L-asparagine [7]. Hence, these cancerous cells are dependent on L-asparagine present in the blood of the patient. Tumor cells require an enormous quantity of L-asparagine for their rapid growth. L-asparagine is limited in the circulating pool when the enzyme L-asparaginase is present resulting in the death of tumor cells [8]. This approach helps in the use of L-asparaginase as an anti-cancerous agent [9]. In addition to clinical applications of L-asparaginase have potential application in food industry. It can be used in eliminating acrylamide formation during frying of starchy foods. Amino acids and reducing sugars present in starchy food, when heated, give a characteristic aroma and flavor. When L-asparagine and reducing sugars react, it leads to the formation of acrylamide. Addition of L-asparaginase enzyme before baking or frying the food leads to conversion of L-asparagine

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to aspartic acid. Thus, the enzyme reduces the risk of acrylamide formation in food [10].

Various actinomycetes such as *Streptomyces griseus* [11], *Nocardia* sp. [12], and Actinomycete strain LA-29 [13] were explored for L-asparaginase production. L-asparaginase enzyme purification was carried out from various organisms such as actinobacteria [14], *Streptomyces halstedii* [15], *Streptomyces tendae* [16], and marine *Streptomyces* spp. [17]. Different purification techniques such as salt precipitation [13,17] and column chromatography [13,14,18] were used during purification steps. Enzyme was characterized with respect to various parameters such as temperature, pH, and molecular weight. Furthermore, studies were undertaken to assess the effect of metal ions and kinetic parameters [14,15,17-23]. Production and purification of extracellular L-asparaginase are easier compared to intracellular enzymes. Hence, the present study aims at isolation of actinomycetes, screening for L-asparaginase production and purification, and characterization of extracellular L-asparaginase.

2. MATERIALS AND METHODS

2.1. Isolation, Screening, and Characterization of Actinomycetes

Soil samples from different farms, in and around Hubballi, Karnataka, were collected. 22 isolates of actinomycetes were obtained from soil by dilution plate technique using starch casein (SC) agar plates (Composition: Soluble starch-1%; K_2HPO_4-0.2%; KNO_3-0.2%; NaCl-0.2%; casein-0.03%; $MgSO_4.7H_2O$-0.005%; $CaCO_3$-0.002%; $FeSO_4.7H_2O$-0.001%; and agar-1.5%) [24]. The plates were incubated for 7 days at 30°C. Single discrete isolates were screened for L-asparaginase production. The isolates were maintained on SC agar and sub-cultured periodically. Isolates were screened for production of L-asparaginase using the method as described by Mahajan *et al*. [25]. Modified M-9 medium (composition: Na_2HPO_4-0.6%; KH_2PO_4-0.3%, NaCl-0.05%, L-asparagine-0.5%, $MgSO_4.7H_2O$-2ml (1 M), $CaCl_2.2H_2O$-1ml, (0.1 M), glucose-5%, agar-2%, and pH-7) was incorporated with a pH indicator (0.007 % Bromo Thymol Blue) [26]. L-asparaginase activity was identified by a change in the color of medium from yellow to green or blue. Modified M-9 medium without dye served as one control. Another control was Modified M-9 medium without L-Asparagine but supplemented with $NaNO_3$. The isolate was identified according to the standard methods as described by Shirling and Gottileb [27]. Various properties of the isolates were studied as reported earlier [4].

2.2. Production of L-Asparaginase and Enzyme Assay

Production of L-asparaginase was undertaken by the method as described by Peterson and Ciegler [28]. The isolate was inoculated in 100 ml of Tryptone glucose yeast extract medium (composition: Glucose-0.l%; K_2HPO_4-0.l%; yeast extract-0.5%; and tryptone-0.5%, pH-7.0) and incubated in rotary incubator shaker at 30°C, 120 rpm for 120 h. At a fixed interval, known volume of samples was withdrawn and centrifuged in a cooling centrifuge (Eppendorf) at 8000 × g for 15 min at 4°C. The supernatant served as the crude enzyme preparation. Enzyme was assayed using the Berthelot reaction which estimates the amount of ammonia liberated [29,30]. To 0.5 ml of crude enzyme, 0.5 ml of 1 mM L-asparagine and 1 ml of 0.5 M Tris-HCl buffer (pH 6) were added and incubated at 37°C for 10 min. The reaction was stopped by adding 1 ml of phenol reagent, 1 ml of 1N sodium hydroxide, and 1 ml of alkaline hypochlorite. The mixture was kept for 15 min. Absorbance was recorded at 600 nm using ultraviolet-visible spectrophotometer (Make: LABINDIA) using appropriate blank. Quantity of enzyme required to liberate 1 µM of ammonia per min is defined as one international unit (IU) of L-asparaginase enzyme. Lowry's method was used to determine protein content [31].

2.3. Enzyme Purification

L-asparaginase was purified using following steps: Ammonium sulfate salt precipitation and column chromatography (diethylaminoethyl [DEAE]-Cellulose and Sephadex G-50). The culture medium was centrifuged in a cooling centrifuge (Eppendorf) at 6000 × g for 8 min. The supernatant obtained was precipitated using ammonium sulfate at a salt concentration ranging from 40% to 80%. It was left overnight at 4°C and then centrifuged at 6000 × g for 20 min at 4°C. The precipitate was dissolved in 0.5 M Tris-HCl buffer (pH 6), and enzyme activity was determined. The precipitate with the highest activity was dialyzed overnight against the same buffer at 4°C and assayed. This was used for further purification. The crude enzyme was applied to a column of DEAE-Cellulose column (15 cm × 2 cm), and protein was loaded. The column was equilibrated with 0.5 M Tris-HCl buffer (pH 6). The elution was carried out using gradient of NaCl (0.1 M–0.5 M) with a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. It was followed by monitoring the A_{280} of each fraction to determine protein content. The active fractions were assayed for enzyme activity. L-asparaginase active fractions were pooled and desalted by dialysis against 0.5 M Tris-HCl buffer (pH 6). Purification of L-asparaginase was continued with Sephadex G-50 at room temperature. The column (15 cm × 2 cm) was equilibrated with 0.5 M Tris-HCl buffer, and then enzyme was loaded onto the column. It was eluted with the same buffer in the beginning and then using a linear gradient of KCl (0–200 mM) in 0.5 M Tris-HCl, buffer. Flow rate of 0.5 ml/min was maintained. 1 ml fractions were collected and estimated for protein content and assayed for enzymatic activity. Absorbance measurement (A_{280}) method was used to estimate protein content. L-asparaginase active fractions were pooled and used for further analysis [13,17,21,23].

2.4. Characterization of the Purified L-Asparaginase

2.4.1. Effect of pH, temperature and substrate concentration on enzyme activity

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to evaluate the molecular weight of the purified L-asparaginase enzyme [32]. The gel was stained with Coomassie Brilliant Blue R-250. Standard molecular weight marker (Bangalore Genei, India) was used to compare the molecular weight of L-asparaginase [21]. The effect of temperature on L-asparaginase activity was studied using Berthelot reaction [18,30]. The temperature of incubation was varied between 20°C and 50°C with step increase of 5°C, and the optimum temperature was evaluated. The pH was varied between 3 and 9 with a step increase of 1 unit. Acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 8.0–9.0) at a concentration of 100 mM were used during the study [14,18,21]. Commercial L-asparagine of concentration between 0.2 mM and 2 mM was used as the standard substrate. The enzyme assay was performed by Berthelot reaction. The evaluation of Michaelis–Menten and Lineweaver–Burk plots yielded the kinetic parameters for the L-asparaginase activity.

2.4.2. Effect of metal ions and additives

Different salt solutions - sodium chloride (Na^+), potassium chloride (K^+), magnesium sulfate (Mg^{2+}), calcium chloride (Ca^{2+}), ferrous sulfate (Fe^{2+}), copper sulfate (Cu^{2+}), mercury chloride (Hg^{2+}), and chemicals, namely, dithiothreitol (DTT), SDS, ethylene diamine tetra acetic acid (EDTA), and mercaptoethanol (ME) were used

to determine their effect on enzymatic activity. This mixture was incubated at room temperature for 30 min, and then enzyme assay was performed [17-19,21].

3. RESULTS AND DISCUSSION

3.1. Isolation and Characterization of Actinomycetes and Screening for L-Asparaginase

Twenty-two actinomycetes were isolated from different soil samples. All the isolates were screened for L-asparaginase production using modified M-9 media with bromothymol blue (BTB) as an indicator. L-asparaginase catalyzes the breakdown of asparagine to aspartic acid and ammonia. Due to the production of ammonia, pH of the media increased. Hence, addition of the pH indicator changed its color and this forms the basis of assay. BTB was used as pH indicator in the medium. Plates/broth containing BTB were yellow at lower pH value and changed to dark blue at higher pH value. Hence, a dense dark blue color is produced if microorganism produces L-asparaginase [25]. In control plate without dye, organism growth was observed with no change in media color. In another control plate, wherein L-asparagine is substituted with $NaNO_3$, growth was not observed indicating, organism was unable to utilize $NaNO_3$. Of all the isolates, only eight actinomycetes were able to produce L-asparaginase as indicated by a change in the color of the media from yellow to blue. Among these isolates, one isolate (A-164) had the ability to change the media color in short duration [Figure 1], and enzyme production was more (9.8 IU/ml) and hence was selected for further studies. Based on the phenotypic, microscopic and biochemical properties of the organism [Figure 2 and Table 1], the isolate was identified as genus *Streptomyces*. Figure 2a shows typical colony morphology for the isolate *Streptomyces* sp. Figure 2b and c shows microscopic view of the organism highlighting the presence of hyphae and asexual spores. The color of mature aerial mycelium was white, and that of substrate mycelium was light yellow without any melanoid pigmentation. The spores were observed to be knob-shaped. The isolate could able to degrade casein, tyrosine, and xanthine, typical properties of genus *Streptomyces* [33,34]. Various researchers have reported production of L-asparaginase by *Streptomyces* spp. such as *S. halstedii* [15], *S. tendae* [16], and *Streptomyces ginsengisoli* [35].

3.2. Production and Purification of L-Asparaginase

The isolated *Streptomyces* sp. was inoculated in production media and incubated for 120 h. Samples were drawn and assayed for enzyme activity. Enzyme activity was carried out using Berthelot reaction, wherein ammonia is estimated by phenol nitroprusside method. This reaction is based on the principle that the ammonia released reacts with Berthelot's reagent to form blue colored product and absorbance is measured [36]. Here, ammonia reacts with hypochlorite and two molecules of phenol to form indophenols which produce characteristic

Table 1: Biochemical characteristics of *Streptomyces* sp.

Characteristics	
Gram staining	**Gram-positive**
Motility	Nonmotile
Acid-fast	Negative
Biochemical characteristics	
Catalase	+
Oxidase	+
Cellulose degradation	+
Gelatin utilization	+
Starch degradation	+
Casein hydrolysis	+
Nitrite reduction	+
Utilization of carbon sources	
Glucose	+
Galactose	+
Xylose	+
Sucrose	+
Fructose	+
Xanthine	+
Arabinose	−
Lactose	−
Rhamnose	−
Utilization of nitrogen sources	
L-Arginine	+
L-glutamine	+
Tyrosine	+
L-Tryptophan	+
L-Tyrosine	+
L-Glycine	+
L-Asparagine	+
L-Cystine	+
L-phenyl alanine	+
L-Histidine	+
L-Hydroxyproline	+
L-Lysine	+

+Positive test, −Negative test.

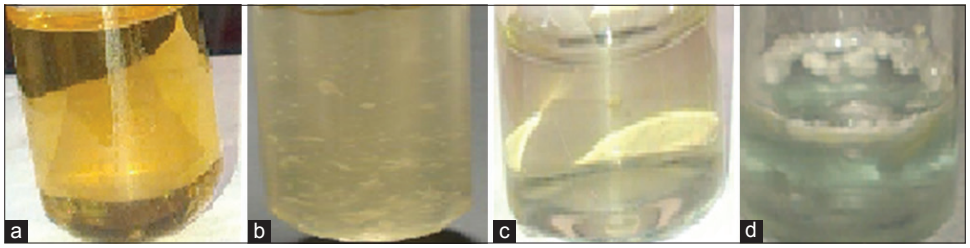

Figure 1: Qualitative screening for L-asparaginase production by *Streptomyces* sp. The broth shows change in color of modified M9 media from yellow to blue due to the production of L-asparaginase. (a) M-9 media containing both L-asparagine and bromothymol blue (BTB) pH indicator without inoculum, (b) control tube containing M-9 media without BTB pH indicator, (c) control tube containing M-9 media without L-asparagine, (d) M-9 media containing L-asparagine, BTB pH indicator, and inoculum.

blue color. The absorbance of indophenols can be measured at 600 nm. It was observed that *Streptomyces* sp. had a highest enzyme activity of 26.67 IU/ml at 72 h [Figure 3] and then the activity decreased. This decrease may be due to substrate limitation or product inhibition. Thaer and Ellaiah [37] also observed that L-asparaginase activity from *Streptomyces* strain A2 was highest after 72 h of incubation. L-asparaginase enzyme from culture media of *Streptomyces* sp. was purified by salt precipitation, ion exchange chromatography, and size exclusion chromatography. Enzyme was precipitated at 85% salt saturation. The precipitate was dissolved and dialyzed against buffer overnight at 4°C and assayed for enzyme activity. This was used for further purification steps. The elution profile of the chromatography on DEAE-cellulose column [Figure 4] showed one peak with L-asparaginase activity. This was applied onto a Sephadex G-50 column [Figure 5]. In the elution profile, L-asparaginase enzyme was eluted at the initial stage of chromatography, indicating that the enzyme was of high molecular weight. The purified enzyme exhibited the specific activity of 390 IU/mg with 60-fold purification and yield of 42% [Table 2]. Various researchers have used different techniques for purification of L-asparaginase enzyme from different actinomycetes and obtained specific activity in the range of 660–5035 IU/mg and yield in the range of 25–60% [14-17,21].

3.3. Characterization of the Purified L-asparaginase

The molecular weight of purified enzyme was found to be 34 kDa [Figure 6]. Prista and Kyridio [38] reported 33 kDa as the apparent molecular weight of L-Asparaginase from *Thermus thermophilus.* Kumar and Selvam [21] observed that the subunits of L-asparaginase had a molecular mass of approximately 33.3 kDa. L-asparaginase was characterized for various parameters. At pH 6, enzyme had a maximum activity of 32.8 IU/ml [Figure 7]. Above or below this pH, activity decreased indicating optimum pH for L-asparaginase is 6.0. The optimum pH of L-asparaginase from *Streptomyces radiopugnans* MS1 was also 6.0 [21]. The optimum pH for L-asparaginase from various sources is in the range of pH 7.5–9.0 enzymes show higher catalytic activity at their optimum pH value (or pH range). At higher or lower pH, enzyme activity decreases due to change in ionization states of amino acids. In the active site of enzyme, side chains of amino acids act as weak acid or base depending on their maintenance of ionization state. In a protein/enzyme, the ionized side chain plays an important role in the interactions that maintain the three-dimensional structure. Removal of a proton disturbs an ionic interaction essential to stabilize the native conformation of the enzyme. This destabilization results in a decrease in enzyme activity. Any increase or decrease in hydrogen ions concentration [H+], changes pH of the reaction mixture leading to drastic changes in the three-dimensional structure of protein which results in enzyme denaturation [39]. Furthermore, pH affects the enzyme activity by changing the ionization state of the substrate [40]. To analyze the effect of temperature, the enzyme was incubated at temperatures 20–50°C during enzymatic reaction. The optimum temperature for L-asparaginase was 35°C, at which maximum enzyme activity (28.8 IU) was demonstrated [Figure 8]. Similar observations were reported for L-asparaginase from *Erwinia carotovora* in which higher enzyme activity was reported at 35°C [22]. As temperature increases, L-asparaginase enzyme activity from *Streptomyces* sp. increased in the beginning reached an optimum and then decreased. It was observed that at a temperature of 50°C, L-asparaginase activity was retained indicating that it is thermostable enzyme. Several other researchers reported that the optimum temperature for L-asparaginase was between 35 and 37°C [14,15,17,18,20,23]. Heat denatures protein mainly by affecting the weak interaction like hydrogen bonds. When temperature is slowly increased, a protein's conformation generally remains stable. But at a narrow temperature, there is an abrupt loss of structure (and function). Further, increase

Table 2: Purification table of L-asparaginase from *Streptomyces* sp.

Stages for purification	Total enzyme activity (IU)	Total protein(mg)	Specific activity (U/mg)	Yield of enzyme (%)	Purification fold
Cell-free extract	2930	450	6.5	100	1
Ammonium sulfate precipitation	2076	102	20.3	70.8	3.12
Dialysis	1725	28	61.6	59.7	9.47
DEAE-cellulose chromatography	1524	18	84.7	52.0	13.03
Sephadex G-50 chromatography	1230	3.15	390	41.9	60

IU: International unit, DEAE: Diethylaminoethyl.

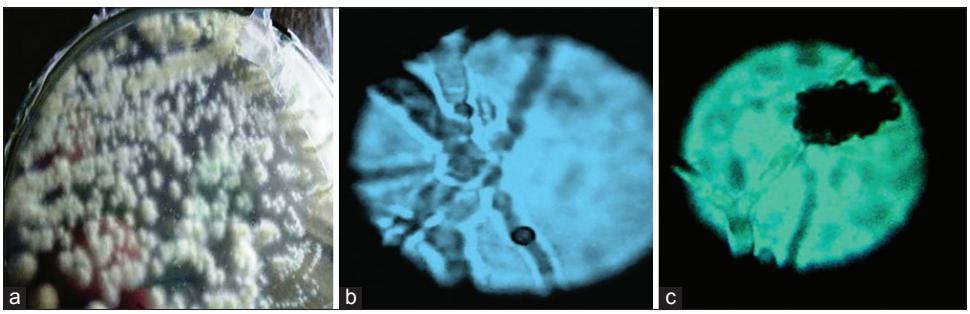

Figure 2: Photographic images of *Streptomyces* sp. (a) Colonies of isolate after 5 days of incubation on glucose asparagine medium, (b) and (c) microscopic view of the isolate (×100).

in temperature results in destabilization of protein structure. The temperature stabilities of L-Asparaginase enzyme from various sources range from 37°C to 80°C due to variations in their primary, secondary, and tertiary structures. Kinetic parameters of the enzyme were evaluated by plotting Michaelis–Menten and Lineweaver–Burk plots for different concentrations of commercial L-asparaginase. K_m (Michaelis-Menten constant) and V_{max} of purified enzyme were 0.065 mM and 20.8 IU/ml, respectively [Figure 9]. Lower K_m value indicates higher affinity of the enzyme to the substrate L-asparagine. Kumar *et al*. [41] obtained K_m of 0.0598 mM for L-Asparaginase from *S. radiopugnans* MS1. Basha *et al*. [17] obtained a lower K_m value 0.024 mM from marine *Streptomyces* sp. L-asparaginase from different microorganisms have different K_m values. Higher K_m values of 2.5 mM, 3.5 mM, 0.074 mM, and 6.72 mM were reported from *Corynebacterium glutamicum*, *Escherichia coli, Vibrio succinogenes,* and *Proteus vulgaris,* respectively [18,23,42]. Investigation on L-asparaginase activity in the presence of various additives was carried out [Figure 10]. Here, Na^+, K^+, and EDTA acted as inducers. Significant loss of activity was observed for other ions, namely, Cu^{2+}, Ca^{2+}, Fe^{2+}, Hg^{2+}, and Mg^{2+}, indicating inhibition of enzyme activity. The study indicates that L-asparaginase might not be a metalloenzyme. The results are in line with that observed by Bhagat *et al*. [19]. Enzyme inhibition in the presence of Cu^{2+}, Fe^{2+}, and Hg^{2+} might indicate the presence of essential vicinal sulfhydryl groups in the catalytic site of the enzyme. In addition, the activity of the enzyme with reducing agents such as 2-ME and DTT provided additional information on the role of sulfhydryl groups in the active site of the enzyme. Reducing agents prevent oxidation of free sulfhydryl groups and maintain three-dimensional structure of the enzyme and hence retain the activity. The enzyme entirely lost its activity in the presence of SDS. The results are in agreement with those obtained by Kumar and Selvam [21].

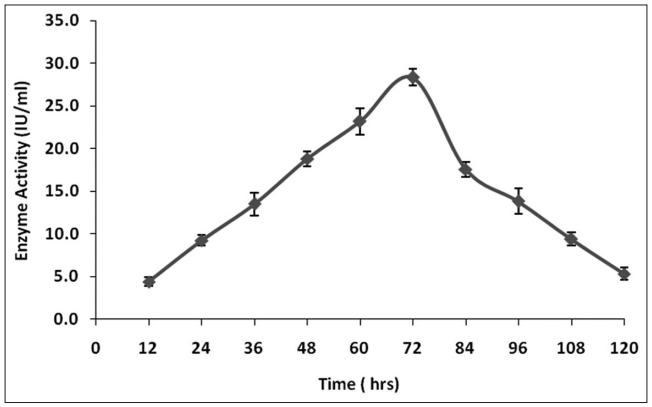

Figure 3: Production profile of L-asparaginase from *Streptomyces* sp.

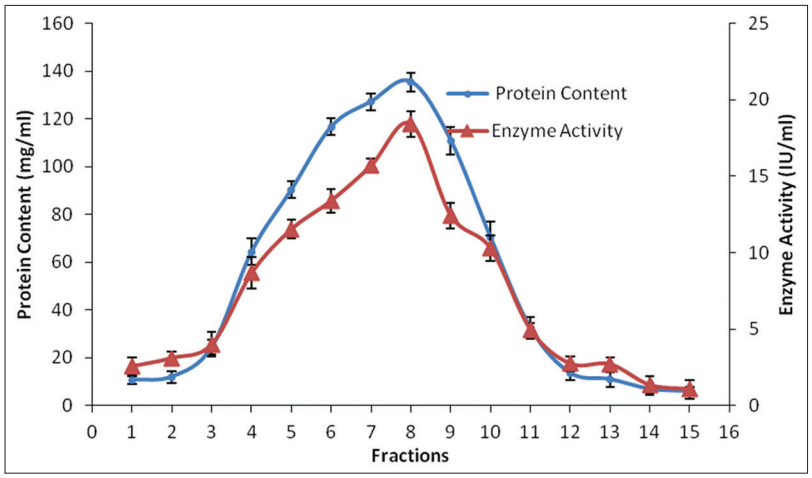

Figure 4: Elution profile of L-asparaginase on diethylaminoethyl-cellulose.

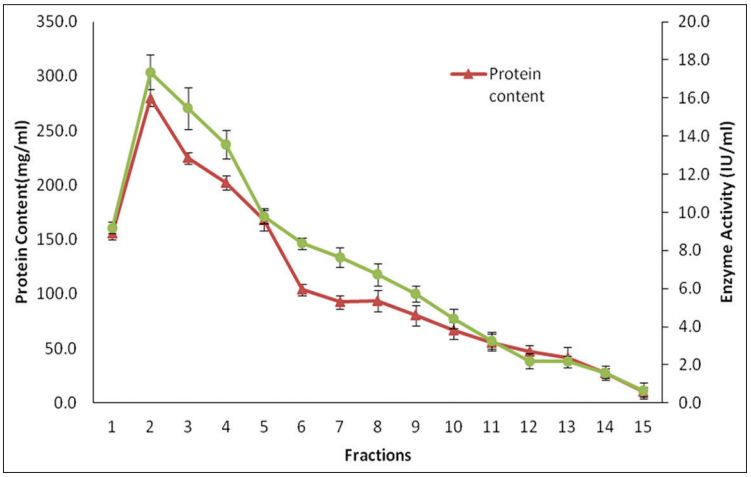

Figure 5: Elution profile of L-asparaginase on Sephadex G-50.

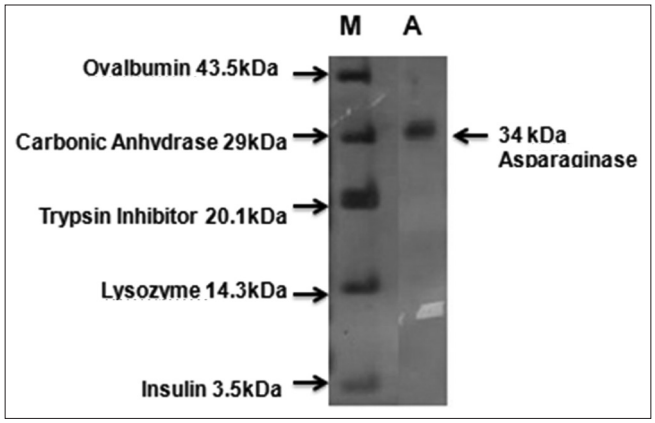

Figure 6: Determination of molecular weight of L-asparaginase by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (M: Molecular weight markers; A: L-asparaginase).

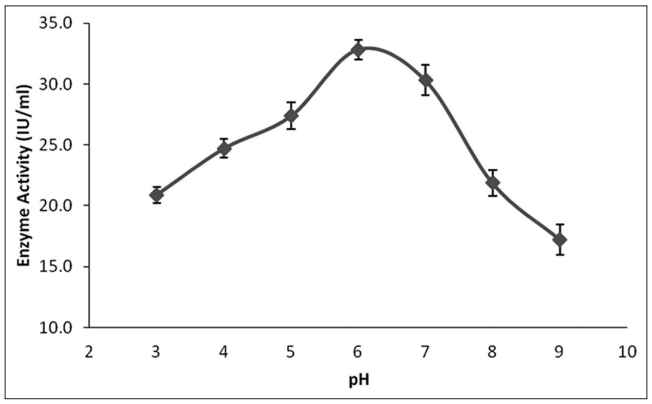

Figure 7: Effect of pH on L-asparaginase enzyme activity from *Streptomyces* sp.

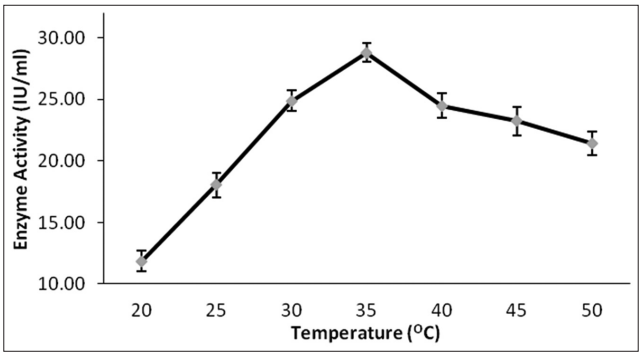

Figure 8: Effect of temperature on L-asparaginase activity from *Streptomyces* sp.

4. CONCLUSION

In the present investigation, isolates of actinomycetes were screened for L-asparaginase production. Based on enzymatic activity, a potent actinomycete was selected and was identified as *Streptomyces* sp., further, L-asparaginase enzyme was purified by salt precipitation, anion exchange chromatography, and size exclusion chromatography. L-asparaginase from *Streptomyces* sp. was purified to apparent homogeneity having a molecular size of 34 kDa. The enzyme was characterized for its various properties. This L-Asparaginase is active at a broad range of temperature and hence can be used in processes involving high temperatures. The kinetic parameters of L-asparaginase indicated that enzyme has high affinity to the substrate. Effect of various metal ions and additives indicated that the enzyme may not be a metalloenzyme and may contain free sulfhydryl groups near the catalytic site. The enzyme L-asparaginase could be of potent use in various biotechnological and industrial applications.

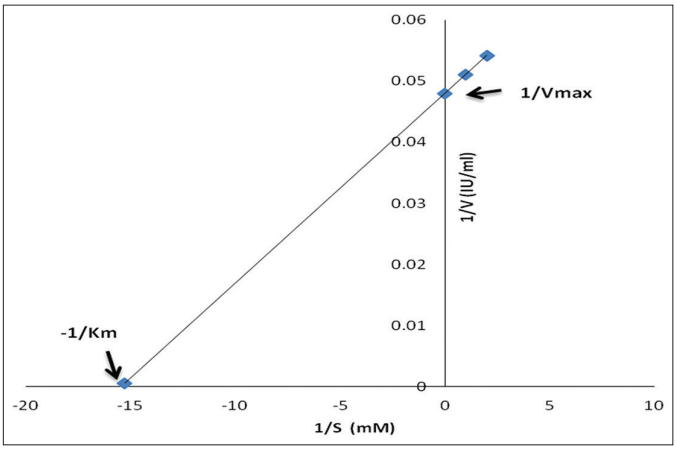

Figure 9: Lineweaver–Burk plot for L-asparaginase from *Streptomyces* sp.

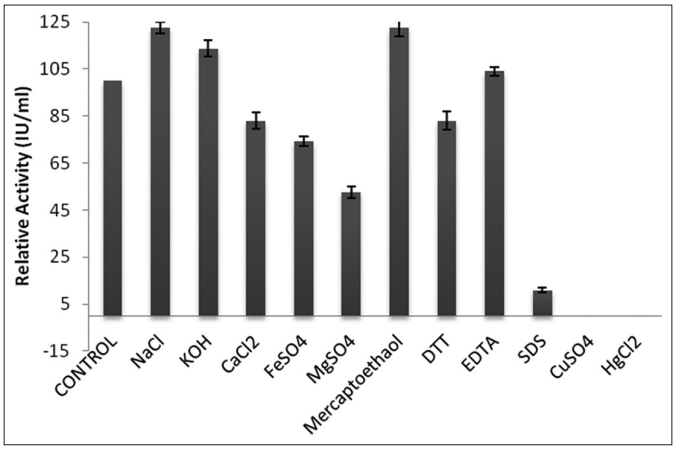

Figure 10: Effect of additives on L-Asparaginase from *Streptomyces* sp.

5. ACKNOWLEDGMENT

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