Kinetic Studies of partially purified L-asparaginase from *Streptomyces albogriseolous* NGP 2

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ABSTRACT

This study was focused on partial purification and characterization of L-asparaginase from *Streptomyces albogriseolous* NGP 2, isolated from marine sediment of south Indian coastal region. In purification steps, 1.75 fold purification was achieved after 85% ammonium precipitation of the with 5.94 recovery. In further purification steps, 11.77 fold purified L-asparaginase was recovered by Sephadex G-100 chromatography with 5.04 of recovery. The specific activity of purified enzyme was 58.53 U/mg. Zymogram of crude enzyme on native-PAGE presented bands with L-asparaginase activity of molecular weight and Isoelectric point were 50 kDa and 8.3. These findings suggested that the L-asparaginase have medical applications.

KEYWORDS: Marine sediment, Molecular weight, Isoelectric point, Zymogram

INTRODUCTION

L-asparaginase may be a therapeutic protein that depletes L-asparagine and also the tumour cells die because of their inability to synthesize this aminoacid. Recent studies proved that Lasparaginase is also used commercially to cut back the formation of amide in cooked foods [1].The prospective of the L-asparaginase in cancer was first discovered by Kidd (1953), who detected the anti-lymphoma activity of the guinea pig serum [2].

Some of the commercially available L-asparaginase is existed beneath the whole name A-ase, ASN-ase, Colapase, Crasnitin, Elspar, Crisantas, Pasum, PEG-asparaginase and Pegasparagasum. At this time those medicine are used as a combined formulation with therapy agent immune suppressant drug, however the facet effects have terribly limited by exploitation the commercially out there Lasparaginase. So, this ends up in the assembly of Lasparaginase from new or less explored sources such as marine and deep land sources [3][4] . Actinomycetes are the group of gram positive bacteria with high G+C (Guanine + Cytosine) content which form branching filaments or hyphae and asexual spores[5]. Generally, actinomycetes are recognized as less explored supply for L-asparaginase. Several species of actinomycete such as *S. karnatakensis, S. venezuelae, S. longsporus* and a marine actinomycete sp. PDK 2 are reported to produce detectable quantity of L-asparaginase [6]. In addition, the assorted concentrations of changed medium ingredients with completely different carbon and nitrogen sources were tested to optimize the medium for optimum production of L-asparaginase [7]. The enzyme was purified from *Erwinia carotovora* using ammonium sulphate precipitation, sephadex G100, CM-cellulose and DEAE Sephadex chromatography [8].

The analysis meted out for the assembly of Lasparaginase from Penicillium sp. had done purification, crystallization of protein and has additionally given the accelerator properties with effective anti-oxidant and cytotoxic activity [9]. Anti-oxidants are extensively studied for their capacity to shield organism and cell injury that are elicited by aerobic stress. aerobic injury plays a considerably pathological role in human disease like cancer, emphysema, cirrhosis, atherosclerosis and inflammatory disease [10].

The Food and Drug Administration (FDA) and World Health Organization (WHO) have approved L-asparaginase for the effective treatment of cancer and there conjointly report concerning theanti-oxidant property [11].

The current study presents sequential optimization strategy to improve L-asparaginase production using *Streptomyces albogriseolous* NGP 2. Partial purification and characterization of the partially purified enzyme were investigated and these parameters are necessary to use the enzyme efficiently in all medicinal applications.

METHODS

ISOLATION OF ACTINOMYCETE

The marine sediment was collected from the coastal region of Marina, Tamilnadu, India at 2-3m depth by using grab sampler. The collected sediment was subjected for enrichment prior to serial dilution. One gram of enriched sample serially diluted using sterile distilled water and spread over starch casein Agar (SCA) plates and incubated at 28 + 2^0 C for 7 days [12].

L-ASPARAGINASE SCREENING

The marine actinomycete isolate was subjected for rapid screening of L-asparaginase production by plate assay method using minimal M9 medium [13]. A 2.5 per cent stock solution of phenol red was prepared in ethanol (pH 6.2) and 3.0 ml of the stock solution was added to 1000 ml of minimal M9 medium. A point inoculation was carried out in a petri plate containing 20 ml of this medium and incubate at 30ºC for 7 days. After incubation period, the appearance of pink colour zone around the colony in the medium indicated presence of L-asparaginase activity.

GENETIC IDENTIFICATION

The genetic level identification of potential actinomycete isolates were carried out. Phylogenetic relationships with closely related species were determined by using MEGA version 4.0. Distance matrices were determined and were used to elaborate a dendrogram by the neighbor-joining method [14][15].

GROWTH KINETICS AND ENZYME PRODUCTION

To determine the optimum culture conditions for enzyme production, the actinomycete isolates were grown in basal liquid medium in an orbital shaker at 120 rpm.

AMMONIUM SULPHATE PRECIPITATION

The culture filtrate was filtered through whatmann No. 1 filter paper and centrifuged at 8000 rpm for 10 min at 4ºC. The culture filtrate (crude enzyme) was brought to 45.0 per cent saturation with ammonium sulphate at pH 8.4 and kept overnight in a cold room at 4ºC. It was thereafter subjected to centrifugation at 8000 rpm for 10 min at 4ºC. The precipitate

was discarded, while the supernatant was brought to 85.0 per cent saturation with ammonium sulphate and centrifuged at 8000 rpm at 4ºC for 10 min. The precipitate from this step was collected and stored at 4ºC. The precipitate was dissolved in 1 M tris - HCl buffer and dialysis against the same buffer for overnight. At each step, the enzyme activity and protein content of the culture filtrate were estimated [12].

An aliquot of the lyophilized sample (1 ml) was loaded onto a sephadex G 100 column (45 X 1.5 cm) equilibrated with 0.05 M trisHCl buffer (pH 8.4) and eluted against 0.1 M KCl (total volume 600 ml). A total of 30 fractions were collected at the flow rate of 5 ml/30 min. All the steps were performed at 4ºC and in each fraction enzyme activity and protein content were estimated.

OPTIMUM SUBSTRATE CONCENTRATION

The optimum substrate concentration for the maximum activity of the enzyme determined in terms of maximum reaction velocity (V_{max}) and michaelis constant (Km at which reaction velocity is half maximum). For this, various concentrations of specific substrates were prepared and incubated with purified enzyme preparations. For L-asparaginase activity, L-asparagine (0 - 2.5 mM) in tris-HCl buffer (0.05 M; pH 8.4) was used as substrate[16]. V_{max} and Km were estimated graphically by plotting substrate concentration in µM on X axis against enzyme activity U/mg protein on the Y axis. The accurate values of V_{max} and Km were obtained by double reciprocal Line Weaver-Burk plot and Eadie-Hofstee plot. The protein content was estimated[17] and the molecular weight of the sample was determined by SDS-PAGE [18].

DETERMINATION OF PI VALUES

The pI values of each enzyme fraction were determined by Iso electric focusing (IEF) technique[19].

RESULTS AND DISCUSSION

SCREENING AND IDENTIFICATION

The actinomycetes growth occured on the SCA plate was subjected for L-asparaginase screening in the minimal M9 medium and it produced maximum enzyme of about 3.32 U/ml. The genetic level analysis of the 16S rRNA gene is the most important tool for correct identification of microbial species. The isolate was identified as *Streptomyces albogriseolous* and the sequence was submitted to Gen-Bank (JX843531). A phylogenetic tree constructed by MEGA 4 software based on 16srRNA partial sequence. Similarly, 16SrRNA phylogenetic analysis of actinomycetes isolated from Eastern Ghats was carried out neighbor-joining algorithm[20]. In the screening of L-asparaginase from marine actinomycetes by plate assay in which the pink zone was appeared around the colony in the medium, indicated L-asparaginase activity [21].

GROWTH KINETICS AND ENZYME PRODUCTION

The results showed that the culture filtrate of *S. albogriseolus* NGP 2 had L-asparaginase total activity of 1.59 U/ml with a protein content of 0.32 mg/ml; the specific activity was 4.97 U/mg protein. When concentrated by ammonium sulphate, the specific activity was increased to 8.74 U/mg protein with a purification factor of 1.75. The protein content was decreased to 0.27 mg/ml. The enzyme recovery was 5.94 per cent. When passed through sephadex G 100 column, the fraction 13 exhibited L-asparaginase activity. In fraction 13,

the protein content was 0.19 mg/ml; specific activity was 58.53 U/mg protein, the purification fold and recovery yield were 11.77 and 5.04 per cent (Fig. 1 and Table 1). Besides, the specific activity and recovery of L-asparaginase from marine actinomycetes by solod state fermentation were reported as 55.52 and 4.97 per cent respectively [12].

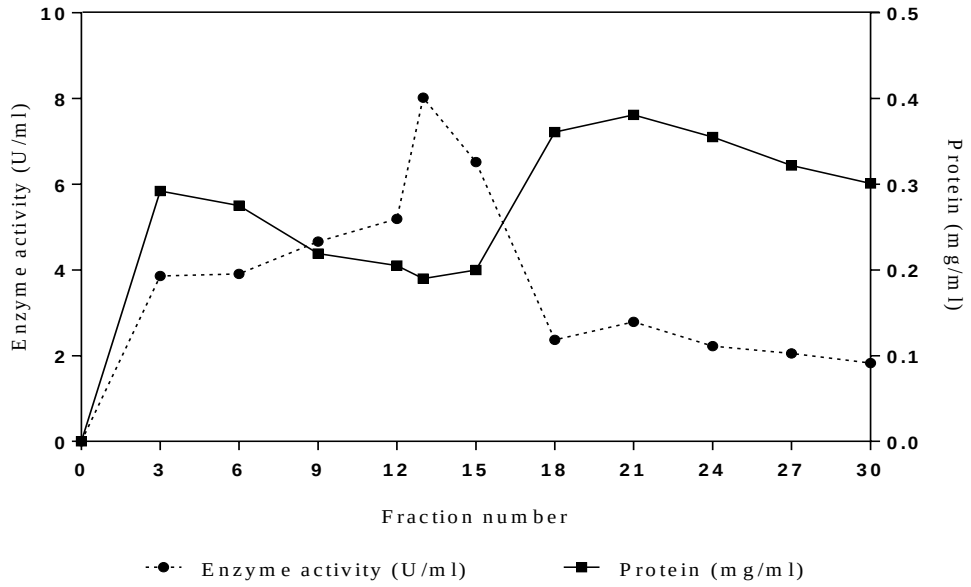


Fig 1: Purification of L-asparaginase of *S. albogriseolous* NGP 2 on Sephadex G 100 column

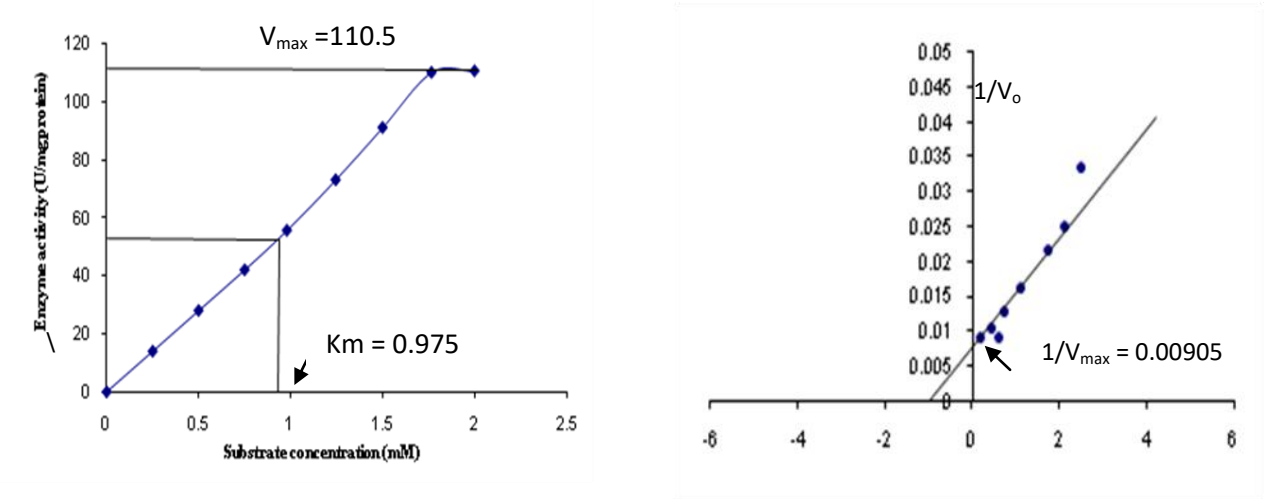
Table 1. Purification of L-asparaginase from the culture filtrates of *S. albogriseolous* NGP 2

Sample	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Recovery Yield (%)	Purification factor
Culture filtrate	500	1.59	0.32	795	4.97	100	1.00
Ammonium sulphate precipitation	20	2.36	0.27	47.2	8.74	5.94	1.75
Column Chromatography Sephadex G -100 Fraction 13	5	8.02	0.19	40.10	58.53	5.04	11.77

The kinetic properties of L-asparaginase were obtained using L-asparagine as substrate in a Lineweaver-Burk plot. In L-asparaginase, Vmax and Km values were estimated by using L-asparagine in tris-acetate buffer (0.2 M; pH 7.0) as substrate; Vmax and Km for *S. albogriseolous* NGP 2 L-asparaginase were 110.5 U/mg protein and 0.975 mM respectively (Fig. 2). The apparent Km of purified L-asparaginase for L-asparagine from *E. coli* was 1.25×10-5 mM and Vmax was 2.5×10-3 M/min [22]. Based on the kinetic parameters for the enzyme from *Enterobacteriaceae* were calculated from Lineweaver-Burk

plot, and Km) and Vmax were 0.89 mM and 0.18 U/mg, respectively [23].

V max **Double reciprocal plot**



Eadie-Hofstee plot

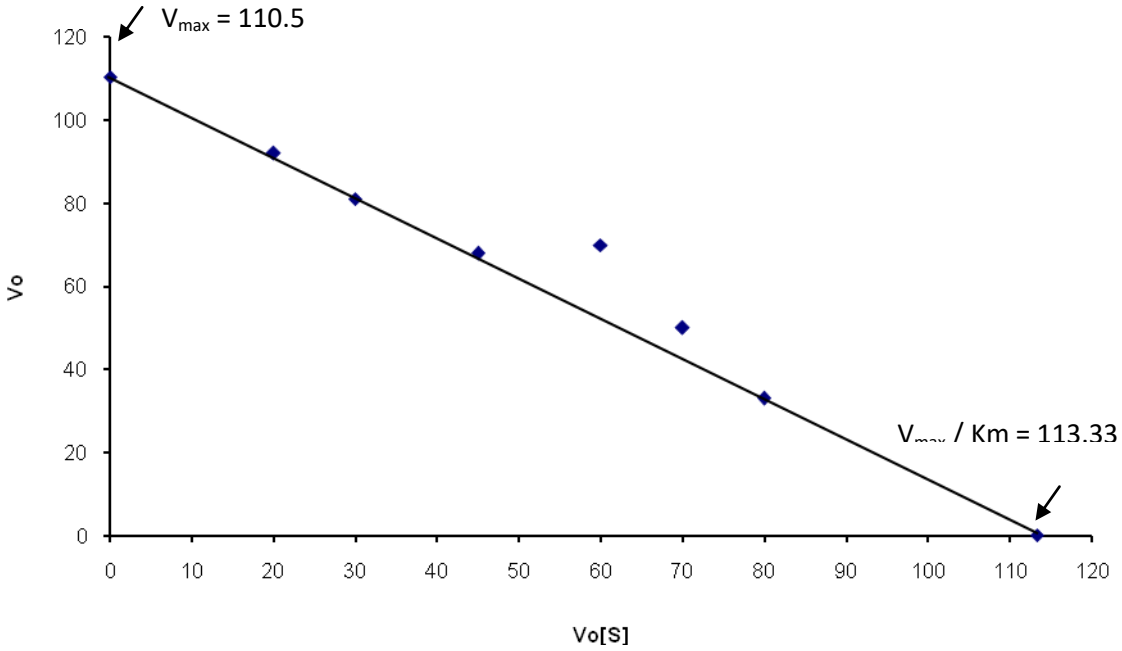
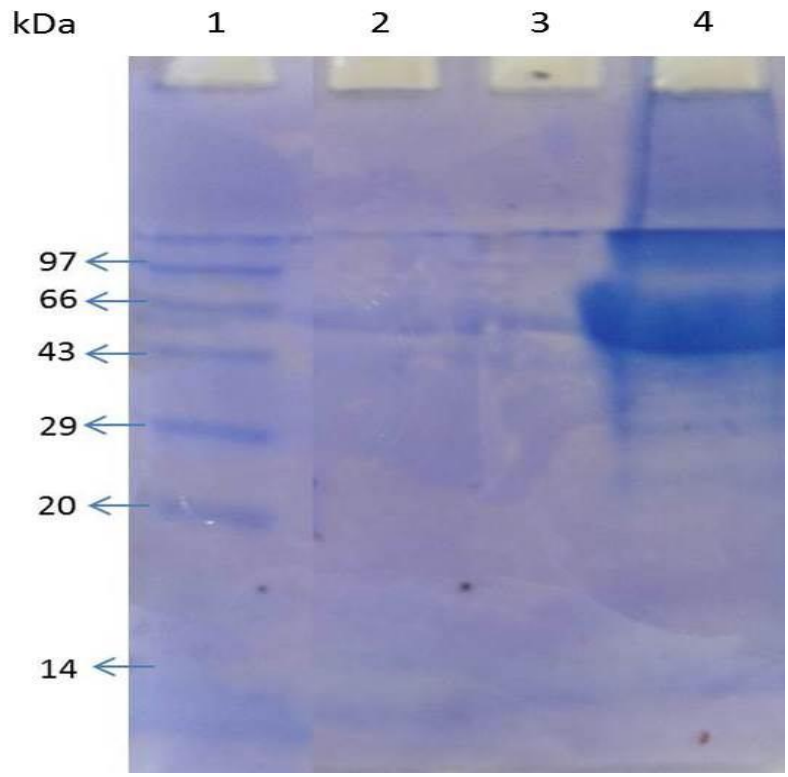


Fig 2: Kinetics of *S. albogriseolus* NGP 2 L-asparaginase

pI and Molecular weight

The enzyme L-asparaginase of actinomycete NGP 2 exhibited single fraction of pI. The pI value was found to be 8.3. The molecular weight of the the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A single band was exhibited, had a molecular weight of 50 kDa (Plate 1).



Lane 1: Molecular weight markers

Lane 2: L-asparaginase

Plate 2: Molecular Weight of L-asparaginase

Similarly, the isoelectric point (pI) of the L-asparaginase produced from *Erwinia carotovora* was between 7.1 and 8.2. The molecular weight of the L-asparaginase from *E.coli* VRY - 15 was found to be 56.0 kDa [24]. Protein profiling by SDS-PAGE revealed the molecular weight of the protein to be 45.0 kDa [10].

CONCLUSION

The present study revealed the kinetic parameters of L-asparaginase produced from actinomycetes, isolated from the South Indian coastal region. According to the kinetic studies, the enzyme will be optimized efficiently to carry out the medicinal applications.

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