Research Article

Purification and Characterization of L-Asparaginase from *Streptomyces griseoluteus* WS3/1

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Accepted on: 26-09-2013; **Finalized on:** 30-11-2013.

ABSTRACT

To purify and characterize L-asparaginase produced from a marine actinomycete, *Streptomyces griseoluteus* WS3/1. L-asparaginase from marine actinomycete was isolated and identified based on a 16S ribosomal DNA sequence analysis and named *Streptomyces griseoluteus* WS3/1. The enzyme was purified by gel filtration chromatography using Sephadex LH-20 column. It had a molecular weight of 54 kDa. The kinetics, optimum pH and temperature of the purified enzyme were determined. The present work for the first time reported the production, purification and characterization of L-asparaginase by marine actinomycetes *Streptomyces griseoluteus* sp. These results provided more information on L-asparaginase production.

Keywords: L-asparaginase, Sephadex LH-20, *Streptomyces griseoluteus*, 16S ribosomal DNA sequence.

INTRODUCTION

The marine biosphere is one of the richest habitats of microorganisms. Marine microbes appeared to have adapted excellently to the marine environment, thus have gained special attention because of their diverse metabolic capabilities and their divergent physiologic lifestyles. Marine ecosystems contain several unique features that set them apart from other aquatic ecosystems, the main factor being the presence of dissolved compounds in sea water, particularly sodium chloride.

Microbial L-asparaginase has attracted considerable attention since the demonstration that L-asparaginase from *E.coli* has anti-tumor activity.[1,2] This enzyme has been the subject of several reviews on its distribution, biochemical and immunological properties. Since then a large number of bacteria and fungi have been screened for L-asparaginase producing potential.[3-5] L-asparaginase catalyzes the conversion of asparagine to aspartic acid and ammonia. Asparagine is a nutritional requirement of both normal cells and cancer cells. Low levels of the non-essential aminoacid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirements for asparagine. This is because normal cells produce enzyme asparagine synthetase, which is able to synthesize asparagine from aspartic acid, whereas, in cancer and tumor cells this enzyme is present in low levels.

MATERIALS AND METHODS

Isolation of L-asparaginase enzyme producing isolate

Microorganisms isolated from sea sediments and corals collected from Tamil Nadu were screened for L-asparginase activities on Asparagine Dextrose Agar (ADA) medium and Asparagine Dextrose Salts (ADS) broth containing 0.009%w/v of phenol red. The colour change of the medium from yellow to pink is an indication of the extracellular L-asparaginase production by the colony[6]. Among several potential L-asparaginase enzyme producing isolates, an isolate named WS3/1 showed highest enzyme activity and is utilized for further work.

Enzyme assay

Step 1: The reaction mixture containing 0.5 mL of 0.04 M L-asparagine, 0.5 mL of 50 mM Tris buffer, 0.5 mL of an enzyme preparation and distilled water to a volume of 2.0 mL was incubated at 37ºC for 30 min. The reaction was then stopped by adding 0.5 mL of 1.5 M TCA. Blank tubes were run by adding the enzyme preparation after the addition of TCA.

Step 2: To 3.7 mL of distilled water, 0.1 mL of the above mixture (Step 1) and 0.2 mL of Nessler's reagent were added at room temperature and the absorbance was measured immediately at 450 nm using spectrophotometer and the amount of liberated ammonia was determined. One international unit of L-asparaginase is defined as the amount of enzyme that liberates 1 µ mole of ammonia per minute under the conditions of the assay.[7]

Purification of L-asparaginase enzyme

The purification of fermented broth was carried out at 4ºC[8] by the following steps.

Ammonium sulphate precipitation

The crude enzyme prepared was brought to 80% saturation with ammonium sulphate at pH 8.5 and kept overnight in cold room. Then it was centrifuged at 5000 rpm, at 4ºC for 15 min. The precipitate was then collected separately and stored at 4ºC for further purification.

Dialysis

The dialysis of the ammonium sulphate was carried out in a pre-treated dialysis tube. The precipitate was dissolved in 1M Tris buffer pH 8.0, until the dialysate was negative when tested for ammonia. Precipitate formed during dialysis was removed by centrifugation and was discarded. For the present study dialysis membrane-100 (Hi-Media) was used. After dialysis, the samples were used for protein estimation and enzyme assay.

Gel filtration on Sephadex column

The dialyzed fraction was applied to a Sephadex LH-20 column that was pre-equilibrated with a 0.01 M Tris buffer pH 8.5. Fractions showing L-asparaginase activity were pooled and concentrated by lyophilization.

Characterization of purified L-asparaginase

The purified fraction obtained in the purification process was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for the determination of molecular weight of L-asparaginase by using standard molecular markers and the lyophilized enzyme samples were used for the characterization of L-asparaginase.

Effect of substrate concentration on L-asparaginase activity

In order to characterize the L-asparaginase produced by the strain of WS3/1, the lyophilized enzyme (1mg/mL) was incubated at a time interval of 30 min with different concentrations of asparagine. The asparagine concentration was varied from 0.01 M to 0.07 M.

Effect of temperature on L-asparaginase activity

The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures ranging from 10-70ºC. Maximum activity was noticed in the range of 35-40^{0}C. The enzyme activity was gradually declined beyond 50^{0}C. At higher temperatures (above 70ºC) L-asparaginase activity was unstable.

Effect of pH on L-asparaginase activity

The activity of L-asparaginase was evaluated at different pH values. Lyophilized enzyme was incubated with 0.04M L-asparagine and 50 mM Tris buffers of pH 4.5 to 10, under assay conditions and the amount of ammonia liberated was determined. For stability check, the enzyme was incubated at different pH in the absence of substrate. The pre-incubation was carried out for 30 min and then the residual activity was measured.

Kinetic Characterization

Calculation of K_m and V_{max} values

The K_m and V_{max} values of L-asparaginase were calculated from the reciprocal plots of substrate concentration versus reaction velocity. The Lineweaver-Burk plot (LB plot) was constructed and it was found to be linear, suggesting a simple Michealis-Menten (MM plot) kinetics.

RESULTS AND DISCUSSION

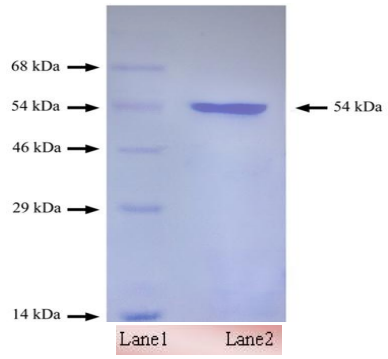
Isolation of L-asparaginase enzyme producing isolate

Marine microorganisms isolated from different sea sediments and corals collected from Tamilnadu were screened for L-asparaginase producing capability.[9] The isolates were streaked on Asparagine Dextrose Agar (ADA) medium and Asparagine Dextrose Salts (ADS) broth containing 0.009% w/v of phenol red to assess the extent of extracellular L-asparaginase production. The colour change of the medium from yellow to pink is an indication of the extracellular L-asparaginase production by the isolate. This colour change is due to change in the pH of the medium, as L-asparagine causes the breakdown of amide bond in L-asparagine and liberates ammonia. After 5 days of incubation, the isolates producing pink coloured zones were used for further work. The 16S rDNA gene sequence of the isolate WS3/1 was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN programme. The high scoring similar to 16S rDNA gene sequences were identified from the BLASTN result and retrieved from Gene Bank database. Phylogenetic trees were inferred by using the neighbour joining Bootstrap analysis with the help of MEGA 3.0 software package. Phylogenetic analysis based on 16S rDNA gene sequencing showed that the strain WS3/1 was grouped into genus *Streptomyces* with 99% bootstrap support.[10]

Purification of L-asparaginase from the strain WS3/1

The proteins from the crude enzyme extract were precipitated by the ammonium sulphate (80%) precipitation and most of the enzyme activity was retained in the precipitate. The specific activity of the enzyme increased to 1042.6 and 1380 IU/mg after dialysis and Sephadex LH-20 column chromatography respectively. Purity of L-asparaginase increased up to 81.75 with 60.09 % recovery in Sephadex LH-20 purification step.[11-15] The purification steps for L-asparaginase were summarized in Table 1.

By using different molecular markers with known molecular weights, it was determined that the molecular weight of L-asparaginase from the strain WS3/1 was found to be 54 kDa [16,14] as shown in Figure 1.



Lane 1: Molecular markers Lane; 2: Purified L-asparaginase

Figure 1: SDS-PAGE of L-asparaginase obtained from the strain WS3/1

Table 1: Purification of L-asparaginase from the strain WS3/1

Purification Steps	Total activity (IU)	Total protein(mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Fermented broth	114.82	6.8	16.88	----	100
Ammonium sulphate Precipitation	92.45	0.1	924.5	54.76	80.51
Dialysis	83.41	0.08	1042.6	61.76	72.64
Sephadex LH-20 chromatography	69.0	0.05	1380	81.75	60.09

Effect of substrate concentration on L-asparaginase activity

In order to characterize the L-asparaginase produced by the strain of WS3/1, the lyophilized enzyme (1mg/mL) was incubated at a time interval of 30 min with different concentrations of asparagine. The asparagine concentration was varied from 0.01 M to 0.07 M. The substrate profiles of L-asparaginase activity were shown in Table 2 and Figure 2. From graph it was observed that at 0.04 M concentration, L-asparaginase showed the maximum velocity. Further increment of the L-asparagine concentration did not enhance the activity significantly. At 0.04 M substrate concentration it reached the stationary phase.

Table 2: Effect of substrate concentration on L-asparaginase activity

Substrate concentration [S]	L-asparaginase activity (IU/mL)
0.01	1.98
0.02	3.46
0.03	4.42
0.04	5.12
0.05	5.56
0.06	5.92
0.07	5.96
0.08	5.96
0.09	5.96

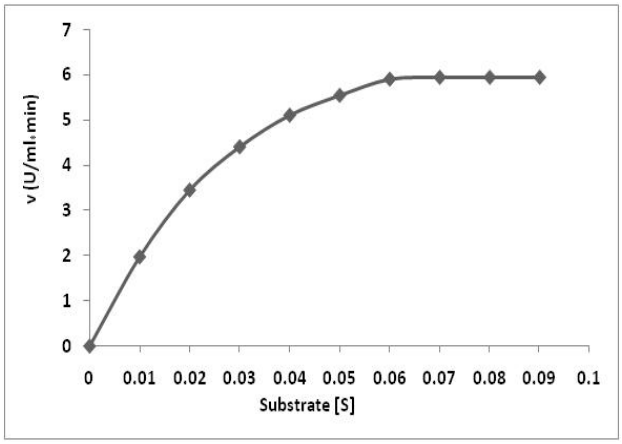


Figure 2: Effect of substrate concentration on L-asparaginase activity

Effect of temperature on L-asparaginase activity

The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures ranging from 10-70ºC. Maximum activity was noticed in the range of 35-40^{0}C. The enzyme activity was gradually declined beyond 50^{0}C. At higher temperatures (above 70ºC) L-asparaginase activity was unstable [8,14] and the results were shown in Table 3 and Figure 3.

Table 3: Effect of temperature on L-asparaginase activity

Temperature (°C)	L-asparaginase activity (IU/mL)
10	1.25
20	2.51
30	3.773
40	5.75
50	5.52
60	4.99
70	1.2

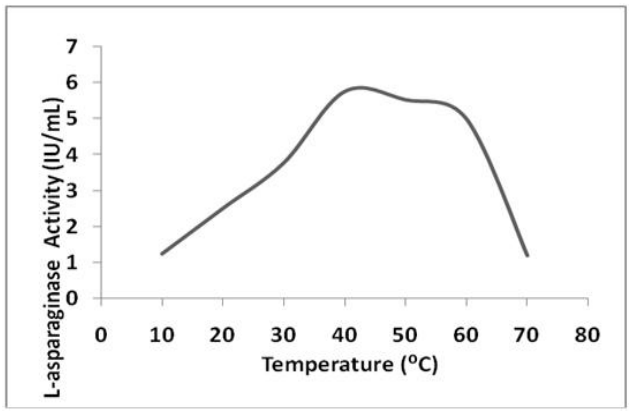


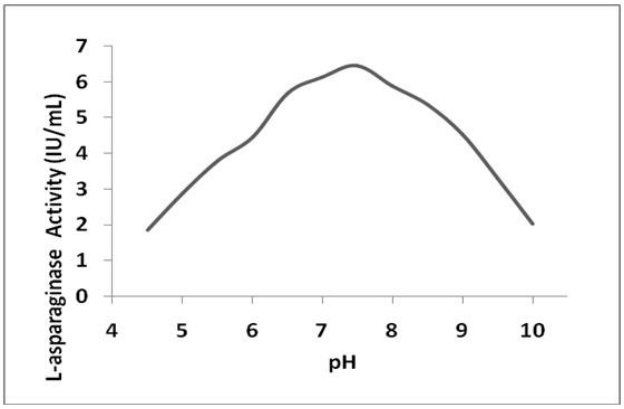
Figure 3: Effect of temperature on L-asparaginase activity

Effect of pH on L-asparaginase activity

The activity of L-asparaginase was evaluated at different pH values. Lyophilized enzyme was incubated with 0.04M L-asparagine and 50 mM Tris buffers of pH 4.5 to 10, under assay conditions and the amount of ammonia liberated was determined. For stability check, the enzyme was incubated at different pH in the absence of substrate. The pre-incubation was carried out for 30 min and then the residual activity was measured [17,18]. The maximum L-asparaginase activity was observed between pH 7.0 to 7.5 and the results were shown in Table 4 and Figure 4.

Table 4: Effect of pH on L-asparaginase activity

pH	L-asparaginase activity (IU/mL)
4.5	1.86
5.0	2.89
5.5	3.8
6.0	4.46
6.5	5.68
7.0	6.12
7.5	6.45
8.0	5.89
8.5	5.36
9.0	4.53
9.5	3.31
10.0	2.02



Calculation of K_m and V_{max} values

The K_m and V_{max} values of L-asparaginase were calculated from the reciprocal plots of substrate concentration versus reaction velocity. The Lineweaver-Burk plot (LB plot) was constructed and it was found to be linear, suggesting a simple Michealis-Menten (MM plot) kinetics. From the LB plot the equation y=0.0039x + 0.1032 gives $1/V_{max}$ value 0.103 and $1/K_m$ value 26.525. So the calculated values of K_m and V_{max} values were 0.0377M and 9.69 IU/mL*min respectively and the results were shown in Table 5, Figure 5 and Figure 6.

Table 5: K_m and V_{max} values of L-asparaginase from the strain of WS3/1

	MM plot	LB plot
K_m	0.03M	0.03M
V_{max}	5.96 IU/mL*min	9.69 IU/mL*min

The proteins from the crude enzyme extract were precipitated by the ammonium sulphate (80%) precipitation and most of the enzyme activity was retained in the precipitate. The enzyme was purified by using sephadex LH-20 column chromatography and lyophilized. The specific activity of the L-asparaginase from WS3/1 increased to 1042.6 and 1380 IU/mg after dialysis and Sephadex LH-20 column chromatography respectively. Purity of L-asparaginase increased upto 81.75 with 60.09 % recovery in the final purification step. By conducting SDS-PAGE with known molecular markers the molecular weight of L-asparaginase from the strain WS3/1 was found to be 54 kDa. Maximum enzyme activity was noticed in the temperature range of 35-40^{0}C, between pH 7.0 to 7.5. The Lineweaver-Burk plot was constructed and it was found to be linear, suggesting a simple Michealis-Menten kinetics. The calculated values of K_m and V_{max} values were 0.0377M and 9.69 U/mL*min respectively.

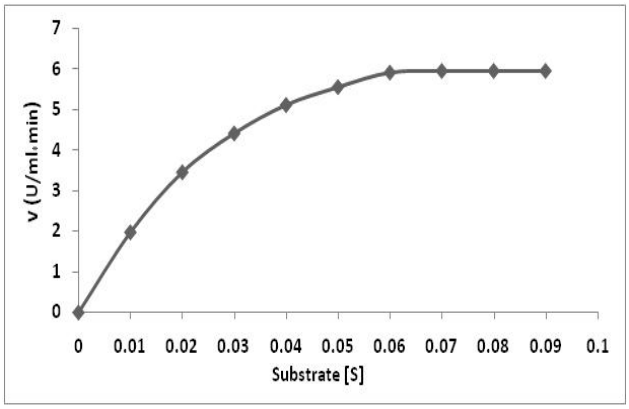


Figure 5: Michaelis- Menten plot of L-asparaginase

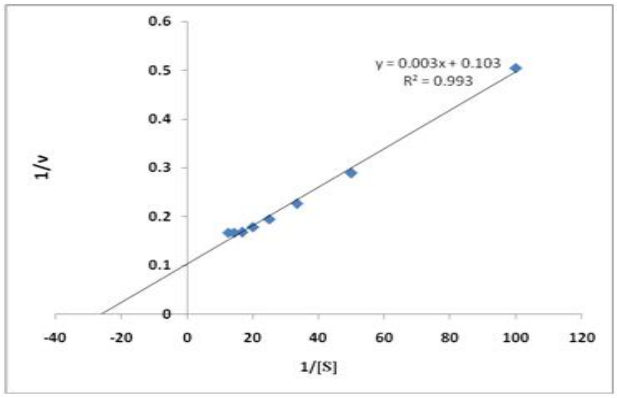


Figure 6: Lineweaver- Burk double reciprocal plot of L-asparaginase

CONCLUSION

In this study, L-asparaginase enzyme from a marine actinomycete named *Streptomyces griseoluteus* sp. WS3/1 was isolated. From the culture filtrate of this strain, an extracellular L-asparaginase enzyme was purified by column chromatography using Sephadex LH-20 and molecular weight was determined by SDS-PAGE. The enzyme substrate specificity, kinetic parameters were also determined. Although there have been many reports on the subject of L-asparaginase enzyme producing microorganisms [5], to the best of our knowledge, this is the first report on L-asparaginase production by a marine actinomycete, *Streptomyces griseoluteus* sp. WS3/1.

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Source of Support: Nil, **Conflict of Interest:** None.