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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE FROM *STREPTOMYCES GULBARGENSIS*

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ABSTRACT

L-asparaginase is an anti-neoplastic agent used in the lymphoblastic leukaemia chemotherapy. In the present study a novel strain, *Streptomyces gulbargensis* was explored for the production of extra-cellular L-asparaginase using groundnut cake extract. The optimum pH, temperature, inoculum size and agitation speed for enzyme production were pH 8.5, 40°C, $1x10^8$spores/ml and 200 rev/min respectively. Maltose (0.5%) and L-asparagine (0.5%) proved to be the best carbon and nitrogen sources respectively. The enzyme was purified 82.12 fold and the apparent molecular weight of the enzyme was found to be 85 kDa. The optima pH and temperature for the enzyme were 9.0 and 40°C respectively. The enzyme was more stable at the alkaline pH than at the acidic one and it retained 55% of the activity at 80°C for 60 min.

Key words: L-asparaginase, *Streptomyces gulbargensis*, Groundnut cake extract, Optimization, Purification.

L-asparaginase (L-asparagine aminohydrolase EC 3.5.1.1), the enzyme which converts L-asparagine to L-aspartic acid and ammonia has been used as a chemotherapeutic agent. It has received increased attention in recent years for its anti-carcinogenic potential (11). The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation. The enzyme is produced by a large number of micro organisms that include *Enterobacter cloacae* (15), *Serratia marcescens* (2) and *Enterobacter aerogenes* (13). The enzymes isolated from *E.coli* and *Erwinia carotovora* are now being used in the treatment of acute lymphoblastic leukaemia (4). However, due to the prolonged administration of L-asparaginase, the corresponding antibodies are produced in man, which causes an anaphylactic shock or neutralization of the drug effect. Therefore there is a continuing need to screen newer organisms in order to obtain strains capable of producing new and high yield of L-asparaginase. Among the actinomycetes several *Streptomyces* species such as *S. karnatakensis*, *S. venezualae*, *S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-asparaginase production (14). The enzyme is produced throughout the world by both submerged and solid-state cultures. Extra-cellular asparaginases are more advantageous than intracellular since they could be produced abundantly in the culture broth under normal conditions and could be purified economically. In this paper, we report the production of an extra-cellular L-asparaginase by a novel isolate, *Streptomyces gulbargensis* (3) under submerged culture using groundnut cake extract. Attempts were made to study the optimization of L-asparaginase production, its purification and characterization from *S. gulbargensis.*

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The strain *S.gulbargensis* was obtained from the Department of Microbiology, Gulbarga University, Gulbarga, India. The isolate was identified as a novel strain at Yunnan Institute of Microbiology, China (3). It was maintained on starch casein agar slants (pH 7.2) containing (gL^{-1}) Starch 10, K_2HPO_4 2.0, KNO_3 2.0, NaCl 2.0, Casein 0.3, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 20 at a temperature of 4°C. Regular sub culturing of the isolate was performed at an interval of every 4 weeks.

Inoculum preparation and Production of L-asparaginase

Spore suspension was prepared from 5 day old culture grown on starch casein agar slant by adding 10ml of sterile distilled water containing 0.01% of Tween 80 and suspending the spores with a sterile loop (9). One ml of this spore suspension was used as an inoculum for production studies. Production of L-asparaginase was carried out in groundnut cake extract. The groundnut cake was obtained from the local market in Gulbarga city. The extract was prepared using ten grams of the powdered substrate dissolved in 100 ml of distilled water, taken in 250 ml Erlenmeyer flask. The contents of the flask were heated for about ten minutes, cooled to room temperature and then filtered using Whatman filter paper No.1. The extract thus obtained was used for bioprocess studies. The parameters studied for submerged culture were initial pH (6.5-9.0) of the medium, incubation temperature (25-55°C), inoculum size ($1x10^5$-$1x10^9$ spores/ml) and agitation speed (140-200 rev/min). Once a given parameter was optimized, it was kept constant at that level while varying the other parameters individually. The effect of addition of various carbon and nitrogen sources on L-asparaginase production was determined in groundnut cake extract. The carbon sources were studied at a concentration of 0.5%. The effect of different concentrations of nitrogen sources was studied by adding a nitrogen source to the extract supplemented with 0.5% maltose. The bioprocess was carried out in 250 ml Erlenmeyer flask containing the optimized medium, kept in a shaker incubator (Remi Orbital Shaker Incubator). Samples were withdrawn at regular intervals of 24 h and assayed for L-asparaginase activity. All the experiments were performed independently in triplicates and the results given here are the mean of three values.

Assay of L-asparaginase

To determine the enzyme activity, 5ml of the culture broth was withdrawn aseptically from the flasks at an interval of every 24 h. The broth was filtered using Whatman filter paper No.1 and then centrifuged (Sigma 3K30) at 9,000 g for 8 min (5). The supernatant thus obtained was used as crude extract for L-asparaginase assay. Assay of enzyme was carried out as per Imada *et al*. (7). The enzyme activity was expressed in IU. One IU of L-asparaginase is the amount of enzyme which liberates 1µmole of ammonia per ml per min (µmole/ml/min).

Purification of L-asparaginase

The purification was carried out using crude enzyme extract (6). The enzyme was purified by the following steps at 0-4°C, unless otherwise mentioned.

Finely powdered ammonium sulfate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 40-60% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in 50mM Tris-HCl buffer pH 8.6 and dialyzed against the same buffer. The dialyzed fraction was applied to a Sephacryl S-200 column (1cm x 50cm) that was pre-equilibrated with Tris-HCl buffer pH 8.6. The protein elution was done with the same buffer at a flow rate of 5ml/30 min. The active fractions were pooled, dialyzed and concentrated. The concentrated enzyme solution was applied to the column of CM Sephadex C-50 that was pre-equilibrated with 50mM Tris-HCl buffer pH 8.6. It was eluted with NaCl gradient (0.1-0.5 M) and 0.1 M borate buffer pH 7.0. The active fractions were collected, dialyzed and concentrated. The L-asparaginase was assayed by the direct Nesslerization method as described earlier. Protein estimation was done with Folin-Phenol reagent using BSA as a standard (10). SDS-PAGE was performed according to the method of Laemmli (8), with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained

with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The following standard proteins were used for molecular weight determination 97.4kDa (Phosphorylase b), 66kDa (Bovine serum albumin), 43kDa (Ovalbumin), 29kDa (Carbonic anhydrase), 18.4kDa (Lactoglobulin) and 14.3kDa (Lysozyme).

pH and Temperature Studies

The activity of L-asparaginase was evaluated at different pH values and temperature. Partially purified enzyme was incubated with 0.04M L-asparagine and 0.05M buffers of pH 4-10, 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. Buffers used were potassium phosphate (pH 4.0-7.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10). The pre-incubation was carried out for 60 min and then the residual activity was measured. The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures ranging from 10-100^{o}C. Thermostability studies were carried out by pre-incubating the enzyme at different temperatures for 60 min.

The analysis of L-asparaginase production was carried out at every 24 h interval after inoculation with the maximum activity obtained at 120 h of incubation (Table 1). The yield of L-asparaginase increased with increase in initial pH of the medium up to 8.5 and thereafter it decreased. The maximum yield (9.8 IU) was obtained at pH 8.5 and minimum (6.4 IU) at pH 6.5. Narayana *et al*. (14) have reported the optimum pH for L-asparaginase production by *Streptomyces albidoflavus* to be 7.5. Maximum yield of L-asparaginase (10.5 IU) was observed at a temperature of 40^{o}C, and the lowest (4.9 IU) at temperature 55^{o}C. Maximum production of L-asparaginase by *S. albidoflavus* was found to be at 35^{o}C (14). An inoculum size of 1x10^{8}spores/ml showed the highest yield (11.0 IU) of L-asparaginase, while the lowest yield of enzyme was observed with an inoculum size of 1x10^{5} spores/ml, producing 6.9 IU of L-asparaginase. Optimization of inoculum size is necessary because too few spores lead to insufficient biomass, whereas too many spores lead to overproduction of biomass resulting in quick depletion of nutrients. The yield of enzyme increased with increase in agitation speed from 140-200 rev/min and decreased later. Maximum L-asparaginase production (12.1IU) was obtained at an agitation speed of 200rev/min and lowest yield (7.1 IU) at 140 rev/min. Table 2 shows the effect of various carbon sources on L-asparaginase production by *S.gulbargensis*. Among all the carbon sources tested, maltose proved to be the best for L-asparaginase production yielding 19.5 IU of enzyme. Maximum L-asparaginase production using maltose as a sole carbon source has been reported in *S. albidoflavus* (14). However glucose, fructose, galactose, mannitol and arabinose proved to be inferior for L-asparaginase synthesis. Glucose is known to lower the enzyme yield by acting as a repressor (13). Repression of L-asparaginase synthesis by glucose has been shown in bacteria such as *Serratia marcescens* (2) and *E.coli* (1). On the other hand, enhancement of L-asparaginase production by glucose was observed in *Aeromonas* sp. (16). The L-asparaginase production pattern using various nitrogen sources at different concentrations is presented in table 2. Highest enzyme activity (25 IU) was recorded when groundnut cake extract was supplemented with 0.5% L-asparagine. Maximum L-asparaginase production using 0.1% L-asparagine as the sole source of nitrogen has been observed in *Enterobacter cloacae* (15) and *Aeromonas* sp. (16). On the contrary, Narayana *et al*. (14) reported yeast extract (2%) as the best nitrogen source for L-asparaginase production by *S. albidoflavus.*

The purification of L-asparaginase was carried out by 4 steps as shown in table 3, with a final yield of 32% and a purification fold of 82.12. L-asparaginase from *S. albidoflavus* has been purified in CM Sephadex C-50 column up to 99.3 fold with 40% recovery (14). Dhevagi and Poorani (4) reported 85 fold purification of L-asparaginase from *Streptomyces* sp.PDK2 by a final Sephadex G-200 gel filtration. SDS-PAGE analysis of the purified enzyme showed the apparent molecular weight of *S. gulbargensis* L-asparaginase to be 85 kDa. Purified L-asparaginase from *Streptomyces* sp.PDK2 (4) and *S.*

albidoflavus (14) exhibited a molecular weight of 140 kDa and 112 kDa respectively.

The optimal pH determined for L-asparaginase activity of *Streptomyces gulbargensis* was 9.0. Similar observations have been reported for asparaginase from *Pseudomonas stutzeri* MB-405 (11). Dhevagi and Poorani (4) reported the maximum L-asparaginase activity of *Streptomyces sp.* PDK7 between pH 8.0 and 8.5. Regarding the pH stability, the enzyme retained more than 80% of the activity in the pH range of 7-10. Our results are in good agreement with those of Manna *et a*l. (11).The enzyme was found to be maximally active at 40^{o}C. This optimum L-asparaginase activity at 40^{o}C is similar to that of *Corynebacterium glutamicum*, reported by Mesas *et al.* (12). Regarding the thermal stability, at 80^{o}C, the enzyme retained 55% of the activity.

The present study revealed that, all the selected parameters examined, showed a considerable impact on L-asparaginase production by the novel isolate, *S.gulbargensis* in groundnut cake extract. To the best of our knowledge, this is the first report on the utilization of groundnut cake extract as a substrate/medium for the production of L-asparaginase by *S.gulbargensis.* Further, the high catalytic activity of the enzyme at physiological pH and temperature and its considerable stability over a wide range of pH and temperature makes it highly favorable to be exploited as a potent anticancer agent.

Table 1. Effect of various parameters on L-asparaginase production by *S.gulbargensis*

Parameter	**L-asparaginase activity (IU)**					
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**
pH						
6.5	3.0	3.9	4.8	5.7	6.4	6.0
7.0	3.2	4.2	5.5	6.6	7.3	6.8
7.5	3.5	5.2	6.5	7.3	8.1	7.2
8.0	3.9	6.6	7.8	8.2	8.9	8.2
8.5	4.2	6.9	8.1	9.0	9.8	9.3
9.0	3.8	6.3	7.4	8.1	8.7	8.1
Temperature (^{0}C)						
25	2.9	4.0	4.8	5.5	6.9	5.7
30	4.2	5.6	6.1	7.4	8.2	7.4
35	5.3	6.2	7.7	8.3	9.7	8.8
40	5.9	7.3	8.7	9.8	10.5	10.0
45	4.3	5.7	6.3	7.4	8.5	8.0
50	2.7	3.9	4.4	5.1	6.3	5.6
55	1.8	2.1	3.0	3.9	4.9	4.0
Inoculum Size (Spores/ml)						
$1x10^{5}$	3.2	4.4	5.0	6.0	6.9	5.8
$1x10^{6}$	4.5	5.8	6.4	7.6	8.4	7.5
$1x10^{7}$	5.1	6.0	7.5	8.1	9.6	8.5
$1x10^{8}$	6.4	7.9	9.1	10.2	11.0	10.4
$1x10^{9}$	4.9	6.0	7.2	8.0	9.1	8.3
Agitation speed (rev/min)						
140	3.6	4.7	5.2	6.0	7.1	6.1
160	4.7	5.7	6.8	7.7	8.5	7.6
180	6.2	7.1	8.8	9.2	10.6	9.5
200	6.8	7.9	9.7	11.0	12.1	11.3
220	6.5	7.4	8.7	9.4	11.4	9.8

Table 2. Effect of various carbon and nitrogen sources on L-asparaginase production by *S.gulbargensis*

	L-asparaginase activity (IU)				
	0.25%	**0.5%**	**0.75%**	**1.0%**	**1.25%**
Carbon Source					
Glucose	-	11.0	-	-	-
Fructose	-	11.4	-	-	-
Sucrose	-	17.8	-	-	-
Maltose	-	19.5	-	-	-
Starch	-	18.0	-	-	-
Galactose	-	10.4	-	-	-
Arabinose	-	10.9	-	-	-
Mannitol	-	10.8	-	-	-
Xylose	-	17.3	-	-	-
Lactose	-	16.8	-	-	-
Nitrogen Source					
Peptone	17.2	18.1	17.3	16.2	15.7
Beef Extract	20	22.4	19.8	18.1	14.3
Yeast Extract	22.8	23.1	21.0	19.0	15.8
Tryptone	14.7	16.3	16.9	14.8	13.6
Soyabean meal	21.0	23.6	23.9	22.0	19.3
Cornsteep liquor	22.8	24.0	23.3	20.9	18.6
Ammonium chloride	15.1	16.8	15.0	14.3	11.3
Ammonium nitrate	18.5	18.3	17.5	16.7	12.6
Ammonium sulfate	23.2	21.8	19.8	17.3	16.4
L-asparagine	23.1	25.0	24.6	21.2	19.1

Table 3. Purification profile of L-asparaginase from *S.gulbargensis*

Step	**Total activity (IU)**	**Total protein (mg)**	**Specific activity (IU/mg)**	**Purification fold**	**Yield (%)**
Crude Extract	3200	128	25	0	100
Ammonium sulfate precipitation	1620	36	45	1.8	50.6
Sephacryl S-200 gel filtration	1210	1.8	672.2	26.88	37.8
CM Sephadex C-50 chromatography	1026.5	0.5	2053	82.12	32

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