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ORIGINAL ARTICLE

L-asparaginase production by *Streptomyces albidoflavus*

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Abstract Attempts were made to optimize the cultural conditions for the production of L-asparaginase by *Streptomyces albidoflavus* under submerged fermentations. Enhanced level of L-asparaginase was found in culture medium supplemented with maltose as carbon source. Yeast extract (2%) was served as good nitrogen source for the production of L-asparaginase. The optimum pH for enzyme production was 7.5 and temperature was 35°C. The release of L-asparaginase from the cells of *S. albidoflavus* was high when strain was treated with cell disrupting agents like EDTA and lysozyme. The enzyme produced by the strain was purified by ammonium sulfate, Sephadex G-100 and CM-Sephadex C-50 gel filtration and the molecular weight was apparently determined as 112 kDa.

Keywords *Streptomyces albidoflavus* · L-asparaginase · Cell disrupting agents

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Introduction

L-asparaginase produced by microorganisms has been widely used as effective therapeutic agent against acute lymphoblastic leukemia and lymphosarcoma [1]. *Escherichia coli, Erwinia carotovora, Bacillus* sp., *Enterobacter aerogenes, Corynebacterium glutamicum, Pseudomonas stutzeri* and *Candida utilis* have been employed for the commercial production of this enzyme. L-asparaginase from two bacterial sources like *E. coli* and *E. carotovora* is currently in clinical use for the treatment of acute lymphoblastic leukemia [2]. Actinomycetes also serve as good source for L-asparaginase. Several *Streptomyces* species such as *S. karnatakensis, S. venezuelae, S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 are capable of producing detectable amount of L-asparaginase [3–6]. In the present work, attempts were made to study the optimization of L-asparaginase production from *Streptomyces albidoflavus*.

Materials and methods

An actinomycete strain was isolated from the laterite soils of Acharya Nagarjuna University campus and the culture was identified as *Streptomyces albidoflavus* by 16S rRNA analysis and gene sequence was submitted to NCBI genbank with accession number EF 142856. The strain was cultivated on yeast extract-malt extract-dextrose agar medium at 30°C for 7 days. Culture suspensions prepared from slants were inoculated to 100 ml of asparagine-dextrose-salts broth that contained L-asparagine (1.0%), dextrose (0.2%), K_2HPO_4 (0.05%), $MgSO_4.7H_2O$ (0.01%) and pH was adjusted to 7.2 [7]. The medium was incubated at 30°C for 5 days.

Production of biomass and L-asparaginase were monitored at 24 h interval.

Assay of L-asparaginase

Culture broth was centrifuged and the cells of *S. albidoflavus* obtained were suspended in 0.05 M Tris-HCl buffer (pH 7.4). The cells were ground in Tris-HCl buffer by homogenizer and cell debris was removed by filtration. Samples (0.2 ml) of filtered cell extract were made up to 2.0 ml with 0.05 M Tris-HCl buffer (pH 7.4) containing 20 μM of L-asparagine. This reaction mixture was incubated for 15 min at 37°C in a water bath shaker. The reaction was then stopped by the addition of 0.1 ml of 1.5 M trichloroacetic acid. Precipitated protein was removed by centrifugation and the liberated ammonia was determined spectrophotometrically at 500 nm by nesslerization. One IU of asparaginase is the amount of enzyme, which liberates 1 μM of ammonia in 1 min at 37°C [8]. After enzyme extraction from cells by using Tris-HCl, the cell pellets were obtained by centrifugation and dried in oven at 90°C. After 24 h, the cell dry weight was determined.

The effect of different carbon and nitrogen sources on cell growth and L-asparaginase production was studied in the basal medium consisting of K_2HPO_4 (0.05%), $MgSO_4.7H_2O$ (0.01%) and pH 7.2. Carbon compounds (1%) were added to the basal medium supplemented with L-asparagine (1%) as nitrogen source. The effect of different concentrations of nitrogen sources was studied by adding a nitrogen source (1%) to the basal medium containing maltose as carbon source. The effect of pH and temperature on L-asparaginase production was studied by cultivating the strain in fermentation medium (Maltose 0.1%, L-asparagine 1%, yeast extract 0.5% and K_2HPO_4 0.05%) at different pH levels ranging from 5.0–9.0 and temperature (15–45°C) for 72 h. Efficacy of cell disrupting agents like EDTA, SDS, Penicillin-G and Lysozyme were tested on the release of L-asparaginase from the 72 h old culture of *S. albidoflavus* and compared with physical methods of extraction such as grinding and rapid freeze-thawing [9]. Different concentrations of cell disrupting chemical agents prepared in Tris-HCl were used to treat the cells twice. Samples (0.2 ml) of treated cells were used as enzyme source.

Purification of L-asparaginase

The purification of L-asparaginase was carried out from the crude enzyme extract [10]. Finely powdered ammonium sulfate was added to 80% saturation for getting the protein precipitate instead of 50% saturation suggested by Distasio *et al.* [10]. The mixture was left for 12 h at 4°C, followed by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in a 0.01 M phosphate buffer (pH 8.0) and dialyzed against the same buffer at 4°C. The dialyzed ammonium sulfate fraction was applied to a Sephadex G-100 column that was pre-equilibrated with a 0.01 M phosphate buffer, pH 8.0. The protein elution was done with the same buffer at a flow rate of 5 ml/min. The fractions were collected manually and assayed for protein at 280 nm as well as for L-asparaginase activity. The active fractions were pooled, dialyzed and concentrated against the 0.01 M phosphate buffer (pH 8.5). The concentrated enzyme solution was applied to the column of CM-Sephadex C-50 that was pre-equilibrated with a 0.1 M sodium borate buffer (pH 7.0). It was eluted with the NaCl gradient (0.1–0.5 M) and 0.1 M sodium borate buffer (pH 7.0). The active fractions were collected, dialyzed and concentrated. The purified fraction was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the identification of molecular weight of L-asparaginase by using standard proteins [11].

Results and discussion

Production of L-asparaginase began after 24 h of cultivation and reached to maximum levels after 72 h of incubation. Maximum biomass production obtained with 72 h old culture could also be correlated with high levels of L-asparaginase production. L-asparaginase formation has been shown a firm link to the active cell growth [2].

Effect of carbon and nitrogen sources on L-asparaginase production

The results presented in table 1 revealed the effect of carbon sources on L-asparaginase production from *S. albidoflavus*. The biosynthesis of L-asparaginase was high when the strain was cultivated in basal medium with maltose as carbon source followed by starch, glucose, trehalose and glycerol. The final pH levels of the fermentation broths containing maltose, starch and trehalose became alkaline (7.5–8.0). When glucose, mannitol and fructose were added to the basal medium, L-asparaginase production decreased because of the acidic nature of fermentation broths. The acidic nature of fermentation medium could inhibit asparaginase biosynthesis. Glucose is reported to be a repressor of asparaginase synthesis due to the acid production [12].

Data on biomass and L-asparaginase production using different concentrations of nitrogen sources are presented

Table 1 Effect of different carbon sources on L-asparaginase production by *Streptomyces albidoflavus* after 72 h of incubation time

Carbon source (1%)	Final pH	L-asparaginase (IU/mg of cell dry wt.)	Cell dry weight (mg / ml)
Glucose	6.5	1.29	30.4
Fructose	6.9	0.48	8.7
Maltose	8.0	3.74	34.9
Starch	7.9	3.03	29.1
Mannitol	6.8	0.36	8.2
Trehalose	7.7	2.12	35.4
Glycerol	7.4	2.26	26.6

Table 2 Effect of nitrogen sources on L-asparaginase production by *Streptomyces albidoflavus* after 72 h of incubation time

Nitrogen source	Concentration (%)	L-asparaginase (IU/mg of cell dry wt.)	Cell dry weight (mg / ml)
L-asparagine	0.1	2.92	18.3
	0.5	3.35	24.7
	1.0	4.77	30.9
	1.5	2.21	30.2
L-glutamine	0.1	1.82	7.8
	0.5	2.98	14.2
	1.0	3.46	24.8
	1.5	2.84	24
Peptone	1	1.70	20.4
	2	3.06	34.7
	3	4.34	39.2
	4	2.87	29.8
	5	1.63	20.6
Yeast extract	1	2.40	33.6
	2	5.93	46.4
	3	5.58	43.6
	4	4.25	32.5
	5	1.82	26.7

in table 2. A high level of asparaginase production was obtained when yeast extract (2%) was used as nitrogen source followed by asparagine (1%). Yeast extract at 2% concentration supported good growth of the isolate reflected by its biomass. With increasing concentrations of peptone and yeast extract from 4% to 5%, there was a decline in enzyme production. This might be due to the presence of high substrate concentration and induction of proteolytic enzymes. Verma *et al.* [1] also stated that yeast extract is important for cell growth and L-asparaginase synthesis, but in high concentration L-asparaginase production was inhibited. Yeast extract (2%) was found to be the best organic nitrogen source for L-asparaginase synthesis from *S. albidoflavus* when compared to peptone (3%). Liu and Zajic [13] reported yeast extract as the best nitrogen source for maximum yield of L-asparaginase. Production of L-asparaginase by *Streptomyces griseus* ATCC 10137 was 0.36 IU per mg of cell dry weight as reported by De Jong [14]. In the present study, 5.93 IU of L-asparaginase per mg of cell dry weight was recorded when *S. albidoflavus* was cultivated in the medium amended with maltose (1%) and yeast extract (2%) as carbon and nitrogen sources respectively.

Effect of pH and temperature on the production of L-asparaginase

The effect of pH and temperature on the production of L-asparaginase was studied by cultivating the strain at different pH levels between 5.0–9.0 and temperature ranges between 15–45°C (Fig. 1). Optimum pH for asparaginase production was found to be 7.5 and 8. Optimum temperature for enzyme production ranged from 30–35°C. Extreme pH and temperature did not favor cell growth as well as L-asparaginase production from *S. albidoflavus*. Mostafa and Salma [3] reported that the 6-day old culture of *Streptomyces collinus* produced high amount of L-asparaginase when grown at pH 8.5 and temperature 28–30°C. But in the present study, 3-day old culture of *S. albidoflavus* was found to produce maximum amount of enzyme when cultured at pH 7.5 and temperature 35°C.

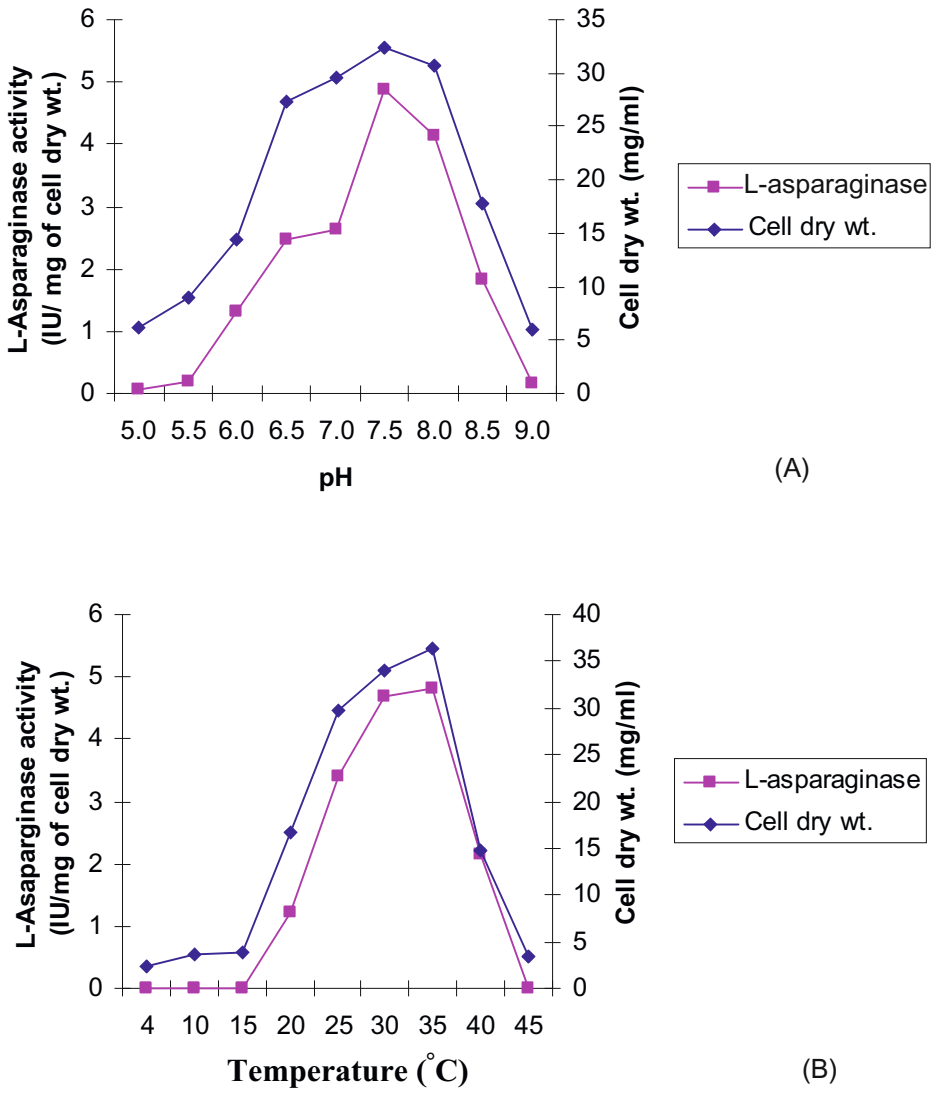

Fig. 1 Effect of A) pH and B) Temperature on L-asparaginase production by *Streptomyces albidoflavus* after 72 h of incubation time

Influence of cell disrupting agents on the release of L-asparaginase from the cells of *S. albidoflavus*

Most of the microbial L-asparaginases are intracellular in nature except few, which are secreted outside the cells [2]. Data on the effect of cell disrupting agents on the release of L-asparaginase from the cells of *S. albidoflavus* are presented in table 3. SDS (20 µg/ml), EDTA (40 µg/ml), lysozyme (50 IU/ml) and Penicillin-G (75 IU/ml) were found to enhance the release of L-asparaginase from the cells. Among these, effect of Penicillin-G was low on the release of L-asparaginase from the cells when compared to other agents. Mostafa [4] found that *S. karnatakensis* produce L-asparaginase intracellularly. The present study revealed that the cell disrupting agents like EDTA and lysozyme are

Table 3 Influence of cell disrupting agents on the release of L-asparaginase from 72 h old culture of *Streptomyces albidoflavus*

Cell wall affecting agents	L-asparaginase (IU/mg of cell dry wt.)
Physical methods	
Cell grinding	5.93
Rapid freeze-thawing	3.42
***Chemical methods**	
SDS (20 µg/ml)	5.14
EDTA (40 µg/ml)	7.1
Penicillin-G (75 IU/ml)	2.95
Lysozyme (50 IU/ml)	7.51

* Optimized concentration of chemical agents

Table 4 Purification profile of L-asparaginase from *Streptomyces albidoflavus*

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	623	2750	4.4	0.0	100
Ammonium sulphate precipitation	49	1032	21	4.7	72.4
Sephadex G-100	6.1	617	101	23	43.5
CM-Sephadex C-50	1.3	568	437	99.3	40

useful to extract intracellular L-asparaginase besides physical treatment. De Jong [14] reported the release of L-asparaginase from the cells of *Streptomyces griseus* ATCC 10137 when treated with lysozyme. But high concentrations of these agents could affect the enzyme activity. The release of L-asparaginase from the cells of *S. albidoflavus* increased from 5.93 IU to 7.1 and 7.51 IU per mg of cell dry weight when shifted from physical treatment (cell grinding) to chemical treatment with EDTA and lysozyme respectively. Rapid freeze-thawing method was less effective than cell grinding for the extraction of L-asparaginase from the cells.

Purification of the L-asparaginase

The purification steps for L-asparaginase are summarized in table 4. The proteins from the crude enzyme extract were precipitated by ammonium sulphate (80%) and most of the enzyme activity was retained by this precipitation. The specific activity of the enzyme increased to 101 and 437 IU/mg after purification with the Sephadex G-100 and CM-Sephadex C-50 respectively. Purity of L-asparaginase was increased up to 99.3-fold with 40% recovery in CM-Sephadex C-50 purification step. L-asparaginase from *Pseudomonas aeruginosa* 50071 has been purified in CM-Sephadex C-50 column up to 106-fold with 43% yield [11]. L-asparaginase from a marine *Streptomyces* sp. PDK2 has been purified 85-fold with 2.18% recovery in the final Sephadex G-200 purification step [6]. SDS-PAGE analysis of purified L-asparaginase from CM-Sephadex C-50 gel filtration exhibited a distinct protein band above 97 kDa might be L-asparaginase with electrophoretic mobility of 0.33. By using different standard proteins with known molecular weights, it was discovered that the apparent molecular weight of L-asparaginase from *Streptomyces albidoflavus* was 112 kDa. Molecular weight of asparaginases produced by microorganisms may vary. Molecular weights of L-asparaginase from *C. glutamicum, Streptomyces* sp.-PDK2 and *P. aeruginosa* 50079 have been determined as 80 kDa, 140 kDa and 160 kDa respectively by SDS-PAGE [2, 6, 11]. The present work clearly revealed that maximum production of L-asparaginase from *S. albidoflavus* could be achieved by cultivating in the medium contain maltose and yeast extract as carbon and nitrogen sources at pH 7.5 and temperature 35°C. Use of cell disrupting agents like EDTA and lysozyme significantly enhanced the release of L-asparaginase from *S. albidoflavus.*

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