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Screening and Characterization of Actinomycetes for Production of Anti-Leukemic L-Asparaginase

KEYWORDS: L-asparaginase, actinomycetes, screening, characterization

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ABSTRACT *Asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) is an anti-neoplastic agent, used in the lymphoblastic leukaemia chemotherapy. However L-asparaginase from bacterial origin can cause hypersensitivity in the long-term used, leading to allergic reactions and anaphylaxis. The search for other asparaginase sources, like eukaryotic microorganisms like fungi, actinomycetes etc. can lead to an enzyme with less adverse effects.*

Various microorganisms' viz. bacteria, fungi, actinomycetes and yeast, have ability for production of L-asparaginase. In the present investigation actinomycetes have been screened for L-asparaginase production. Primary and secondary screening has been done for 10 actinomycetes cultures for L-Asparaginase production. Assay was carried out for selection of maximum L-Asparaginase producers. The isolates exhibited extracellular as well as intracellular activity. Amongst the screened actinomycetal cultures 60% actinomycetal cultures showed remarkable extracellular enzyme activity. The actinomycetal isolate Act10 showed 202.88 µM/min enzyme activity which was screened as maximum L-asparaginase producer which was used for characterization.

Introduction

Microbial L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) have attracted considerable attention since the demonstration that L-asparaginase from different bacteria and fungi have antitumour activity. L-asparaginase is produced by large number of microorganisms. (Gulati *et.al.* 1997) It catalyses the hydrolysis of L-asparagine into aspartate and ammonia and this reaction is essentially irreversible under physiological conditions (Lubkowski *et al.* 1996). Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine (Swain *et al.* 1993; Manna *et al.* 1995). Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from the circulating plasma pools. Supplementation of L-asparaginase results in continuous depletion of L-asparagine. Under such environment, cancerous cells do not survive. This phenomenal behavior of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase. This enzyme is also a choice for acute lymphoblastic leukemia, lymphosarcoma and in many other clinical relating to tumor therapy in combination with chemotherapy (Prakasham *et al.* 2006). The excellent properties of this enzyme, such as the activity at the alkaline pH range at 37°C make it extremely valuable in the chemotherapeutic treatment of leukemia. (Ashraf *et al.* 2004). L- asparaginase is proved to be a potent antineoplastic agent in animals and has given complete remission in some human leukemias.

Bacterial L-asparaginases from E.coli, *Erwinia carotovora* and *Erwinia chrysanthemi* are effective in treating acute lymphosarcoma and lymphoblastic leukemia (Tiwari and Dua, 1996). Ever since Escherichia coli L-asparaginase anti-tumour activity was demonstrated, its production using microbial systems has attracted considerable attention owing to their cost effective and eco-friendly nature. In general, it is noticed that biochemical and enzyme kinetic properties vary with the microbial source. Moreover, L-asparaginases of bacterial origin can cause hypersensitivity in the long term use, leading to allergic reactions and anaphylaxis (Reynold and Taylor, 1993). This suggests that there is a need to discover new L-asparaginases that are serologically different, but have the similar therapeutic effects. The search for other asparaginase sources, like eukaryotic microorganisms like yeast and fungi have a potential for asparaginase production (Pinheiro *et.al.*2001), and the enzyme may have less adverse effects.

In the present investigation actinomycetes were isolated and screened for L-asparaginase production and the optimization of nutritional parameters at flask level to achieve maximum L-asparaginase production. Different pH, incubation conditions, temperature were studied. Effects of different nutritional parameters to examine the optimal condition for L-asparaginase production were performed.

Materials and Methods

Primary Screening- The microorganisms were isolated from the soil by enrichment method. Primary screening of L-asparaginase producing actinomycetal isolate was done by using Modified Czapek Dox's medium (Gulati *et.al.* 1997).

Three consecutive enrichments were carried out by serial dilution method. The flasks of last enrichment were used for isolation of L-asparaginase producing organism on above media containing L-asparagine and phenol red. Several microbial strains were isolated. L-asparaginase positive cultures were screened based on the color change from yellow to pink. The isolates were maintained in the aforementioned medium slants at 4°C after isolation.

Secondary screening: The L-asparaginase producing isolates selected by primary screening were used further for quantitative production of enzyme. In secondary screening the strains were selected based on maximum production of L-asparaginase determine by performing enzyme assay.

L-asparaginase Assay: L-asparaginase enzyme assay was measured by colorimetric method using UV-visible spectrophotometer, by estimating the ammonia produced during L-asparaginase catalysis using Nessler's reagent. Reaction mixture consisted of 50mM Tris Buffer,1ml; 189mM L-Asparagine solution,0.1ml; Distilled water, 0.9ml; incubate at 37°C for 5min. then enzyme solution, 0.1ml was added incubate at 37°C for 30min 1.5mM Tri Chloro Acetic acid,0.1ml was added to stop the reaction. The ammonia produced in the supernatant was estimated by Nesseler's method.

Enzyme Unit :- one unit of L-asparaginase is defined as that amount of enzyme capable of producing 1µmole of ammonia

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per minute at 37^{0}C.

Protein Estimation:- Protein was determined by Biuret method, using BSA as a standard by spectrophotometric measurements of absorption at 520nm. Standard graph was prepared with crystalline BSA.

Location of enzyme: The supernatant and cell free extract were assayed for enzyme activity in order to locate the enzyme.

Preparation of cell free extract: - The cells from 100ml of culture broth were harvested by centrifugation and pellet was suspended in lysis buffer (9 g glucose, 3g Tris, 20ml of 0.25M EDTA per 1000ml distilled water. After autoclaving, stored at 4^{o}C. 0.02% lysozyme). After overnight incubation this cell free extract used for the assay of L-asparaginase.

Optimization of cultural conditions:- The actinomycetal strain which showed highest activity was used further for optimization of cultural conditions viz. pH, temperature, incubation conditions. The pH of the medium was varied form pH 5.0 to pH 10.0, the incubation temperature was varied from 30^{0}C to 60^{0}C. The incubation condition (Shaking and Stationary) for all the isolates viz. Act1-Act10 were studied.

Similarly Carbon source and nitrogen source for maximum production of L-asparaginase were optimized. The various C-sources (0.5%) used were Fructose, maltose, starch, lactose, casein, oat meal, corn flour, molasses, Carboxy Methyl Cellulose (CMC) and sucrose. The various nitrogen sources (1%) used were beef extract, meat extract, peptone, tryptone, casein. Flasks were incubated at the optimum temperature.

Results and discussion: -
In the present investigation 10 actinomycetal isolates were isolated as a potential source of L-asparaginase (Fig.1; Table1). After primary screening 4 actinomycete L- asparaginase producing cultures were selected for secondary screening.

The isolates viz. Act1, Act7, Act8 and Act10 showed extracellular L-asparaginase activity (Table2). SVA 1 showed 89% extracellular activity, this makes the extraction, purification and downstream processing of the enzyme easier and economical. Optimization of incubation condition of screened isolates revealed that maximum L-asparaginase production was obtained when the flasks were incubated in stationary condition (Table2). Actinomycetal isolate Act10 showed 5 times greater enzyme activity when the flask was incubated in stationary state as compared to shaking condition. This may be due to extreme labiality of the enzyme resulting into surface denaturation of the enzyme due to vigorous shaking with concomitant loss of activity, as reported by Wriston and Yellin (1973). For effective utilization of any microbial system at bioprocess level, it is essential to screen and evaluate various nutritional and environmental requirements for microbial growth and subsequent enzyme production. Nutritional requirements for maximal synthesis of enzyme vary from one microorganism to the other. The rate of synthesis varies in the same organism as a function of cultural conditions reported by Barnes *et.al* (1977).

L-asparaginase from *E.coli* with antitumor activity has a pH optimum of 8.4 to 8.6. Assessing the pH activity of the actinomycetal isolate revealed that the enzyme is most active between pH 8.0 to 9.0. This similarity with *E.coli* L-asparaginase may hold a lot of promise to be harnessed for its therapeutic potential. Higher L-asparaginase activity was obtained at pH 9.0 for Act10. At physiological pH about 60% enzyme activity was obtained. Similarly the temperature optimum was found to be 30^{0}C this is closer to the physiological temperature (Table3).

Optimization of nutritional parameters namely carbon and nitrogen source resulted in maximum enzyme activity with glucose and peptone respectively. Maximum enzyme activity was obtained with glucose as carbon source as compare to the organic sources. However, tryptone and beef extract as a nitrogen source gave maximum enzyme activity. (Table3).

In view of hypersensitivity reactions associated with presently used *E.coli* and *Erwinia carotovora* L-asparaginase, the actinomycetal isolate Act10 hold a lot of promise for the production of L-asparaginase. L-asparaginase activity and its antilymphoblastic activity on human tumor cell lines will be explored.

Fig.1: Control with positive Actinomycetes isolates

Table 1: Screening of actinomycetal cultures on the basis of enzyme activity:

Culture	Conc. of Ammonia (µg/ml)	Enzyme Activity (µM/min)
Act 1	140.412	275.31
Act 2	5.118	10.03
Act 3	82.294	161.36
Act 4	47.882	93.88
Act 5	6.941	13.61
Act 6	92.294	180.96
Act 7	121.412	238.06
Act 8	123.471	242.09
Act 9	92.059	180.50
Act 10	103.471	202.88

Table2: Effect of incubation condition on intracellular and extracellular enzyme production

Actinomycetal isolates	L-Asparaginase activity			
	Intra-cellular	Extracel-lular	Stationary condition	Shaking condition
Act1	4.40	11.01	1.10	0.12
Act7	2.18	9.80	1.96	1.18
Act8	6.32	13.29	3.10	1.11
Act10	3.24	28.34	4.21	1.67

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Table3: Optimization of cultural conditions for the production of L-asparaginase

Cultural conditions	pH & Temp	Enzyme activity (µM/min)	Cultural conditions	C sources	Enzyme activity (µM/min)	% Activity	Cultural conditions	N sources	Enzyme activity (µM/min)	% Activity
pH	5	0.72	Carbon sources	Manitol	1.77	33.45	Nitrogen sources	Tryptone	7.76	82.9
	6	0.45		Fructose	2.32	43.85		Meat extract	1.56	16.66
	7	1		Glucose	5.29	100		Peptone	9.36	100
	8	1.38		Starch	1.85	34.97		Beef extract	5.88	62.82
	9	1.96		Lactose	1.6	30.24		Casein	5.95	33.56
	10	1.27		Oat Meal	1.88	35.53		Soyabean meal	6.22	66.45
Temperature	30°C	1.77		Corn Flour	3.08	58.22		Gelatin	4.89	52.24
	40°C	1		Molasses	3.95	74.66		Malt extract	2.11	22.54
	50°C	0.81		CMC	1.66	31.37		Yeast extract	3.90	41.66
	60°C	0.86		Maltose	2.54	48.01		Urea	5.10	54.48
	70°C	0.42		Sucrose	3.95	61.43		Albumin	3.67	39.20

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