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RESEARCH ARTICLE

Production of Tumor Inhibitory L-asparaginase by wild and mutant strains of *Pseudomonas fluorescens*

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Abstract

The production of L-asparaginase using wild and mutant strains of *Pseudomonas fluorescens* was studied to ascertain optimal nutritional conditions for large scale production leading to enzyme purification studies. Five bacterial isolates were screened for the production of L-asparaginase. All the five isolates have the ability to produce L-asparaginase. The optimal culture conditions for L-asparaginase production was found at pH 8.0, Incubation time 48 hrs and 37^{o}C temperature. Glucose was the best carbon source and beef extract was found to be sole nitrogen source. The L-asparaginase activity was 0.6 fold higher (168.4 IU/ml) using mutant *P.fluorescens* Pf1 UV60 than the wild strain and 0.54 fold more activity was recorded using Pf1 EMS 90 mutant than the wild strain. The molecular mass of the purified L-asparaginase was found to be 160,000 dalton.

Introduction

L-Asparaginase (L-Asparaginase amino hydrolase, EC 3.5.1.1) is an amidase group of enzyme involved in the catabolism of amino acid asparagine i.e. hydrolysis of the asparagines into aspartic acid and ammonia. This therapeutic enzyme is mainly used in treatment different forms of cancer (acute lymphoblastic leukemia). Asparagines are an essential amino acid which is essential for cell growth but it is not synthesized inside the body by any metabolic pathway. The reason it is preferred for the purpose is it is biodegradable, non-toxic and can be administered at the local site quite easily. Other agents are found quite painful when administered to the patient and also these are quite costly. Current clinical studies indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell diseases in man.

The antineoplastic activity is attributed to the depletion of L-asparagine by the action of L-asparaginase from *Erwinia carotovora* is reported by Lee *et al*. (1989). Mashburn and Wriston (1963) purified *E. coli* L- asparaginase and demonstrated its tumour inhibitory activity. Yasser *et al*. (2002) worked on *Pseudomonas aeruginosa* for L-asparaginase production in a solid state culture and also done an evolution of culture condition using factorial design. Younes Ghasemi *et al.* (2008) have studied the various concentrations of modified medium ingredients and various carbon sources were tested to optimize the medium for expression and identification of L-asparaginase in *E. coli*. Other bacterium includes *Pseudomonas aeruginosa* (Manikandan *et al.,* 2010), *Citrobacter* sp (Shah *et al.,* 2010) and *Bacillus* species (Maysa *et al.,* 2010); Basha *et al.,* (2009) screened the actinomycetes for enzyme activity. Microbial asparaginase have been particularly studied for their application as therapeutic agents in the treatment of certain types of human cancer (Gallagher *et al*., 1989). L-asparaginase from two bacterial sources (*E.coli* and *Erwinia carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukaemia (Keating *et al*., 1993). In the present research, we are focusing the enhanced production of biomedically valuable L-asparaginase production by strain improvement through UV and EMS treatment and its optimization conditions using *Pseudomonas fluorescens*.

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Material and Methods

Preliminary screening

Rapid plate assay for priliminary screening of L-asparaginase producing bacterial strains was carried out according to Gulati *et al*. (1997).

Qualitative assay

Modified M-9 agar medium was used for qualitative analysis of *Pseudomonas fluorescens* bacteria. Which contain (per 1000 ml of distilled water) $Na_2HPO_4.2H_2O$, 6.0g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; LAsparagine, 5.0 g; $MgSO_4.7H_2O$,2.0 ml; $CaCl_2$ $.2H_2O$,0.1 ml; 20% glucose stock,10.0 ml; agar, 20g; 2.5% dye (phenol red) stock solution was prepared in ethanol and pH was adjusted to 7.0. From this, 0.3 ml of dye (stock solution) was added to 100ml of modified M-9 medium. All *Pseudomonas fluorescens* strains were placed on the Modified M-9 agar medium and NaNO3 (nitrogen source) was added as control to M-9 medium instead of L-Asparagine. All plates were incubated at 37°C for 48 h. Formation of pink zone around the bacterial colonies indicate the L- asparaginase production by bacteria because at alkaline pH (due to accumulation of ammonia in medium) phenol red indicator was converted to pink.

Asparaginase activity assay

Modified M-9 liquid medium was used to determine Asparaginase activity. 50 ml of medium was taken in 250 ml Erlenmeyer flask and inoculated with *Pseudomonas fluorescens* strains which shown positive result in qualitative assay. This flask was incubated at 37°C at 250 rev min-1 for 48 h. Modified method of Mashburn and Wriston (1963) was used to assay L-Asparaginase activity in which 0.1 ml of cell suspension was taken to which 0.9 ml of 0.1 M sodium borate buffer and 1 ml of 0.04 M Asparaginase were added. This mixture was incubated for 10 minutes at 37°C. After 10 min 0.5 ml of 15% trichloro acetic acid was added and centrifuged at 8000 rpm for 10 min. supernatant was collected. 0.2 ml of supernatant was diluted to 8 ml with distilled water. This mixture was treated with Nessler's reagent and 1 ml of 2M NaOH. This mixture was incubated at 37°C for 15 min and absorbance determined at 500 nm was compared with Standard curve which was prepared from ammonium sulfate solution in different concentrations. 1 I.U (International Unit) of L-Asparaginase is equal to amount of ammonia liberated from L-Asparagine per minute.

Protein assay

The amount of Asparaginase produced from *Pseudomonas fluorescens* strains was determined by using Lowry's method (1951) and absorbance was compared with standard curve which was prepared from bovine serum albumin.

Optimization of culture conditions

Effect of pH on L-asparaginase production

To determine the pH influence on L-asparaginase production was studied with varying medium pH such as 2, 4, 6, 8, and 10 for 48 h incubation period. After incubation, the sample was collected by centrifugation for the determination of L-asparaginase activity and protein concentration.

Effect of temperature on L-asparaginase production

The effect of temperature was studied by cultivation of *P.fluorescens* strains in M9 medium and incubated at different temperature such as 25, 30, 37, 40, and 45^{o}C for 48 h incubation. After incubation, the L-asparaginase activity and protein concentration were determined.

Effect of Incubation time on L-asparaginase production:

To evaluate the effect of various incubation time was carried out by culturing the *P.fluorescens* strains in the production medium and incubated at varying incubation time such as 12, 24, 36, 48, 60 and 72 h. After incubation, the L-asparaginase activity and protein concentration were determined.

Effect of carbon source on L-asparaginase production

Various carbon source such as glucose, sucrose, maltose, xylose, mannose, and starch at 0.1% concentration were supplemented to the production medium, then introduced the culture and incubated at 48 hours. Afterthat, the enzyme activity and protein concentration were detected by extracting sample from the fermented medium.

Effect of nitrogen source on L-asparaginase production

To study the effect of various nitrogen sources on L-asparaginase production was determined with 0.5% of nitrogen sources such as beef extract, tryptone, yeast extract, peptone, and glycine were incorporated in the production medium and incubated at 48 hours. After incubation, the L-asparaginase activity and protein concentration were determined.

Mutagenesis of *Pseudomonas fluorescens* isolates (Pf1 to Pf5) by UV

The UV treatment for L-asparaginase production was done according to Ellaiah *et al*.

(2002). The L-asparaginase producing microorganisms are exposed to UV for 30, 60, and 90 seconds. After exposing the plates to UV the plates were kept at room temperature for 24 h. After incubation, the L-asparaginase activity and protein concentration were determined.

Mutagenesis of *Pseudomonas fluorescens* isolates (Pf1 to Pf5) by EMS (Ethyl Methane Sulfonate)

The effect of EMS treatment for L-asparaginase production was done according to Ellaiah *et al*. (2002). The culture was pelleted by centrifugation, then washed it by using citrate (0.1M; pH 5.5) and Phosphate (0.1 M; pH 7.0) buffers separately 0.2 ml of EMS (10mg/10 ml) was added at different time intervals such as 0, 30, 60, 90, and 120 seconds. Afterthat, the plates were incubated at 37^{o}C for 24 h. After incubation, the L-asparaginase activity and protein concentration were determined.

Purification of L-asparaginase

The purification of produced enzyme was carried out at 4^{o}C on the crude extract according to the modified method of Distasio *et al*. (1976). Finely powdered ammonium sulphate salt was added to the crude eenzyme at 80% saturation level. The mixture was left for 12 h at 4^{o}C followed by centrifugation at 8000 rpm for 20 min at 4^{o}C. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4^{o}C. The dialysed sample was collected in a sterile container and stored at -20^{o}C for further studies.

Molecular weight determination

The molecular weight determination of the purified asparaginase enzyme was done according to El-Bessoumy *et al*. (2004). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3 mm slab gel of 12%. The gels were stained with 0.025 Coomassie brilliant blue R-250 and destained with destaining solution. The standard proteins were used as marker protein for the molecular weight determination (Bangalore Genei).

Results

Screening of L-asparaginase producing microorganism

The preliminary selection revealed that all the five *Pseudomonas fluorescens* isolates (Pf1 to Pf5) showed pink zone around the colony on M9 medium containing phenol red as indicator (Fig 1), indicating the increase in pH which originated from ammonia accumulated in the medium.

Optimization of culture conditions

Effect of pH on L-asparaginase production

Fig. 2 represents the data for the effect of pH on L-asparaginase production by *Pseudomonas fluorescens* isolates (Pf1 to Pf5). It revealed that maximum L-asparaginase production was 120 IU/ml at pH 8.0 by *Pseudomonas fluorescens* isolate Pf1. Next to this strain, 80, 72.6, 68.5, and 44.2 IU/ml of L-asparaginase were produced by *P.fluorescens* isolates Pf2, Pf3, Pf4 and Pf5 respectively. Below and above level of pH 8.0 showed decreased level of L-asparaginase enzyme activity.

Effect of temperature on L-asparaginase production

Data on the effect of temperature on L-asparaginase enzyme production by *Pseudomonas fluorescens* isolates (Pf1 to Pf5) is given in Fig.3. The maximum L-asparaginase activity (48.6 – 84.5 IU/ml) was recorded at 37^{o}C. The produced L-asparaginase enzyme ranged from 28.4 to 84.5 IU/ml. Increase and decrease level of temperature from the optimal level (37^{o}C) the L-asparaginase activity was decreased.

Effect of Incubation time on L-asparaginase production

Fig. 4 revealed that the highest L-asparaginase production was 96.2 IU/ml at 48 hrs of incubation by *Pseudomonas fluorescens* isolate Pf1. Next to this strain, 82.5, 74.6, 64.5, and 42.4 IU/ml of L-asparaginase were produced by Pf2, Pf3, Pf4 and Pf5 isolates of *Pseudomonas fluorescens* respectively. Below and above level of optimum incubation time (48 hrs) showed decreased levels of L-asparaginase enzyme activity.

Effect of carbon source on L-asparaginase production

The data for the effect of carbon source on L-asparaginase production by *Pseudomonas fluorescens* isolates (Pf1 to Pf5) is given in Fig.5. It revealed that maximum L-asparaginase production was 140.5 IU/ml by *Pseudomonas fluorescens* isolate Pf1 using glucose as sole carbon source. Next to this strain, 120, 78.6, 70.5, and 62.5 IU/ml of L-asparaginase were produced by Pf2, Pf3, Pf4 and Pf5 isolates of *Pseudomonas fluorescens*, respectively. Compared to glucose the other carbon source showed decreased levels of L-asparaginase enzyme activity.

Table 1: L-Asparaginase activity (IU/ml) by UV mutant strains of *Pseudomonas fluorescens*

***Pseudomonas fluorescens* Isolates**	**L-asparaginase activity (IU/ml)**	**Protein (mg/ml)**	**Specific activity (IU/mg protein)**	**% increase of activity**
Wild Pf1	110.5	98.6	1.120	-
Mutant Pf1 UV 30	120.5	95.6	1.260	9.05
Mutant Pf1 UV 60	178.4	128.5	1.386	61.45
Mutant Pf1 UV 90	130.5	102.0	1.279	18.09
Wild Pf2	90.0	85.4	1.054	-
Mutant Pf2 UV 30	95.5	78.6	1.215	6.1
Mutant Pf2 UV 60	140.6	86.5	1.625	56.2
Mutant Pf2 UV 90	110.4	78.01	1.415	22.7
Wild Pf3	68.6	69.5	0.987	-
Mutant Pf3 UV 30	74.2	69.5	1.068	8.2
Mutant Pf3 UV 60	84.6	74.6	1.134	23.3
Mutant Pf3 UV 90	70.5	68.0	1.036	2.8
Wild Pf4	60.5	64.5	0.938	-
Mutant Pf4 UV 30	64.5	78.4	0.873	13.2
Mutant Pf4 UV 60	69.8	70.6	0.974	15.4
Mutant Pf4 UV 90	62.5	63.75	0.980	3.31
Wild Pf5	60.5	48.6	1.245	-
Mutant Pf5 UV 30	61.5	43.46	1.415	1.7
Mutant Pf5 UV 60	68.5	40.5	1.691	13.2
Mutant Pf5 UV 90	63.5	54.2	1.172	4.9

Table 2 : L-Asparaginase activity (IU/ml) by EMS mutant strains of *Pseudomonas fluorescens*

***Pseudomonas fluorescens* Isolates**	**L-asparaginase activity (IU/ml)**	**Protein (mg/ml)**	**Specific activity (IU/mg protein)**	**% increase of activiy**
Wild Pf1	110.5	98.6	1.120	-
Mutant Pf1 EMS 30	120.6	115.0	1.049	9.14
Mutant Pf1 EMS 60	180.7	168.0	1.076	63.5
Mutant Pf1 EMS 90	220.6	210.0	1.050	99.6
Wild Pf2	90.0	85.4	1.054	-
Mutant Pf2 EMS 30	98.7	75.0	1.36	9.7
Mutant Pf2 EMS 60	140.6	124.0	1.134	56.2
Mutant Pf2 EMS 90	170.7	113.56	1.503	89.7
Wild Pf3	68.6	69.5	0.987	-
Mutant Pf3 EMS 30	70.5	68.0	1.036	2.8
Mutant Pf3 EMS 60	85.6	60.49	1.415	24.8
Mutant Pf3 EMS 90	128.4	69.0	1.861	87.2
Wild Pf4	60.5	58.5	1.034	-
Mutant Pf4 EMS 30	68.5	78.0	0.878	13.2
Mutant Pf4 EMS 60	84.5	65.8	1.284	39.7
Mutant Pf4 EMS 90	110.5	78.08	1.415	82.6
Wild Pf5	60.5	48.6	1.245	-
Mutant Pf5 EMS 30	62.5	72.0	0.868	3.31
Mutant Pf5 EMS 60	78.2	68.5	1.142	29.3
Mutant Pf5 EMS 90	88.6	62.6	1.415	46.4

Table 3: Purification profile of L-asparaginase activity (IU/ml) by *Pseudomonas fluorescens*

Pseudomonas fluorescens Isolates	Purification Steps	Collected Volume (ml)	Total activity (IU/ml)	Total Protein (mg/ml)	Specific activity (IU/mg protein)	Purification factor	Recovery
Pf1	Crude extract	500	48000	180	0.53	1	100
	80% Ammonium sulphate	25	2000	118.6	0.67	1.3	65.9
	Ethyl alcohol	2.5	6	35	0.07	0.10	19.4
Pf2	Crude extract	500	355000	430.6	1.6	1	100
	80% Ammonium sulphate	25	5250	240.8	0.87	0.54	55.9
	Ethyl alcohol	2.5	26.5	48	0.22	0.25	11.1
Pf3	Crude extract	500	320000	380	1.7	1	100
	80% Ammonium sulphate	25	4500	230.4	0.78	0.45	60.6
	Ethyl alcohol	2.5	21.5	46	0.19	0.24	12.1
Pf4	Crude extract	500	160000	280	1.14	1	100
	80% Ammonium sulphate	25	3000	225.4	0.53	1.7	80.5
	Ethyl alcohol	2.5	17	37	0.18	2.5	13.2
Pf5	Crude extract	500	365000	475	1.5	1	100
	80% Ammonium sulphate	25	5500	234	0.94	0.63	49.3
	Ethyl alcohol	2.5	31.5	56	0.23	0.24	11.8

Effect of nitrogen source on L-asparaginase production

Fig. 6 explains the data for the effect of nitrogen source on L-asparaginase production by *Pseudomonas fluorescens* isolates (Pf1 to Pf5). It revealed that the highest amount L-asparaginase production was 96.4 IU/ml by Pf1 isolate of *Pseudomonas fluorescens* using beef extract as sole nitrogen source. Next to this strain, 78.6, 72.1, 54.5, and 52.5 IU/ml of L-asparaginase were produced by Pf2, Pf3, Pf4, and Pf5 isolates of *Pseudomonas fluorescens*, respectively. Compared to beef extract the other nitrogen source showed decreased levels of L-asparaginase enzyme activity.

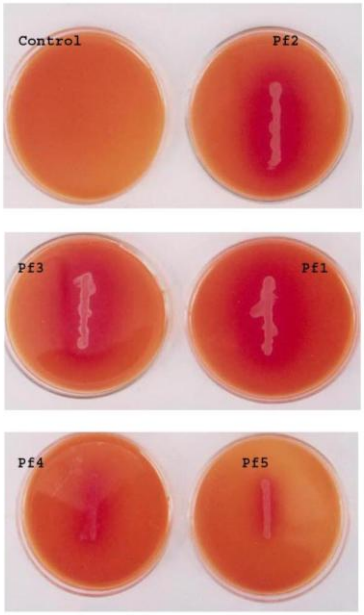
UV mutant on L-asparaginase production

Table 1 provides the data for the UV mutants on L-asparaginase production. The highest L-asparaginase activity (178.4 IU/ml) was observed by using *Pseudomonas fluorescens* Pf1 UV 60. It was 0.6 fold of the Parent strain. Similarly all the mutant strains showed increased L-asparaginase activity. The specific activity observed was ranged from 0.873 to 1.691 IU/mg. The percentage increase of L – asparaginase activity was high compared to the parent strain. The highest percentage increase of 61.45 % was recorded by *Pseudomonas fluorescens* Pf1 UV 60. Similarly all the other strains also showed high percentage of increase of L – asparaginase activity.

EMS mutant on L-asparaginase production

The data for EMS mutant on L-asparaginase production is given Table 2. It revealed that EMS treatment could yield many mutants. Examination of L-asparaginase activity of the mutant showed increased activity (220.6 IU/ml) by *Pseudomonas fluorescens* Pf1 EMS 90. It was also 0.52 fold more of the wild strain (*Pseudomonas fluorescens*). The specific activity observed in the present study was ranged from 0.840 to 1.426. The percentage increase of L–asparaginase activity was high (99.6%) by *Pseudomonas fluorescens* Pf1 EMS 90 strain. Other strains also showed increased activity of L – asparaginase (% increase of activity) compared to the parental strains.

Fig.1: Screening of L-Asparaginase producing Pseudomonas fluorescens isolates (Pf1 to Pf5)



Purification profile of L-asparaginase

The L-asparaginase enzyme of *Pseudomonas fluorescens* isolates Pf1 to Pf5 were recovered following 80% saturation of the culture supernatant with ammonium sulphate showed an increase of specific activity ranged from 0.07 to 1.7 IU/mg protein (Table 3).

Molecular mass determination

The molecular mass of partially purified L-asparaginase sample was run in SDS –PAGE gel using coomassie brilliant blue staining method. The molecular mass of the purified L-asparaginase seemed to be 160,000 dalton (Fig.7).

Fig. 2 : Effect of pH on L-asparaginase production (IU/ml) by various *P.fluorescens* isolates *(Pf1 to Pf5)*

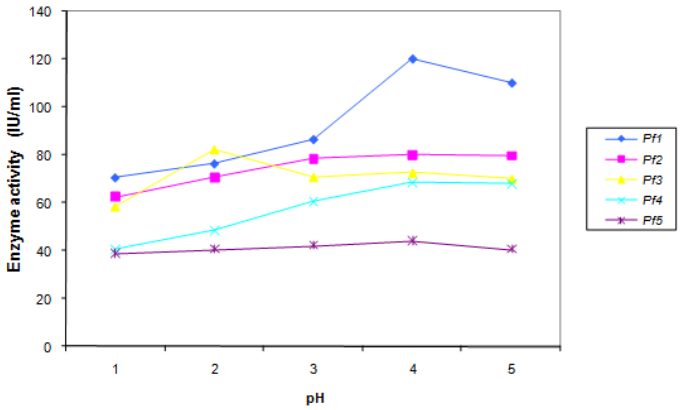


Fig. 3 : Effect of temperature (°C) on L-asparaginase production (IU/ml) by various *P.fluorescens* isolates *(Pf1 to Pf5)*

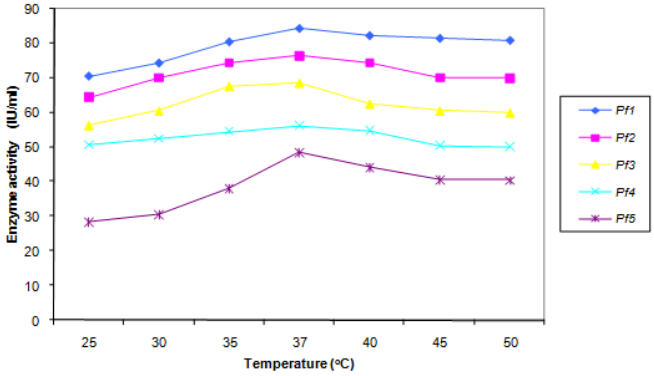


Fig. 4: Effect of incubation time (h) on L-asparaginase production (IU/ml) by various *P.fluorescens* isolates *(Pf1 to Pf5)*

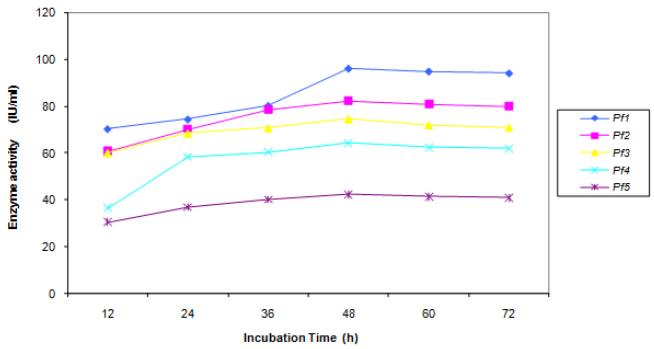


Fig. 5 : Effect of carbon source on L-asparaginase production (IU/ml) by *P.fluorescens* isolates *(Pf1 to Pf5)*

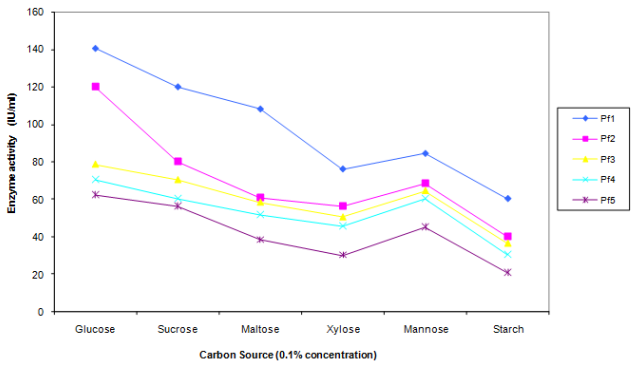


Fig. 6 : Effect of nitrogen source on L-asparaginase production (IU/ml) by *P.fluorscens* isolates *(Pf1 to Pf5)*

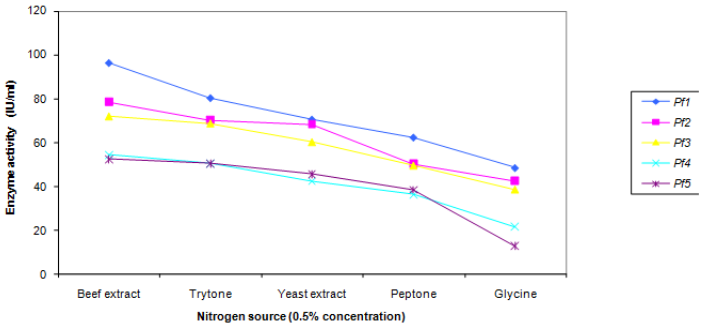
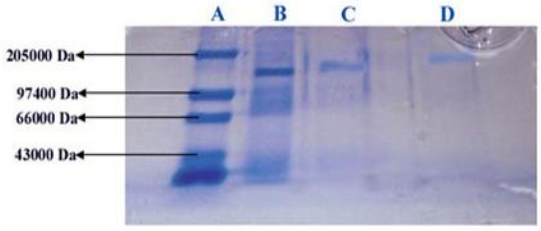


Fig.7: SDS-PAGE electrophoresis of partially purified L-Asparaginase enzyme



Lane A : Standard protein marker
Lane B : Partially purified L - asparaginase of Pf1
Lane C : Partially purified L - asparaginase of Pf2
Lane D : Partially purified L - asparaginase of Pf3

Discussion

All the *Pseudomonas fluorescens* isolates (Pf1 to Pf5) showed positive for L-asparaginase production by producing pink coloration around the colony. The control showed no colour change. Thus, the plate assay is rapid and reliable, it is advantageous and L-asparaginase production can be visualized directly from the plates without performing time consuming assays (Wade *et al*., 1971; Arima *et al*., 1972; Imada *et al*., 1973). The incubation period for bacteria and fungi was 18 – 48 h and 48-72 h, while in broth studies it is 24-48h for bacteria and often exceeds 96 h for fungi (Imada *et al*., 1973). This concept is inagreement with the present experiment. The present result showed that the incubation period for better production of L-asparaginase was 48 h.

As a rule, different nitrogen sources can be used to promote high enzyme production the nitrogen regulation mechanism in filamentous fungi has been carefully studied (Caddick *et al*., 1994). Similarly in the present data showed different nitrogen sources used to promote high L-asparaginase production. Mukherjee *et al*. (1999) reported that the ten fold purification of the L-asparaginase enzyme with a specific activity of 55 IU/mg protein and recovery of 54%. Similarly in the present study showed 1.7 fold purification of the L-asparaginase enzyme with a specific activity of 0.53 IU/mg protein and the recovery of 80.5%.

The effect of different concentration of glucose on enzyme production showed catabolic repression. Glucose at 1% caused almost total inhibition of enzyme activity, while at 0.1% it showed a slightly stimulatory effect on enzyme production, compared with glucose free medium (Geckill and Gencer, 2004). This finding is in agreement with the present result. In the present experiment 0.1% of glucose as carbon source showed a stimulatory effect on L-asparaginase production compared with glucose free medium.

Sobis and Mikucki (1991) reported that the optimum pH of purified Staphylococcal L-asparaginase was found to be 8.6 and 8.8. The

temperature optimum was 30^0C. In contrast to this report, the present data showed the optimum pH of purified L-asparaginase from *P.fluorescens* was found to be 8.0. The temperature optimum was 37^0C. El-Bessouomy *et al.* (2004) suggested that maximum L-asparaginase activity occurred when it was incubated with an optimum substrate concentration at pH 9.0. A similar pH values were obtained for *E.coli* (Castaman and Rodeghiero, 1993 and Liboshi *et al*., 1999) *Pseudomonas aeruginosa* 10143 (Robert *et al*., 1968), and many other microbial asparaginase activity (Balcao *et al*., 2001). In contradiction to the previous report, the present experiment showed maximum L-asparaginase activity recorded at pH 8.

A temperature profile showed that the enzyme had maximum activity at 37^0C. Similar results were recorded for asparaginase from *Pseudomonas aeuroginosa* 50071 (El-Bessoumy *et al*., 2004), *Pseudomonas stulzeri* MB-405 (Manna *et al.*, 1995), *Erwinia carotovora* (Maladkar *et al.*, 1993), and *Staphylococcus* (Sobis and Mikucki,1991).

Ellaiah *et al*. (2002) reported that UV induced mutant AUV 4 showed highest enzyme activity it was 1.64 of the parent strain. Similarly the present experiment also showed higher enzyme activity by using mutant strains than the parent strains. Shu-Gui Caob and Ke – Chang Zhanga (2000) reported an increasing enzyme production of 3.25 fold by using *Pseudomonas* mutant of UV and EMS. This finding is in agreement with the present report it also showed an increase in L-asparaginase production by using various isolates (Pf1 to Pf5) of *Pseudomonas fluorescens* mutants of UV and EMS.

Conclusion

From the observations carried out clearly indicate that *Pseudomonas fluorescens* strains have the ability to produce significant amount of L-asparaginase enzyme. Fermentation studies carried out indicate that the process is an efficient and economically viable for the production of L-asparaginase especially in the developing countries. Critical factors influence the selection of asparaginase as a therapeutant such as temperature, pH, incubation time, carbon and nitrogen sources. The present study indicates that large quantities of the enzyme with highest activities reported can be obtained at pH 8.0, Incubation time 48 hrs and 37^oC. Glucose was the best carbon source and beef extract was found to be sole nitrogen source which makes it attractive for further optimization and characterisation studies.

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