Optimization of Process Parameters for Enhanced Production of L-Asparaginase from *Erwinia carotovorum* MTCC 1428

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Abstract:- **In this study the stepwise optimization strategy was applied to maximize the production of antileukemic enzyme L-Asparaginase from *Erwinia carotovorum* MTCC 1428. I studied the effect of one parameter at a time and other parameter kept constant, by this way the result was that among different carbon & nitrogen sources. Lactose & Yeast extract respectively gave maximum enzyme activity and the individual optimum temperature , initial pH of the medium , incubation period , inoculums size and rotation of shaking incubator were found to be 3%(w/v), 2% (w/v) , 30ºC , 7.0 , 48 hrs , 6% (v/v) and 150 rpm respectively. The maximum specific activity of L-Asparaginase in the optimized medium was 16.18 U/mg of protein resulting in overall 3.8-fold increase in the production compared to un-optimized medium.**

Keywords: L-Asparaginase, Antileukemic&Glutaminase.

I. INTRODUCTION

L-Asparginase (L-aspargineamidohydrolase; E.C. 3.5.1.1.) catalyze the deamination of L-Aspargine to L-Aspartic Acid and Ammonia. Although Clementi in 1922 had reported its presence in guinea-pig serum,the anti-tumor properties of the enzyme were only recognized some time later .Tsuji first reported deamination of L-asparginase by extract of *E.Coli*in 1957.L-asparaginase is used as a chemotherapeutic agent for acute lymphocytic leukaemia (ALL) andless frequently for acutemyeloblasticleukaemia, chronic lymphocytic leukaemia, Hodgkin's disease, melonosarcomaand non- Hodgkin's lymphoma. Since several types of tumour cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase. Effective depletion of L-asparagine results in cytotoxicity for leukaemic cells (Basha S., et.al. 2009).Current clinical studies indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man (Peterson and Ciegler, 1969). Various bacteria such as, *E.coli*,*Erwiniaaroideae, Proteus vulgeris ,Streptomyces griseus, Vibrio succinogenes, Citrobacterfreundi, Thermusaquaticus, Enterobacteraerogenes,ThermusthermophilusZymomonasmobilis and Pseudomonas aeruginosa* found to produce L-asparaginase(Baskar&Renganathan, 2009).L-asparaginase is also tried for reducing the acryl amide content in roasted and friedfoods.Based on sequence homology analysis (Borak D. &Jaskolaski et. al., 2007) as well as biochemical (Cedar H. et al,1968 ,Campbell HA. et al,1967& Dunlop PC et al,1980) and crystallographic data (Miller M. et al,1993 & Yao M.,Yasutake et al, 2005)available L-Asparginase sequences can be divided in to three families.Bacterial type l-asparaginase, Plant type l-asparaginase& Rhizobium etli l-asparaginase.Bacterial type l-asparaginase can be further classified in to two sub-type; type I and type II. Two type of l-asparaginase found in E.Coli; have been designated EC1 and EC2 in E.Coli B (Campbell HA. et al 1967), and Asn I and Asn II in E.Coli K12 (Cedar H. et al,1968).Type-I was found to be expressed constitutively whereas type-II is induced by anaerobiosis (Cedar H. et al, 1968).Only the type-II l-asparaginase present tumor inhibitory activity and for this reason, have been extensively studied (Cedar H. et al, 1968). L-Asparaginase was produced by the technique of submerged fermentation because if offer many advantages over SSF such as submerged fermentations are generally perfectly mixed reactions, high water content leads to porosity, lower oxygen diffusion limitations & ease of control of environmental parameters etc.

II.MATERIALS & METHODS

2.1. Microorganism

The pure cultures of *Erwinia carotovorum* MTCC 1428 was procured from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh. YBP medium was used for maintenance of pure culture of *Erwinia carotovorum* MTCC 1428. Sub-culturing was done after a period of 30 days. The slants were maintained at 4^0C.

2.2. Media and cultivation condition

The YBP media (50 ml) was dispensed in 250 ml Erlenmeyer flasks and sterilized. Then media was autoclaved at 121º C for 20 min and then inoculated with *Erwinia carotovorum* MTCC 1428. The flasks were incubated in a rotary shaker for 24 h at 180 rpm. YBP medium have following composition for 1 liter media:Beef extract 1g; Yeast extract 2g; Peptone 5g; Agar 15g; NaCl 5g. pH of the medium was adjusted to 7.0 by adding NaOH and HCl (1 N). The production of L-Asparginase

has been studied in modified basal semi-synthetic medium for submerged fermentation was:

Table1. Production media composition

Ingredients	Weight (g/L)
Carbon source	1.0
Nitrogen-source	1.0
(KH_2PO_4)	3.0
Sodium chloride	0.5
Na_2HPO_4	6.0
$MgSO_4.7H_2O$	0.5
$CaCl_2.2H_2O$	0.015

pH 6.5 of the medium was adjusted by adding NaOH and HCl (1 N).

2.3. Medium and process optimization of L-asparginase production

Submerged fermentation was carried out for *Erwinia carotovorum* MTCC 1428 using semi synthetic basal medium. The production media were autoclaved at 121ºC for 15 minutes and were cooled to 37ºC. Then medium were inoculated with 1 ml inoculums for 50 ml production media in 250 ml shake flask and incubated at 30ºC for 24 hours and all the production experiments were carried out in triplicate form.

Stepwise optimization strategies was applied for this study, one factor varied and others kept constant and after getting optimum value for the maximum enzyme activity, then it's was taken in to the further studies.

2.4.1 Effect of various carbon sources

To optimize the cultivation conditions yielding maximum l-asparginase, the culturewas grown on various carbon sources at initial temperature 30ºC and incubation time of 24 h and the l-asparginase activity was observed.Glucose, fructose, maltose,lactose and starch each at same concentrations i.e., 1.0% were used as different carbon sources for the production of l-asparginase enzyme and the best carbon source was taken in to further studies.

2.4.2. Effect of various concentration of carbon source

Effect of different concentration of suitable carbon source on production of l-asparginase by *Erwinia carotovorum* was checked. The data was collected for 1 %, 2%, 3%, 4% & 5% and its optimum concentration was taken in to further studies.

2.4.3. Effect of various Nitrogen source

The effect of different nitrogen sources i.e., peptone, yeast extract, urea, beaf extract and urea (each at a conc. Of 1.0%) on l-asparginase production was observed and results are presented and the best nitrogen source was taken in to further studies.

2.4.4. Effect of various concentration of Nitrogen source

Effect of different concentration of suitable nitrogen source on production of l-asparginase by *Erwinia carotovorum* was checked. The data was collected for 1 %, 2%, 3%, 4% & 5% and its optimum concentration was taken in to further studies.

2.4.5. Effect of various inoculumssizesand incubation times

The effect of different inoculums size i.e. from 1-5 ml on l-asparginase production was observed and results are presented and the best inoculums size was taken in to further studies.Effect of fermentation time on the production of l-asparginase by *Erwinia carotovorum* was observed. The data was collected for 12h, 24h, 48h and 60h.

2.4.7. Effect of incubation temperatures

The production medium was incubated at different temperature levels, to study the effect of temperature on production of enzyme. Various incubation temperature *viz.*20ºC-40ºC were screened for the highest enzyme production.

2.4.8. Effect of initial pH and agitation

The influence of initial pH on l-asparginase by *Erwinia carotovorum* was determined in the pH range (5-9). Medium was adjusted to required pH with the addition of Phosphate or Tris buffer.The influence of different rpm of shaking incubator on l-asparginase by *Erwinia carotovorum* was determined in the rpm range (50-300) and suitable rpm is screened for maximum enzyme production.

2.5. Intracellular L-AsparginaseExtraction and its partial purification

Using ultrasonication for release of L-asparginase, first cells were harvested from the production medium and centrifuged at 10000 rpm for 10 min & 4^0C. After this cells were washed with 50 mMTris-HCl buffer pH 8.6 and were resuspended in the same buffer to make a cell suspension of 2% w/v. Cells were disrupted using ultrasonication probe with 30 pulses at 30 se c interval for 10 min. Disrupt thus obtained was centrifuged at 12000 rpm for 15 min at 4 deg C. The cell free supernatant was subjected to enzyme assay.
1M Ammonium stock solution was prepared by dissolving 114 g of anhydrous ammonium sulfate in 1 L D.W., from that working standard was prepared. Working standard was prepared by serial dilution. From 1 µM working standard solution, 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 µM. standards were prepared by taking 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 ml respectively. The total volume was made upto 2.5 ml using distilled water.1 ml of Tris-Hcl buffer was added to each test tube and incubates it for 30 min. at 37ºC, 0.5 ml 15 M TCA was added followed by 1 ml NaOH. Immediately the solution was mixed and allowed to react for 20 min. The

final solutions were red at 480 nm in spectrophotometer. Blank was prepared by without anhydrous ammonium sulfate.

2.6. Assay of L-Asparginase

Assay of enzyme for release of ammonia was carried out as per Marhburn and Wriston(1993).1.7ml of 0.01M L-asparagine was taken in a test tube,0.2ml of 0.05M buffer(Tris-HClpH 8.6)and 0.1ml of enzyme were added to a final volume of 2ml and the reaction mixture was incubated for 10min at 37°C. After the incubation period, 0.5 mL of 1.5M TCA was added to stop the reaction. To 0.5ml of the above reaction mixture 6.5ml of distilled water and 1ml nessler's reagent were added and incubated further for 10min for Nesselerisation. The O.D was measured at 480nm. One unit of L-Asparaginase was defined as the amount of enzyme which liberated 1µmol of ammonia per ml under the above assay conditions. Specific activity was given as units per milligram protein.

2.7. Protein estimation

Protein estimation was done using the method given by Bradford.This method relies on the binding of the dye Coomassie-Brilliant blue G250 to proteins and has been developed from the staining of proteins in gel. The method is both quick and sensitive.0.5 ml protein extract was taken in a test tube.To the protein solution 5 ml of Bradford's reagent was added.A blank was prepared using 0.5 ml buffer instead of the protein solution.The samples were allowed to stand at room temperature for 10-30 min.Readings of O.D. at 595 nm were taken against the blank using spectrophotometer.The specific activity was calculated by the formula,Specific activity (U/mg) = Enzyme activity/protein concentration of reaction mixture.

III.RESULTS & DISCUSSION

The bacterial culture *Erwinia carotovorum* MTCC 1428 was earlier grown on YBP media was then inoculated in various production media vary in different process and medium parameters. The L-asparginase activity was then calculated in these media.

3.1. Enzyme assay of E. carotovorum crude extract

The crude enzyme was extracted and then enzyme assay was done by estimating the amount of ammonia released. The O.D. was then compared with the standard graph for ammonia.Protein estimation of the supernatant received after the centrifugation of the fermented broth was done by using the method given by Bradford. The O.D. was taken at 595 nm in the UV spectrophotometer and compared with the standard BSA curve.

3.2. Effect of different Carbon sources on L-Asparginase production

Using 1g/l (1% w/v) of different carbon sources, L-Asparginase production was highest in lactose medium. The nature and amount of carbon source in culture media is important for growth and production of intracellular L-Asparginase in bacteria. L-Asparginase yield was high in media containing lactose as sole carbon source (Fig 1).lactose medium showing the maximum enzyme activity was assayed for protein estimation by Bradford's method and it was found that it contain 0.605 mg/ml of protein content. The specific activity thus obtained was 3.97 U/mg.

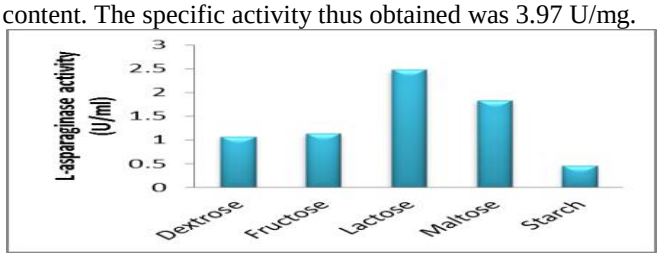

Fig. 1 Effect of various carbon sources.

3.3. Effect of different concentration of lactose on L-Asparaginase production

Using different concentration of Lactose (1-5% w/v), L-Asparginase production was highest in the concentration of 4g/l (4% w/v) of lactose followed by 3% (w/v) but it shows decrease in enzyme activity if lactose concentration in higher than 4%(w/v) (Fig.2).Lactose concentration (4% w/v) showing the maximum enzyme activity was assayed for protein estimation by Bradford's method and it was found that it contain 0.685 mg/ml of protein content. The specific activity thus obtained was 6.11 U/mg.

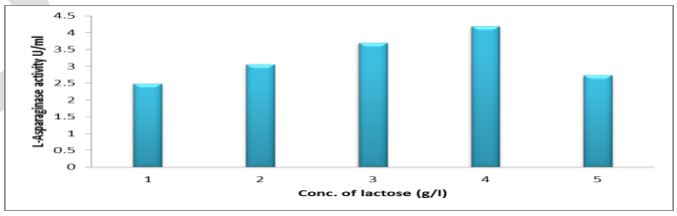

Fig. 2 Effect of different concentration of lactose.

3.4. Effect of different N-Sources on L-Asparginase production

Among the different N-sources (1g/ml) assessed, yeast extract served as the best one followed by Peptone (fig 3) .The yeast extract showing the maximum enzyme activity was assayed by protein estimation by Bradford's method and it was found that it was found that it contains 0.528 mg/ml of protein content. The specific activity thus obtained was 6.19 U/mg.

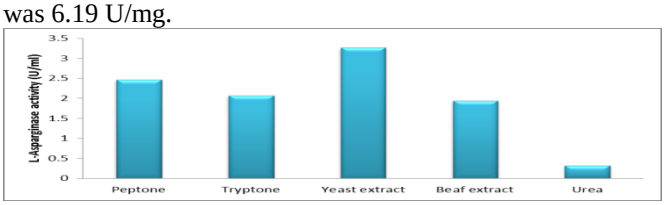

Fig. 3 Effect of various nitrogen sources.

3.5. Effect of different concentration of yeast extract on L-Asparginase production

Among the different concentration of Yeast extract (1-5 g/ml) assessed, 2g/l of yeast extract served as the best one followed by 3g/l of concentration (fig. 4) .The 2g/l of yeast extract concentration showing the maximum enzyme activity was assayed by protein estimation by Bradford's method and it was found that it was found that it contains 0.715 mg/ml of protein content. The specific activity thus obtained was 6.55 U/mg.

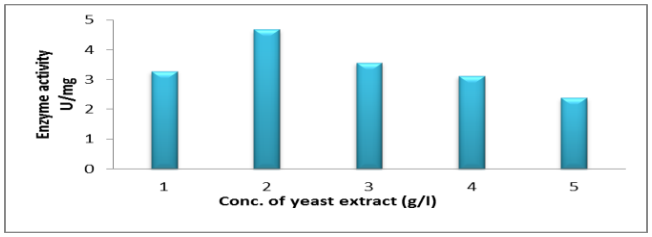

Fig. 4 Effect of various conc. of yeast extract.

3.6.Effect of different inoculum size on L-Asparginase production

The most appropriate inoculums size for the production of L-asprginase by *Erwinia carotovorum MTCC 1428* using submerged fermentation in 50ml media was found to be 3.0 ml (6% v/v) (fig 5) the media containing 4.0 ml inoculums size showing the maximum enzyme activity was assessed for protein estimation by Bradford's method that it contains 0.553 mg/ml of protein content. The specific activity thus obtained was 8.53 U/mg.

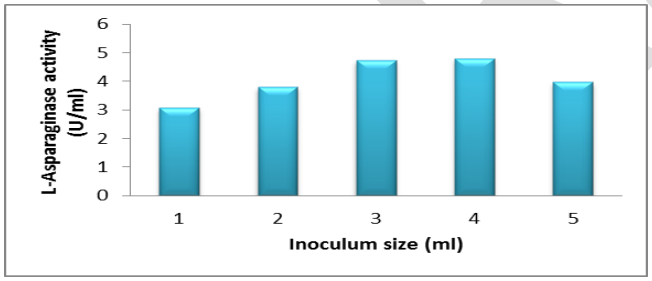

Fig.5 Effect of different inoculums size**.**

3.7.Effect of different Incubation timeon L-Asparginase production

The optimum temperature for the production of L-Asparginase was found to be 30ºC. The further increase in temperature decreases the production of L-Asparginase (fig.6). The incubation temperature of 30ºC showing the maximum enzyme activity was assessed for protein estimation by Bradford's method and it was found that it contain 0.417 mg/ml of protein content. The specific activity thus obtained was 11.23 U/ml.

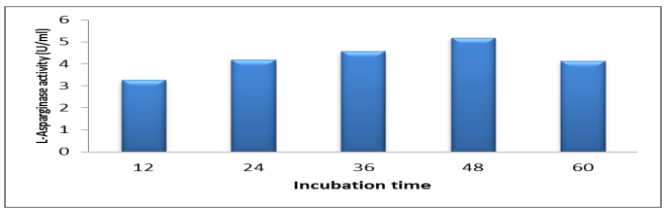

Fig.6 Effect of different incubation time.

3.8. Effect of different rotational speed (RPM) on L-Asparginase production

The bacteria strain *Erwinia carotovorum MTCC 1428* was found to be most active for the production of L-Asparginase using the submerged fermentation method at the 150 RPM followed by 200 RPM and the enzyme production was decreased as rotational speed was going to be increased. So the bacterium is found to be most active at 150 RPM (fig 7).The pH showing the maximum enzyme activity was assessed for protein estimation by Bradford's method and it was found that it contain 0.869 mg/ml .The specific activity thus obtained was 13.09 U/mg.

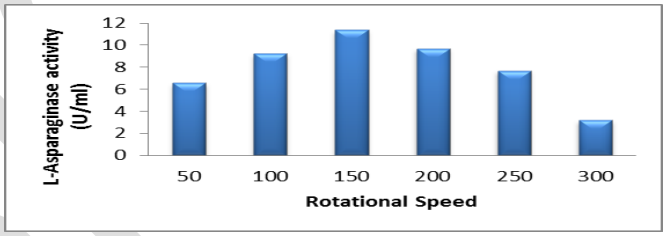

Fig.7 Effect of different shaking speed.

3.9.Effect of different temperature and pH on L-Asparginase production

The optimum temperature for the production of L-Asparginase was found to be 30ºC. The further increase in temperature decreases the production of L-Asparginase (fig. 8). The incubation temperature of 30ºC showing the maximum enzyme activity was assessed for protein estimation by Bradford's method and it was found that it contain 0.417 mg/ml of protein content. The specific activity thus obtained was 11.23 U/ml.

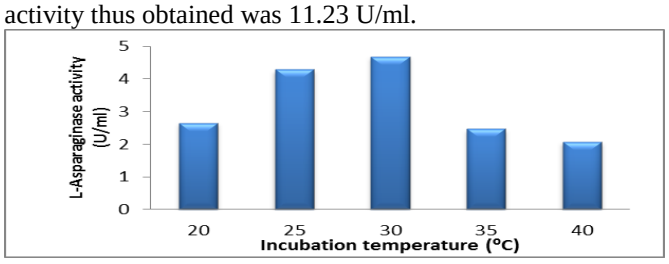

Fig.8 Effect of different incubation temperatures.

The bacteria strain *Erwinia carotovorum MTCC 1428* was found to be most active for the production of L-Asparginase using the submerged fermentation method at the initial pH 7 0f the fermentation media. So the bacterium is found to be most active at the neutral pH (fig 9).The pH showing the

maximum enzyme activity was assessed for protein estimation by Bradford's method and it was found that it contain 0.917 mg/ml .The specific activity thus obtained was 10.22 U/mg.

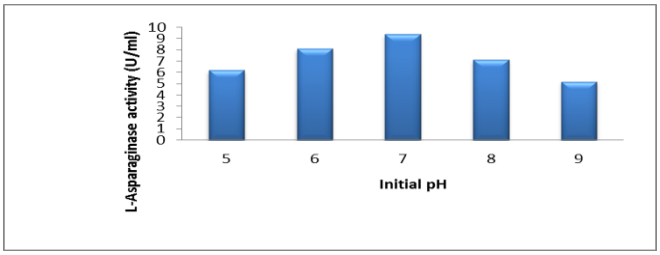

Fig.9 Effect of Initial pH.

4.0.Optimized culture conditions and enhanced production of L-asparaginase

Finally the optimized values for the various process parameters were deduced, when grown under all the optimum conditions for submerged fermentation the bacteria *Erwinia carotovorum* exhibit the increased enzyme production. It was evident from the results that medium under optimized condition showed the increase in enzyme activity and specific activity when compared with individually optimized parameter media. Hence it was concluded that the media were optimized.

The reading for the optimized culture and maximum production is

L-Asparginase activity : 13.96 U/ml
Protein concentration : 0.863 mg/ml
Specific activity : 16.18 U/mg

IV. CONCLUSION

In the optimization of bioprocess variables for L-Asparginase production by *Erwinia carotovorum* MTCC1428,the maximum enzyme activity obtained with lactose (4% w/v), yeast extract (2% w/v), inoculum size (3ml) at 30ºC, neutral pH & 150 rpm.These suggest that medium was optimized and *Erwinia carotovorum* MTCC 1428 could be promosing source for production of L-Asparginase. However further work to understand better control strategies for obtaining high yields, the metabolism of L-Asparginase production and downstream processing for purification of L-Asparginase need to be done.

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