ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

THERMOKINETIC STUDIES OF PRODUCTION OF L-ASPARAGINASE BY BACILLUS LICHENIFORMIS IN BATCH FERMENTATION.

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Manuscript Info

Manuscript History:

Received: 14 April 2016
Final Accepted: 19 May 2016
Published Online: June 2016

Key words:
L-Asparaginase, Bacillus licheniformis, Biomass, Kinetics, Specific growth rate.

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Abstract

Microbial L-Asparaginase having potential application in the chemotherapy of acute lymphoblastic leukemia. Present study was carried out to study the thermokinetics of L-Asparaginase from Bacillus licheniformis. Enzyme production was carried out at different temperature and pH for different duration of time. The kinetics of biomass formation, product formation and substrate utilization was carried out at different conditions. The maximum L-Asparaginase activity of 2.9 U/ml was observed after the 24 hrs of post inoculation. The maximum specific growth rate was calculated and found to be 0.2209 h^{-1}. The productivity was found to be 0.574 U g $cell^{-1}$ h^{-1}.

Introduction:-

Microbial enzymes have been widely used in various industries in the world. L-Asparaginase (L-Asparaginase aminohydrolase EC 3.5.1.1) is the first enzyme having anti leukemic activity to be studied on human cells (Savitri et al 2003). The therapeutic potential of L- Asparaginase for the treatment of malignant tumors has been studied since 1922 (Tozuk et al 1997).L-Asparaginase is a tetramer protein that deaminates asparagine and glutamine to aspartic acid and glutamic acid respectively by releasing ammonia. This reaction is irreversible in nature under physiological condition (Prakash et al 2006).It has been observed that the release of glutamic acid is less as compared to the aspartic acid.

L-Asparaginase has widely been used as a therapeutic cancer for the treatment of patients suffering from ALL (Acute Lymphoblastic leukemia)and other types of cancer too(Verma et al 2007). This enzyme is a promising agent in treating of some forms of neoplastic cell diseases in children (Jaffe et al 1971 and Oettgen et al 1967). Asparaginase is widely distributed in nature and it plays a crucial role in amino acid metabolism and utilization. It is a nutritional requirement of both normal and cancer cells. Cancer cells differentiate themselves from normal cells in diminished expression of L-Asparagine (Mannan et al 1995). In case of acute lymphoblastic leukemia cells can be differentiated on the basis of the requirement of supplement of L-Asparagine. Normal cells produce L-Asparagine with the help of an enzyme asparagine synthetase. Tumor cells are not capable of synthesizing L-Asparagine by their own and hence they depend on the circulating plasma pools to fulfill their nutritional requirements. (Swain et al 1993).This forms the basis for the use of L-Asparagine as a drug which deaminates the asparagine present in the circulating plasma, thus depriving the cancerous cells of this amino acid required for protein synthesis.

L-Asparaginase is known to be produced from a variety of microorganisms. The present study emphasizes on the producing capability of L-Asparaginase by Bacillus licheniformis. Microbial strain of Bacillus licheniformis was subjected to quantitative screening for the production of L-Asparaginase. Quantitative screening was performed in shake flask by using M9 medium. Maximum enzymatic activity was calculated. The substrate utilization study was carried out to predict the kinetics of enzyme production. The production of this industrial enzyme is strongly

influenced by thermal studies. The stability of the enzyme is dependent on various physiological conditions. In this paper, we have performed the thermokinetic studies of L-Asparaginase production.

Materials and Methods:-

Microorganism culture and maintenance:-

Bacillus licheniformis (MTCC 1483) was used as the producer of L-Asparaginase. M9 agar media was used to grow the microorganism in presence of 0.1% of Asparagine as inducer at 37˚C . The microorganism were sub cultured after every 30days. The stock cultures of organism were kept at 4˚C. The bacterial strains of Bacillus licheniformis (MTCC 1483) continue with kinetic studies with the proposed media containing (Na_2HPO_4-6gm, KH_2PO_4-3gm, NaCl-0.5gm, L-asparagine-5gm,1mol/l $MgSO_4.7H_20$-2ml,0.1ml/l $CaCl_2.2H_2O$-1ml, 20%Glucose stock-10ml). All media were sterilized at 121˚C and 1 atmosphere for 15 minutes using an autoclave.

Preparation of seed culture and shake flask production:-

The agar slants were prepared on LA medium as the seed banks. A loop full of culture was transferred to proposed seed medium in a 100ml Erlenmeyer flask and incubated overnight in an orbital shaker at 170 rpm at 37˚C. 5% of seed medium (v/v) was transferred aseptically to production medium and incubated at same conditions. The pH of the medium was set at 7. Quantitative screening was carried out to estimate the L-Asparaginase production potential in shake flask. 100ml of M9 broth (Na_2HPO_4-6gm, KH_2PO_4-3gm, NaCl-0.5gm,L-asparagine-5gm,1mol/l $MgSO_4.7H_20$-2ml,0.1ml/l $CaCl_2.2H_2O$-1ml, 20% Glucose stock-10ml) was prepared in a shake flask and autoclaved. A culture of Bacillus licheniformis was taken and inoculated in the media. After inoculation in sterile conditions the flasks were plugged and moved into shaker rotating at 170 rpm and 37ºC for overnight. The culture broth was removed from shaker at different time intervals and centrifuged at 10000 rpm, 4ºC for 10 minutes. The extracellular enzymes were obtained in the supernatant. The supernatant was preserved and checked for enzymatic activity.

The culture was maintained at 37˚C at 150 rpm for 24 hours and sample was taken at interval of every 2 hours and it was checked for biomass production, substrate utilization by DNS test and enzymatic activity or product formation by Nesselerization method.

Determination of cell biomass:-

Bacterial growth was estimated by measuring the optical densities at 600nm with the help of spectrophotometer between the absorbance of 0.2 and 0.9 with the Beer's law being followed. Whenever required the samples were diluted with double distilled water for the attainment of desired range of absorbance. For attainment of cell dry weight (CDW), the sample cultures were centrifuged at 8000 rpm for 10 minutes and the supernatant obtained was used for residual glucose and product analysis. The retained biomass was washed twice with double distilled water and there after dried in an oven at 110˚C overnight. The differential weight of the membrane gives the dry weight of the cells. A standard graph was plotted for cell dry weight versus absorbance for further estimation of cell dry weight.

Assay of L-Asparaginase:-

Assay of enzyme was done by the Nesselarisation, in which 0.2 ml of enzyme was mixed with 0.2 ml of 0.05 M Tris-hydroxy methylaminomethane buffer and 1.7 ml of 0.01M L-asparagine solution were combined and incubated for 15 minutes at 37^{o}C. The reaction was stopped by adding 0.1 ml of 15% (w/v) tri-chloroacetic acid 0.5ml portion of the supernatant is diluted with 7 ml of distilled water and treated with 1 ml of Nessler's reagent. The reaction solution was allowed to settle down for 10 minutes. The color sample was observed under spectrophotometer at 480 nm (Imada et al 1973). The rate of hydrolysis of asparagine is determined by measuring the released ammonia. One unit of L-Asparaginase releases one micromole of ammonia per minute at 37˚C and pH 8.6.The standardization of the method was performed by pure L-asparagine purchased from SIGMA ALDRICH.

Determination of Glucose concentration:-

The glucose concentration of the samples was carried out by using DNS test for residual sugar estimation. The sample was centrifuged at 10,000rpm at 4C for 10 minutes to remove the insoluble solids before analyzing by DNS test. The samples were dispensed in test tubes, the volume was made up to 1ml with distilled water, followed by addition of 2ml of DNS reagent and the tubes were heated in a boiling water bath for 10 min followed by rapid cooling. The dark orange to brownish color formed was observed at 540nm by using UV-Vis spectrophotometer against blank. The standard plot was prepared by plotting OD 540 of standard against sugar concentration. The

amount of sugar present in the sample was calculated from standard plots. The samples with higher concentration were diluted to this range before the measurement.

Effect of Temperature on the production of L-Asparaginase:-
The experiment was designed to examine in fine detail the responses of temperature on cell biomass and product formations were investigated. Two types of studies were performed. The effect of temperature and pH were studied both during the production process and during performing the enzymatic activity. To check the thermo stability of the enzyme, M9 media was prepared and poured in three flasks containing 50 ml of the broth. A loop full of culture was inoculated in the media and then kept for incubation at three different temperatures 30˚C, 37˚C, 45˚ C at 170 rpm. Assay was performed at different time intervals and was checked for highest activity (Mannan et al. 1995) .

Effect of Temperature on the activity of L-Asparaginase:-
The effect of temperature on the activity of the enzyme was studied by incubating the enzyme at different temperature like 10˚C, room temperature (20˚C), 30˚C, 40˚C,50˚C, 60˚Cand 70˚C. Enzymatic assay was performed with this varying temperature sample (Siddalingeshwara et al 2011).

Effect of pH on the production of L-Asparaginase:-
M9 media was prepared and taken in 5 flask containing 50ml of the broth and the pH was adjusted to 5, 6, 7, 8, 9 respectively. All the flasks were inoculated and incubated at constant temperature 37˚C at 170 rpm. Samples were collected on the next day at several time intervals to check highest activity.

Effect of pH on the activity of L-Asparaginase:-
Enzymatic assay was also performed with pH value of 6, 7, 8, 8.6, 9 in Tris- HCl buffer to check the effect of pH on enzymatic activity.

Results and discussions:-

Shake Flask Production
Batch fermentation in the shake flasks for L-Asparaginase production was carried out in duplicate for almost 28 hours at constant temperature 37˚C and 170 rpm. The experimental data obtained are fitted in some well established models and thus were validated.

Thermokinetic studies:-
Determination of cell biomass:-
There was in steady increase in biomass. The biomass increased exponentially in the beginning, entered a stationary phase almost after 18-20 hours. The maximum volumetric biomass was observed in a range between 24 -30 hours of post incubation. We can see the biomass grows at a steady state. The maximum biomass obtained is 8gm/litre (Fig-1).

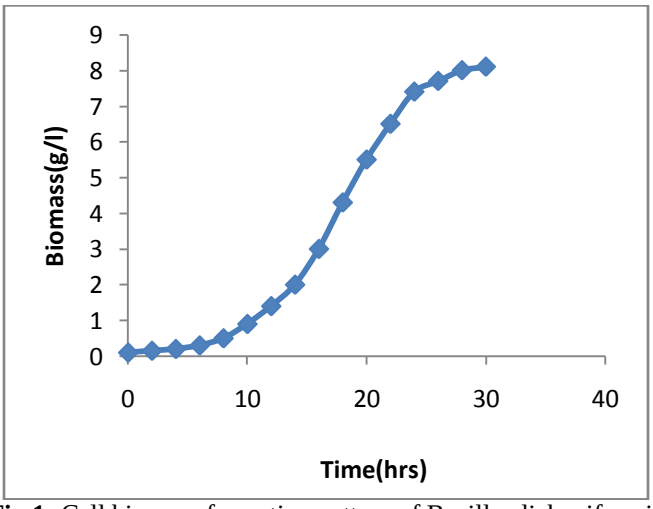

Fig 1: Cell biomass formation pattern of Bacillus licheniformis.

L-Asparaginase production with time:-

The samples were collected in eppendorf at different time intervals and centrifuged. The supernatant obtained was checked for enzymatic assays. The maximum activity of L-Asparaginase was found after 24hours of post incubation at 37˚C and after which its activity start declining slowly. L-Asparaginase is example of growth associated product and is produced simultaneously with microbial growth. So the enzymatic activity was increased continuously with increase in biomass and L-Asparaginase production decline after 24 hours. One unit of ammonia released can be defined in terms of mmol of ammonia released per ml of enzyme. The maximum enzymatic activity was found to be 2.9U/ml (Fig-2).

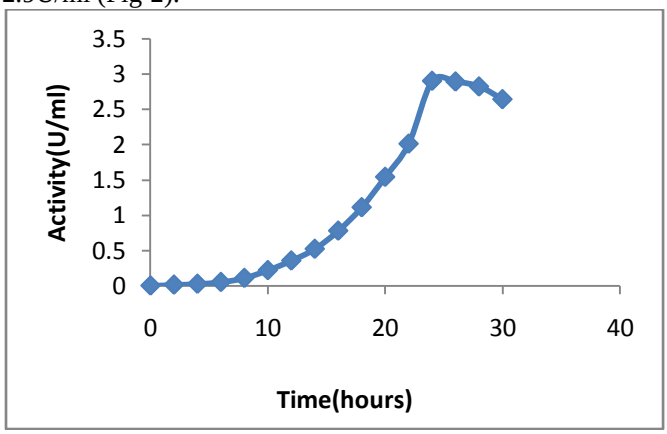

Fig 2: Production of L-Asparaginase with time.

Substrate utilization with time:-

The substrate utilization was checked for residual sugar estimation and it was done by DNS test. The glucose is consumed mainly for cell biomass growth along with the maintenance of cell biomass. The above experimental results revealed the glucose decreased exponentially at the beginning with increased cell biomass exponentially with time. Almost 77% of the substrate was utilized after 24 hours (Fig-3).

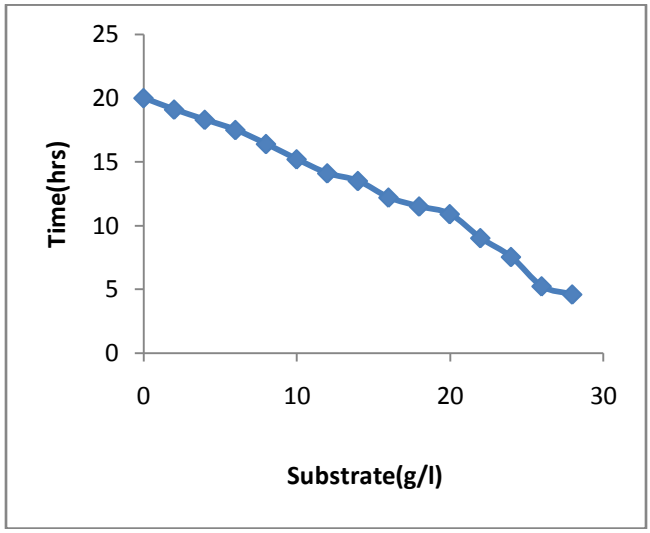

Fig 3: Substrate utilization pattern of Bacillus licheniformis.

Effect of Temperature on L-Asparaginase production:-

The production of L-Asparaginase was studied with variation of temperature. The culture samples were incubated at 30˚C and 45˚C. At 30˚C, the maximum enzymatic activity was achieved after 22 hours of post incubation. But at 45˚C, there was negligible enzymatic activity. The maximum enzymatic activity obtained at 30˚C was 2.70U/ml (Fig-4(a) and (b)).

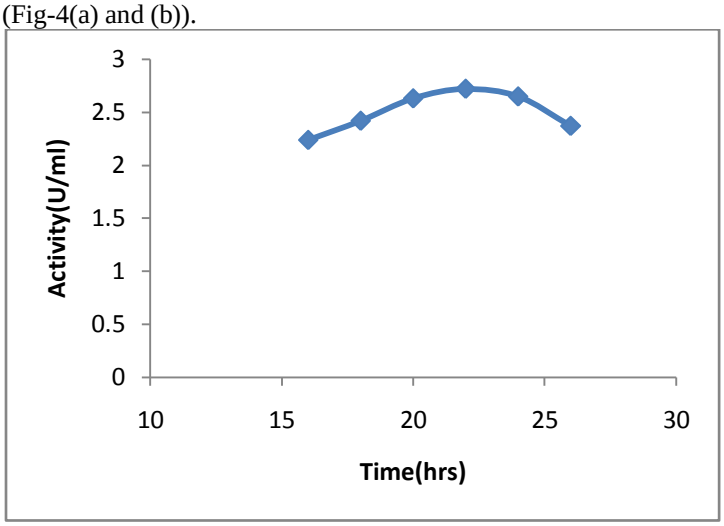

Fig 4(a): Production of L-Asparaginase at 30˚C.

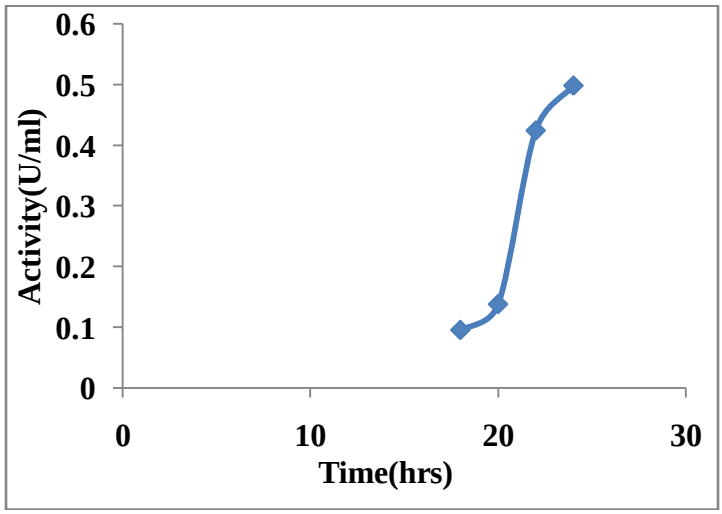

Fig 4(b): Production of L-Asparaginase at 45˚C

Effect of pH on L-Asparaginase production:-

The enzymatic activity was checked with varying pH at three different time intervals. We can see the enzyme activity follows almost similar pattern in all the pH. The maximum activity was found at pH 7 after 24 hours of post incubation. The activity started declining slowly with increase in pH. The activity was found to be 2.88U/ml (Fig-5).

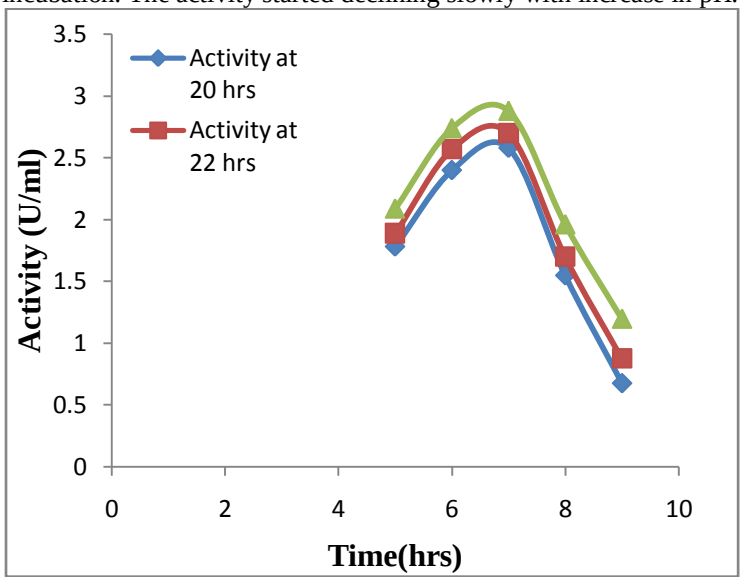

Fig 5: Production of L-Asparaginase with varying pH.

Effect of Variation of temperature on enzymatic activity:-

The enzymes were incubated at different temperatures for 30 minutes. The result on the effect of temperature on enzymatic activity denotes that the highest enzymatic activity at 40 ˚C. The enzymatic activity was almost constant over a range of 40˚C -70˚C and there was no such loss of enzymatic activity even at 70˚C (Fig-6).

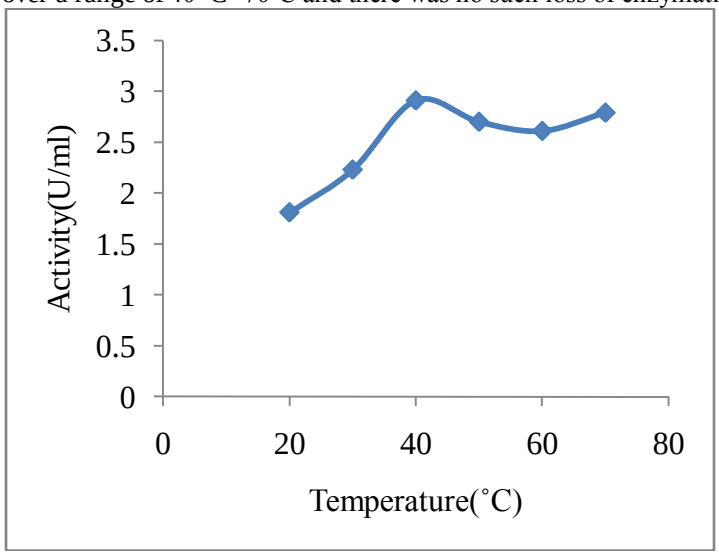

Fig 6: Variation of enzymatic activity with respect to temperature.

Effect of variation of pH on enzymatic activity:-

The enzymatic activity was also performed with different pH of Tris-HCl buffer in the assay. It was observed that the maximum enzymatic activity lies in the range of 8-9 after which there is slight decline in the activity. The optimum pH was found to be 8.6 of Tris-HCl buffer (Fig-7).

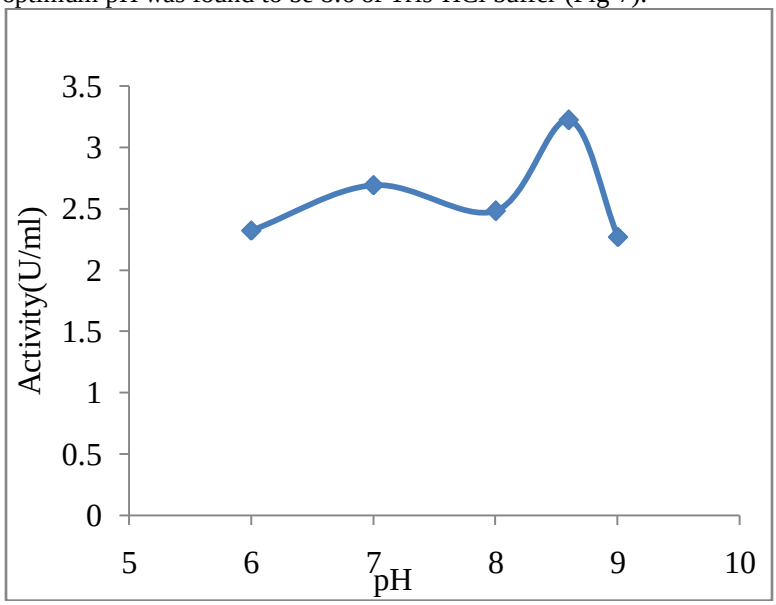

Fig 7: Variation of enzymatic activity with pH.

Conclusion:-

The maximum enzymatic activity was found to be 2.9U/ml. The optimum temperature was found to be 37˚C and 170 rpm. The enzymatic activity was found after 24 hours. The production was L-Asparaginase was very low at 45˚ C. The substrate was utilized up to 77% till 28 hours. The maximum volumetric biomass was found to be 8 grams/litre. The optimum pH for the production of L-Asparaginase was found to be 7. The enzymatic activity remains almost stable till 70˚C. The optimum pH of Tris buffer for Nesselarisation was found to be 8.6. The maximum specific growth rate was calculated and found to be 0.2209 h^{-1} . The productivity was found to be 0.574 U g $cell^{-1}$ $h^{-1.}$

Acknowledgement:-

The authors highly acknowledge the support from AICTE-GATE for fellowship. The authors also gratefully acknowledge the Department of Bioengineering, BIT Mesra for providing all the chemicals and instrumental facilities. I am thankful to my friends and family members for their constant support.

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