Journal of Bioprocessing & Biotechniques

Rajesh, et al. J Bioproces Biotechniq 2011, 1:4
DOI: 10.4172/2155-9821.1000110

Research Article Open Access

Effect of Inducers and Physical Parameters on the Production Of L-Asparaginase Using *Aspergillus Terreus*

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Abstract

An effort has been made to find the best medium and operating conditions for the production of extracellular L-asparaginase by *Aspergillus terreus* in shake culture fermentation through stepwise optimization strategies like studies on the effect of pH and temperature on L-asparaginase, studies on the enzyme activity in various concentration of L-glutamine and study the growth kinetics for L-glutamine medium. Our study shows that L-glutamine combined with L-asparagine influence the asparaginase production level and physical parameter such as pH and temperature has more contribution in enzyme production. The pH 8 and temperature 35°C found to be good environmental condition the production of extracellular L-asparaginase by *Aspergillus terreus*.

Keywords: L-asparaginase; *Aspergillus terreus*; Shake culture fermentation; L-glutamine

Introduction

L-asparaginase (L-asparagine amidohydrolase; EC.3.5.1.1) catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia. L-asparaginase is present in many animal tissues, bacteria, plants and in the serum of certain rodents, but not in mankind. L-asparaginase is produced by a large number of micro-organism include *E. coli* [1], *Erwinia cartovora, Enterobacter aerogenes* [2], *Cornybacterium glutamicum, Candida utilis* [3], *Staphylococcus aureus* [4], *Thermus thermophilus* [5], *Pisum sativum.* Although Clementi in 1922 had reported its presence in guinea-pig serum, the anti-tumour properties of the enzyme were only recognized some time later. Tsuji first reported Deamidation of L-asparagine by extracts of *E. coli* in 1957. Broome in 1961 discovered that the regression of lymph sarcoma transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L- asparagines.

Several micro-organisms including *Serratia marcescens* produce L-asparaginases with antitumor activity. Although extensive studies have been carried out on the isolation and on the anti leukemia properties of this enzyme [6,7], very little information is available on the production of this enzyme by *S.marcescens* [7, 8]. A large amount of research has been conducted upon the biosynthesis of L-asparaginase since Masburn and Wriston demonstrated antitumor activity. Kinetic studies would allow the prediction of fermentation rate, product yield, and the control of the fermentation process. A continuous fermentation has advantages of productivity, ease of control, uniformity and low labor costs. Several species of *Erwinia* have been reported to provide promising sources of L-asparaginase and have proved to be effective against solid tumors [9]. Tumor cells, more specifically lymphatic tumor cells, require huge amounts of asparagines to keep up with their rapid, malignant growth. This means they use both asparagine from the diet as well as what they can make themselves to satisfy their large asparagines demand. L-asparaginase is an enzyme that destroys asparagine external to the lymphatic tumor cells. Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. L-asparaginase is used as a chemotherapeutic agent for acute l*ymphocytic leukemia* and less frequently for acute *myeloblastic leukemia, chronic lymphocytic leukemia*, Hodgkin's disease, *melonosarcoma* and non-Hodgkin's lymphoma. L-asparaginase produced by bacteria leads to adverse side effects in human trials. Therefore there is a search for the other sources of L-asparaginase production. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera such as *Aspergillus, Penicillium* and *Fusarium* are commonly reported in scientific literature to produce asparaginase with less adverse effects. Asparaginase is an anti-neoplastic agent, used in the lymphoblastic leukemia chemotherapy. Neoplastic cells cannot synthetase L-asparagine due to absence of L-asparagine synthetase. For this reason the commonest therapeutic practice is to inject intravenously free enzyme in order to decrease the blood concentration of L-asparagine affecting selectively the neoplastic cells. The importance of micro-organism as L-asparaginase sources has been focused since the time it was obtained from *E. coli* and its antineoplastic activity demonstrated in guinea pig serum [9-11]. The *E.coli* and Erwinia enzymes were isolated, purified, and experimentally used as an anti-leukaemic agent in human patients. It demonstrated high potential against children's acute lymphoblastic leukemia. Several research groups have studied asparaginase production and purification in attempt to minimize impurities that produce allergic reactions. It has been observed that eukaryotic micro-organisms like yeast and filamentous fungi have a potential for asparaginase production. For example, the mitosporic fungi genera such as *Aspergillus, Penicillium,* and *Fusarium* are commonly reported in scientific literature to produce asparaginase. Objective of this research work is to find the best medium and operating conditions for the production of extracellular L-asparaginase by *Aspergillus terreus* in shake culture fermentation through stepwise optimization strategies like studies on the

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Received July 03, 2011; **Accepted** November 18, 2011; **Published** November 21, 2011

Citation: Rajesh MJ, Rajesh L, Veni VS, Thirumurugan G, Sethuraman OS, et al. (2011) Effect of Inducers and Physical Parameters on the Production Of L-Asparaginase Using *Aspergillus Terreus*. J Bioprocess Biotechniq 1:110 doi: 10.4172/2155-9821.1000110

effect of pH and temperature on L-asparaginase, studies on the enzyme activity in various concentration of L-glutamine and study the growth kinetics for L-glutamine medium.

Materials and Methods

Microorganism

The filamentous fungus selected to use throughout this study is *Aspergillus terreus* MTCC 1782 was obtained from Institute of Microbial Technology, Chandigarh, India.

Culture conditions

Stock culture: The spores and mycelial fragments of strain were cultivated in Czapek-Dox medium which containing the following ingredients (g/l of distilled water). Solution A: Sodium Nitrate 40.0; Potassium Chloride 10.0; $MgSO_4.7H_2O$, 0.52; $FeSO_4.7H_2O$, 0.2; dissolved in 1L of distilled water and stored in refrigerator. Solution B: K2HPO4 20.0; dissolved in 1L of distilled water and stored in refrigerator. Solution C: 1 gram of $ZnSO_4$. 7 H_2O dissolved in 100ml of distilled water. Solution D: 0.5 gram of CuSO4. H_2O dissolved in 100ml of distilled water. For 1L of Czapek-Dox medium; 50 ml of solution A, 50 ml of solution B, 1 ml of both solution C & solution D, 900ml distilled water, 30 gram of Sucrose and 20 gram of Agar. *Aspergillus terreus* 1782 strain was cultivated in Czapek agar slants at 37°C for 4 days.

Production and isolation of crude enzyme:Culture suspensions were prepared from the stock culture, using 10 ml of sterile water. 10 ml of this suspension was transferred to conical flask with 100 ml of liquid Czapek-Dox medium prepared based on statistical designs and kept in orbital shaker at 160 rpm. Then culture was filtered on Whatman 2 filter paper and the cell-free filtrate was used as crude enzyme solution.

Variation in medium pH

The pH of the Czapek Dox production media was varied with phosphate buffer having various pH. The experimental pH was selected as 6, 7, 8 & 9. Among the four pH levels, the optimum pH for the maximum enzyme activity was taken into further studies.

Variation in medium Temperature

The production medium was incubated at different temperature levels, to study the effect on production of enzyme. The selected experimental temperatures in the medium were 25°C, 30°C, 35°C and 40°C. Among the four temperature levels, the optimum temperature for the maximum enzyme activity was taken into the further studies.

Variable substrate concentration in media preparation

The spores and mycelial fragments of strain were cultivated in modified Czapek-Dox medium which containing the following ingredients (g/l of distilled water). Solution A: Sodium Nitrate 40.0; Potassium Chloride 10.0; $MgSO_4.7H_2O$, 0.52; $FeSO_4.7H_2O$, 0.2; dissolved in 1L of distilled water and stored in refrigerator. Solution B: K_2HPO_4 20.0; dissolved in 1L of distilled water and stored in refrigerator. Solution C: 1 gram of ZnSO4. 7 H_2O dissolved in 100ml of distilled water. Solution D: 0.5 gram of $CuSO_4$. H_2O dissolved in 100ml of distilled water. For 1L of Czapek-Dox medium; 50 ml of solution A, 50 ml of solution B, 1 ml of both solution C & solution D, 900ml distilled water, different L-glutamine concentration and 0.5 gram of L-asparagine. *Aspergillus terreus* 1782 strain was cultivated in modified medium (at 160 rpm)

The production medium composition modified by the addition of L-glutamine with an inducer L-asparagine; the L-glutamine concentration was varied from 0.5 % to 2.5 % but L-asparagine concentration was 0.5% for all medium. Here the production medium was maintained under optimum pH and temperature condition which were obtained from earlier studies. Enzyme activity and biomass in dry weight were determined at equal time interval upto 72 hours. L-Glu concentration 0.5%, 1.0%, 1.5% and 2.0% represented as medium M1, M2, M3 and M4 respectively. Initially the culture was inoculated at uniform concentration in each production medium.

Standard ammonia preparation

1M Ammonia stock solution was prepared by dissolving 114 g of anhydrous ammonium sulfate in 1 litre deionized water. From that working standard was prepared. Working standard was prepared by serial dilution. From 1µM 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 deionized water. 1ml of Tris-Hcl buffer was added to each test tube and incubates it for 30 minutes at 37°C. 0.5 ml 15% TCA was added followed by 1 ml NaOH. Immediately the solution was mixed and allowed to react for 20 minutes. The final solutions were red at 480nm in spectrophotometer. Blank was prepared by without anhydrous ammonium sulfate.

Assay of L-asparaginase activity

Enzyme activity of the culture filtrates was determined in at the end of cultivation time by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent. A 0.1ml sample of culture filtrate (enzyme solution), 0.9 ml of 0.1 M Tris-hyroxy methyl aminomethane buffer (pH 8.5), and 1 ml of 0.04 M L-asparagine solution were combined and incubated for 10 min at 37°C. The reaction is stopped by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid. After centrifugation, a 1 ml portion of the supernatant fluid is diluted to 3 ml with distilled water and treated with 1.0 ml of Nessler's reagent and 1.0 ml of 2.0 M NaOH. The color reaction was allowed to proceed for 20 min before the OD at 480nm is determined. The OD was then compared to a standard curve prepared from solutions of ammonium sulfate as the source. Blank was prepared by without asparaginase enzyme sample (Produced in production medium).

One unit (IU) of L-asparaginase is that amount of enzyme which librates 1 µmole of ammonia in 1 min at 37°C.

$HCOOCCHNH_2CH_2COONH_2 + H_2O \rightarrow HOOCCHNH_2CH_2COOH + NH_3$

Determination of µmoles of ammonia liberated using the standard curve.

Units/ml enzyme= (µmole of NH_3 liberated) (1) (2.5) / (0.1) (20)

0.1 = Volume of filtrate
1.0 = Volume of supernatant
2.5 = Total volume of reaction mixture
20 = Time of assay in minutes

Biomass estimation

5 ml of sample was collected from the production medium, it was filtered with the Whattman 2 filter paper and dried in hot air oven at 35oC or Centrifuge the sample at 5000rpm for 5 minutes and dried in hot air-oven at 35oC. A graph is plotted of cell biomass (Dry weight g/l) against time intervals of 12, 24, 36, 48, 60 and 72 hrs for different media. Specific growth rate is found using µ= (In xt -In xo)/t and a maximum growth rate chart is drawn.

Results

Standard graph for ammonia estimation

The standard line showed the R2 = 0.9997 (Y = 0.3883 X) Figure 1.

Effect of pH on enzyme production

In the present study, a high enzyme activity was obtained at pH 8, while 48 hr productions. Enzyme production was less in other pH ranges at all the deviations. The maximum production of asparaginase was identified in 48 hr in all the pH levels (0.10, 0.15, and 0.175 IU/ml in pH 6, 7 and 9 respectively).The enzyme production was nil in all pH levels (6, 7 and 9) except pH 8 in 24 hr Figure 2.

Effect of temperature on asparaginase activity

The enzyme activity on the temperature at different time intervals showed in Figure 3. The maximum asparaginase activity (0.25, 0.3, 0.203 and 0.163 IU/ml at 24, 48, 72 and 96 hr, respectively) was found at 35oC throughout culture duration. The minimum enzyme production (0.075 IU/ml) was recorded in 25oC at 72 hr duration. Similarly it was maximum (0.3 IU/ml) in 35oC at 48 hr. During the study it was observed that, the enzyme production was maximum at 48 hr and recorded thereafter in all the temperatures.

Asparaginase activity at different concentration of substrate

The asparaginase activity in different concentrations (M1, M2, M3 and M4) was given in Figure 4 at optimum pH and temperature. The enzyme activity was maximum in the M4 (3.40, 4.55, 4.30 and 3.75 IU/ml in 24, 48, 72 and 96 hr respectively) when compared with other medium. When compared with culture duration, it was recorded maximum at 48th hr (0.95, 2.05, 3.15 and 4.55 IU/ml in M1, M2, M3 and M4 respectively).

Biomass concentration in different substrate concentration

The biomass concentration (Dry weight – g/l) was increased gradually with the culture duration from 12 to 72 hr. At the 36th hr of incubation, M2 showed the maximum (4.90 g/l) biomass production, whereas in the 96th hr, it was maximum (14.6 g/l) in medium M4, when compared with other medium Figure 5.

For each medium the specific growth rate were calculated from Figure6a, 6b, 6c, 6d. The glutamine substrate concentration 1/S (g/l)-1 and 1/Y (hr-1) was plotted and Ym (Maximum specific growth rate) and KS (Substrate constant) were calculated as 0.029 hr-1 and 2.43 (g/l) respectively Figure 7.

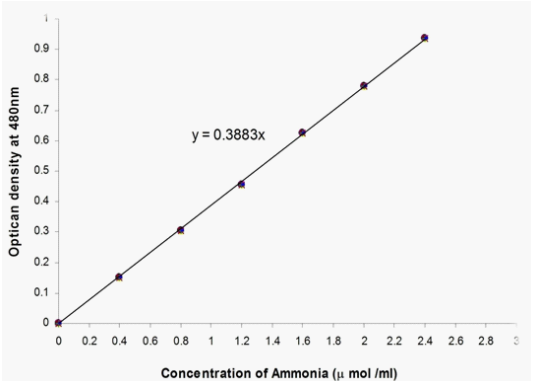

Figure 1: Standard Graph for Ammonia for the Estimation of Asparaginase Activity.

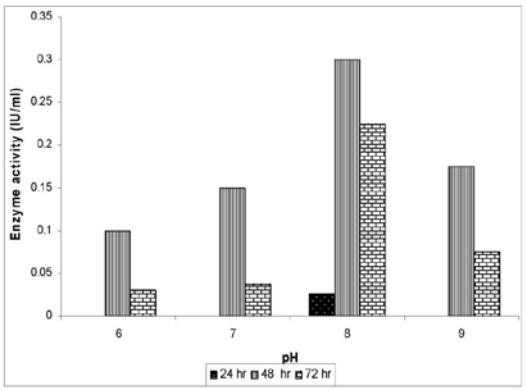

Figure 2: Enzyme asparaginase activity (IU/ml) on various pH levels (6,7,8 and 9) at different time intervals (24, 28 and 72 hrs).

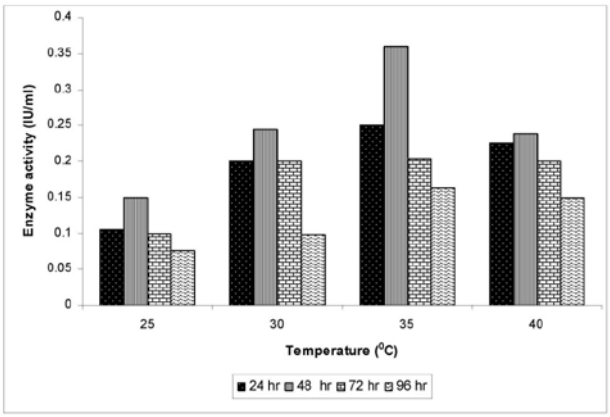

Figure 3: Asparaginase activity (IU/ml) on various temperatures (25, 30, 35 and 40oC) at different time intervals (24, 48, 72 and 96 hrs).

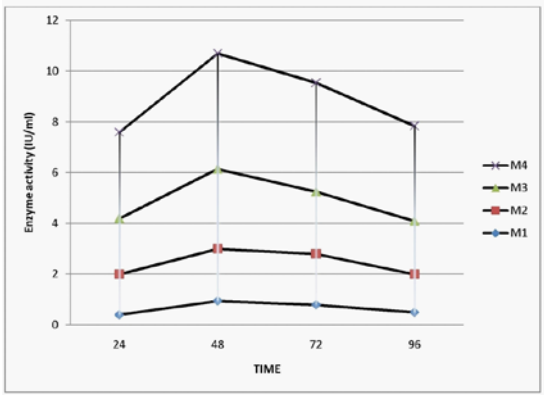

Figure 4: Asparaginase activity (IU/ml) in substrates M1, M2, M3, M4 at different time intervals (24, 48, 72 and 96 hrs).

Discussion

Environmental factors are significant for biosynthesis of asparaginase with *Serratia marcescens* ATCC 60.Aeration of the fermentation medium is necessary for the growth of *Serratia marcescens*, but not for

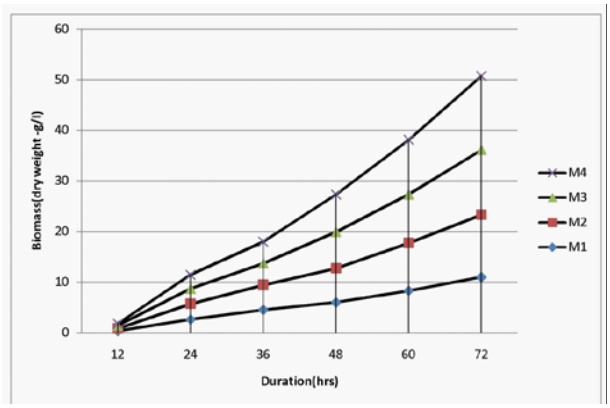

Figure 5: Biomass concentration (Dry weight g/l) in the M1, M2, M3, M4 medium at different time intervals (12, 24, 36, 48, 60 and 72 hrs).

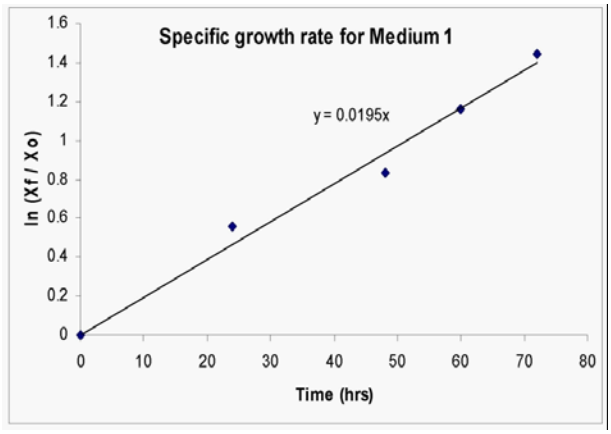

Figure 6a: Biomass specific growth rate for medium M1.

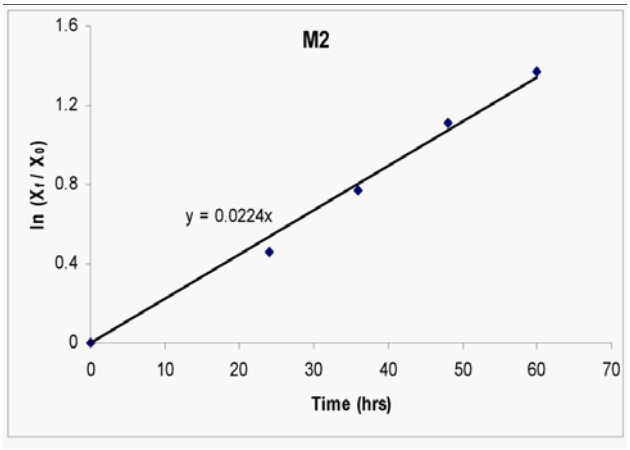

Figure 6b: Biomass specific growth rate for medium M2.

the production of asparaginase. Enhanced biosynthesis of asparaginase by *E. coli* K-12 with anaerobic growth condition has been reported by Cedar and Schwartz [12].As with number of enzymes, formation of asparaginase is inhibited by the addition of sugars, particularly glucose. In the case of asparaginase biosynthesis the depressive effect of carbohydrates may be a function of their ability to lower the pH value of the fermentation beer Heinemann and Howard [6]. The quality of L-asparaginase produced by *Streptomyces griseus* ATCC 10137 in most media did not differ significantly; yields ranged from 0.6 to 1.2 IU of L-asparaginase in cells from 100ml of broth. The glucose asparagines (GA) medium supported only poor growth and poor L-asparaginase production. Heinemann and Howard [6] reported that addition of glucose to the medium of *Serratia marcescens* resulted in substantial reduction in the quantity of L-asparaginase produced.

In *E. coli*, higher aeration rate affect the L-asparaginase production. Maximum activity occurred between pH 7 and 8 [12]; enzyme activity was essentially absent below pH 4.5, reported by Peterson and Ciegler [13]. Maximum L-asparaginase activity of 1.17 IU/100ml of broth found at pH 8.5 [14] and pH 9, *Pseudomonas aeruginosa* 10145 [11], and many other microbial asparaginase activities. Similarly, in the present study the maximum enzyme activity (0.3 IU/ml) was found at pH 8. The enzyme had maximum activity at 37oC, and more than 52% activity was attained at 50oC [15]. Similar results were recorded for asparaginases from *Pseudomonas stutzeri* MI-405, *Erwinia carotovora*, and *Staphylococcus* [16]. However in the present study, the optimum temperature for the L-asparaginase activity was recorded at 350C for *A. terreus*. Biomass production of *Aspergillus tamarii* with different nitrogen sources shows highest amount at urea 0.2% and L-glutamine 1% as nitrogen source. The biomass production of *Aspergillus terreus* with different nitrogen sources presented different growth curves when compared with A. tamarii. The biomass production of *A. terreus* pre-

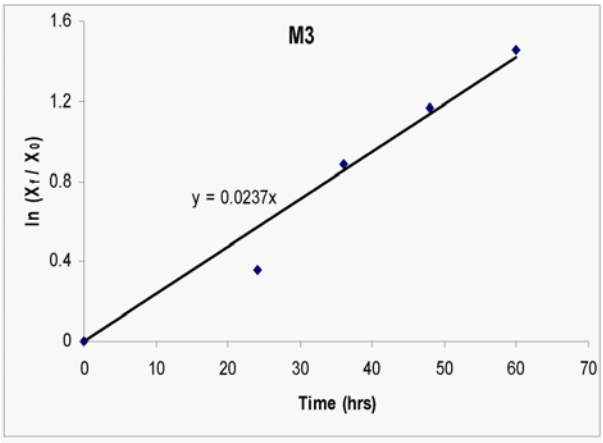

Figure 6c: Biomass specific growth rate for medium M3.

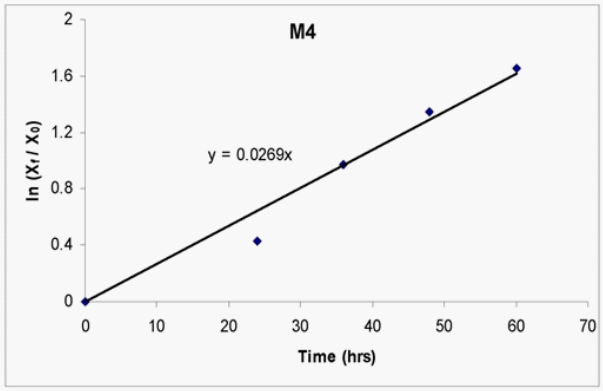

Figure 6d: Biomass specific growth rate for medium M4.

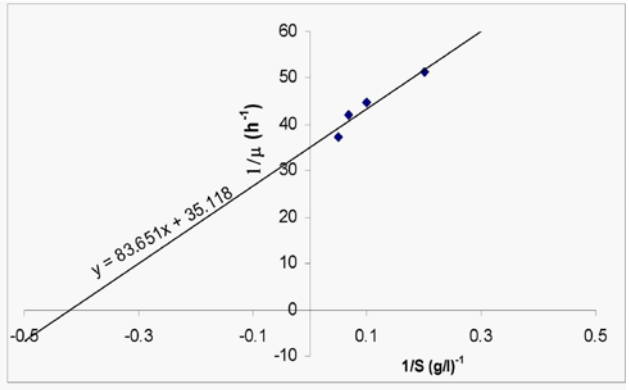

Figure 7: Maximum Specific growth chart.

sented an average of 1.5 times greater than A. tamari and L-glutamine 0.1% medium revealed itself as a good nitrogen source for the *A. terreus*. The protease activity could be responsible for decrease in L-asparaginase activity since protease levels increase when L-asparaginase levels decrease. L-asparaginase enzyme activity was 20 IU/l found at glutamine 1% reported by Maria et al. In the *A. terreus*, the biomass production was maximum in glutamine 2% medium with L asparagine 0.5% was identified in the present study. The L-asparaginase behaves like an intraperiplasmic enzyme, since whole cells possessed equivalent activity as broken cells, the enzyme not being produced extracellularly. The enzyme has been subsequently purified to near-homogeneity and characterized .

Although L-asparagine has been known to be an excellent nitrogen source for the growth of eukaryotic micro algae, the report of an algal L-asparaginase appeared only fairly recently (Paul and Cooksey, 1979). The organism, a marine *Chlamydomonas* species, could use L-asparagine, but no other amino acid for autotrophic growth (Paul and Cooksey, 1979). Nitrogen deprivation stimulated enzyme synthesis and the enzyme was repressed in the presence of combined nitrogen [17]. These are characteristics common to extra cellular [17] enzymes or enzymes found in periplasmic space were condition ionic strength, pH and substrate concentration are poorly controlled. The requirement of ammonium for good enzyme production is interesting because these ions are a product of the asparaginase reaction. It is also apparent that maximum enzyme production occurs in a medium containing ammonium ions and fumarate: asparaginase has no obvious metabolic role in such an environment. The purified enzyme has a specific activity of 200 IU/mg of protein [18]. Therefore, in crude cell extract with specific activity of 10 IU/mg of protein, asparaginase represents 5% of the total protein [19]. It is possible that asparaginase catalysis at 4oC is responsible for the loss of viability. However, the cells grown in the Wolin medium also contains significant amounts of asparaginase. The data obtained in this investigation may be compared with the published data for *E. coli* A-1. Barnas et al. showed that this strain, a good asparaginase producer when, compared with literature reports for other organism, yields a maximum of 27 IU/ml of culture. *Vibrio succinogenes*, in the best medium developed in this investigation, yielded about 2.5 IU/ml of culture. This 10 fold difference must be viewed against the 30 to 40 fold energetic advantage possessed by *E. coli* growing aerobically on glucose compared to anaerobic growth of *Vibrio succinogenes* on formate- fumarate. In *Azotobacter*, the maximal enzyme activity is reached in 30 min and is maintained at the same level during further growth of the organism in the presence of ammonium sulfate. *Erwinia aroideae* is not able to grow in the presence of ammonium nitrogen. The *Azotobactor vinelandii* asparaginase is induced in all stages of growth. Asparaginase from *A. vinelandii* had an optimum pH different from asparaginases of *Bacillus coagulans*, guinea pig serum, and *Serretia marcescens*, which have been reported to have broad pH optima [20]. External asparaginase activity of less than 1 nmol/min per mg of cells would be very difficult to detect this level of activity above the normal background on ammonia using the Nessler method. The current study also proved that, the maximum L-asparaginase production (4.55 IU/ml) found to be at glutamine (2%) and L-asparagine (0.5%) at 48th hour culture. The stationary phase was prolonged up to 72hrs showed the maximum yield of biomass.

L-asparaginase synthesis was increased by addition of L-aspartic acid, L-glutamine, L-glutamic acid and L-asparagine to the level of 20, 20.7, 19.8 and 25 IU/ 10ml respectively using *Serratia marcescens* reported by Sugumaran et al. [21].The addition of L-aspartic acid or L-glutamic acid to the medium containing sodium fumarate and corn steep liquor slightly enhanced enzyme production, but these amino acids may not be considered specific inducers for enzyme formation [22]. At 5.0% Yeast Extract concentration, the higher aeration provided yields of 5.2 IU/ml in this experiment. L-asparaginase levels up to 6.6 IU/ml were observed during these studies. With greater concentration Yeast Extract, viscosity increase could not be compensated for, and subsequent enzyme levels were greatly reduced, reported by Peterson and Ciegler [14]. L-asparaginase is produced throughout the world by sub-merged fermentation (SF). This technique has many disadvantages, such as the low concentration production, and consequent handling, reduction, and disposal of large volumes of water during the downstream processing. Therefore, the SF technique is a cost intensive, highly problematic, and poorly understood unit operation.

Conclusions

L-asparaginase production has been regulated by the physical parameters and the nitrogen sources. L-glutamine combined with L-asparagine influence the asparaginase production level. Physical parameter such as pH and temperature has more contribution in enzyme production. The pH 8 and temperature 35°C found to be good environmental condition for *Aspergillus terreus*. Our studies proves that L-asparagine induce the culture to produce more asparaginase enzyme. We can increase production level of asparaginase by adding inducer, L-asparagine in industrial level.

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