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Production and optimization of L-asparaginase by *E.coli* ATCC10536 from food waste

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ABSTRACT

The current study aimed at utilization of food waste as squid pen (SP) and cooked chicken bone (CCB) for L-asparaginase (LA) production and optimization using E.coli culture. During our investigations, high levels of L-asparaginase were obtained from E.coli using squid pen as a sole carbon source as compared to CCB. Optimization of parameters resulted in 2.5folds increase in the L-asparaginase activity. Optimum L-asparaginase production 4.316IU/mL was observed when production media was supplemented with 1%, (w/v) ammonium sulphate, 2%, (w/v) substrate at pH 9, and 37°C after incubation for 5 days.This study underlines the use of food waste as an alternate substrate for the production of L-asparaginase and to improve the cost effectiveness of this enzyme.

Keywords: L-asparaginase, Squid pen, Chicken bone, Optimization, *E.coli*

INTRODUCTION

L-asparaginase is an important therapeutic enzyme used to treat acute lymphoblastic leukemia in combination with other drugs. L-asparaginase makes use of substantial need, tumour cells have got for amino acid asparagine [1]. For tumour cells, L-asparagine is an essential amino acid while normal cells are self-sufficient and can produce within the cells by an enzyme called asparagine synthetase. L-asparaginase presence deprives tumour cells of an important growth factor by converting all L-asparagine to L-aspartate and ammonia, as a result, they may fail to survive [2].

L-asparaginase is extensively distributed among the animals, plants, and microorganisms. Owing to easy culturing methods and convenient extraction and purification techniques microorganisms are proved to be a better source of L-asparaginase enzyme. L-asparaginase has been reported from both Gram positive and Gram negative bacterial species from the terrestrial and marine environment [3].. L-asparaginases from most of the Gram negative bacteria can be categorized into two main types: type I L-asparaginase that are expressed quantitatively and possess enzymatic activity on both L-glutamine and L-asparagine amino acids, while type II L-asparaginase possess high specific activity on L-asparagine and only induced in anaerobic condition [4]. Type II asparaginases produced from *Escherichia coli* (EcAII), and *Erwinia chrysanthemi*(Er A) has been used as ananti-tumour agent for the effective treatment of (ALL) for over 30 years. L-asparaginase from two bacterial sources like *E. coli and E. carotovora* is currently in clinical use for the treatment of acute lymphoblastic leukemia [5]. L-asparaginase production has been studied in various bacterial and fungal species such as *Escherichia coli, Erwinia carotovora, Pseudomonas eruginosa, Enterobacter aerogenes, Bacillus subtilis, Aspergillus tamari, Aspergillus niger* and *Aspergillus terreus*[6-11].

In recent years, utilization of biodegradable food waste has gained more importance in bioprocess industries because of low cost and high nutrient content. Transferring the waste products into valuable biomolecules like enzyme by

fermentation not only reduces the process expenses but also the risk of environmental pollution. Both squid pen and chicken bone are food wastes obtained as food waste by product. Squid pen and cooked chicken bone contains sufficient amount of crude protein, nitrogen, ash, fat, fibre, calcium, phosphorus and magnesium hence, can be successfully exploited as a prospective substrate in solid state fermentation for production of L-asparaginase enzyme [12, 13].

The current study aimed at the production and optimization of L-asparaginase from *E.coli* through solid state fermentation using squid pen and chicken bone as a substrate. This seems to be the first report on production and optimization of L-asparaginase enzyme using this substrate.

EXPERIMENTAL SECTION

2.1 Microorganisms and Culture Conditions

E.coli ATCC10536was obtained from Biotechnology Postgraduate Lab, Faculty of Industrial Sciences & Technology University Malaysia Pahang. Nutrient agar slants were used to culture *E.coli*. ATCC10536. The preserved culture was revived on fresh nutrient agar slants over a period of 2 weeks.

2.2Chemicals and Substrates

All chemicals used were of analytical grade. The nutrient broth media (NB, pH7) was obtained from LAB M,United Kingdom was used for the growth of bacterial isolates. Two growth supporting natural sources namely squid pen (*Loligo spp*.) and Chicken bone seeds were used as substrates for maximum production of L-asparaginase enzyme during this research. Squid pen (*Loligo spp*.) was collected from various local food markets of Kuantan, Malaysia while the chicken bone was collected from food cafes in Kuantan, Malaysia. Squid pen and cooked chicken bone were washed by running tap water until clean and cleaned squid pen and cooked chicken bone was left to air dry. Once completely dried the both substrate were grounded by the grinding machine (Retsch) into the powder form and stored in moisture free jars and bags in the chiller at 8°C.

2.3 Inoculum Preparation

The inoculum was prepared by adding 50ml of nutrient broth to 100ml capacity Erlenmeyer flask. Aseptically bacterial isolate was transferred from the agar slant to the Erlenmeyer flasks containing nutrient broth and incubated at 37°C for 24 hours. The inoculum size of 5 ml (5×102 cells/ml) was used throughout the experiment for subsequent inoculations unless otherwise specified.

2.4 Screening of Substrates as Carbon Source

50ml of nutrient broth media was prepared into 100 ml conical flask and 0.5 g of squid pen or cooked chicken bone was added to each flask respectively. pH was adjusted to 7 and media was autoclaved at 15lb pressure at 121°C for 20 min. After cooling both flasks were inoculated with 5 ml of inoculum and incubated at 37°C for 48 hours. The control was run only in the presence of nutrient broth media.

2.6 Extraction of Crude Enzyme

After 48hrs incubation, the crude enzyme from the fermented production media was extracted. The fermented media was transferred into 50 ml centrifuge tube and centrifuged at 4500 rpm for 6 minutes at 4 °C by using freeze centrifuge machine (Heraeus Biofuge Primor) to obtain cell-free filtrate (CFF) which later on was filtered on Whatman filter paper number 2 and used as crude enzyme.

2.6Quantitative Assay for the Estimation of L-asparaginase Activity

Imada [14] method was followed to evaluate L-asparaginase activity by measuring the amount of ammonia liberated during Nesslerization. The reaction was initiated by adding 0.5 ml supernatant into 0.5 ml 0.04 M L-asparagine and 0.5 ml 0.5 M acetate buffer, pH 5.4, and incubated at 37°C for 30 min. After the incubation period, the reaction was stopped by adding 0.5 ml of 1.5 M of trichloroacetic acid (TCA). Only 0.1 ml was taken from the above reaction mixture, 3.75 ml of distilled water and 0.2 ml Nessler's reagent was added and incubated for 20 min. The optical density (OD) was measured at 450 nm. The blank was run in all assays. The enzyme activity was expressed in International unit. One IU of L-asparaginase is defined as the amount of enzyme that liberates 1 µmol of ammonia per ml per minute [µmole/ml/min]. A standard curve of ammonium sulphate was used for calculating ammonia concentrations.

3. OPTIMIZATION OF L-ASPARAGINASE

As high yielding organisms is the prime requirement of the biotechnological processes, to satisfy this requirement preliminary optimization of various fermentation parameters are necessary. For this purpose, we optimized various environmental and nutritional parameters.After investigating the best substrate for L-asparaginase production, one

factor at a time (OFAT) method was used to carry out the optimization studies. Different optimization parameters such as effects of substrate concentrations (1- 2% w/v), pH (ranging from 3, 5, 7, 9), incubation period (0-10 days), temperature (4, 25, 37 and 50°C), various inoculum sizes (2,4 6,8,10 mL/50 mL) and different nitrogen sources on L-asparaginase production were studied.

3.1Effect of Different Nitrogen Sources

The effect of additional inorganic and organic nitrogen source on L-asparaginase production was studied using 1% w/v inorganic nitrogen sources viz ammonium chloride, ammonium sulphate and sodium nitrate while two organic nitrogen sources such as malt extract and yeast extract. The nutrient broth was used to prepare production media. Sterile production media was prepared with the0.5g concentration of either squid pen in 100ml Erlenmeyer flask. 1% w/v of above mentioned nitrogen source was added to the production media and inoculated with 5ml of *E.coli* suspension. Production media was incubated at 30°C for 48 hours. Control was run for all four experiments. Fermented media was collected after incubation time of 2 days, centrifuged at 4500 rpm for 6 minutes at 4 °C, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content.

3.2Effect of Different Substrate Concentrations

Optimum substrate concentration required to maximize the production of L-asparaginase enzyme was determined by adding 0.1-1g/50mL of the substrate to production media and incubating for 48 hours. Sterile production media was prepared with addition of 0.1, 0.3, 0.5, 0.7, 0.9 & 1g concentration of squid pen as substrate in 100ml Erlenmeyer flasksa were inoculated with 5ml of *E.coli* suspension and incubated at 30°C for 48 hours. Fermented media was collected after incubation time of 2 days, centrifuged at 4500 rpm for 6 minutes at 4 °C by using cooling centrifuge, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content. Control was run in parallel with replicate samples. The optimum period achieved was taken for further experiments.

3.3Effect of Different pH Values

The optimum pH for L-asparaginase activity was determined over a pH range of 3 -9. For pH studies, the nutrient broth was used to prepare production media. Sterile production media was prepared by addition of 0.5g concentration of squid pen in 100ml Erlenmeyer flasks as a substrate. The pH of flasks was maintained as 3, 5, 7 and 9 using 0.1N HCL or NaOH. Flasks containing squid pen as substrate were inoculated with 5ml of *E.coli* suspension and incubated at 30°C for 24 hours. Control was run along all samples. Fermented media was collected after incubation time of 1day, centrifuged at 4500 rpm for 6 minutes at 4 °C, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content.

3.4Effect of Different Incubation Temperatures

The optimum temperature range for enzyme activity was determined by incubating the assay mixtures at various temperatures viz 4, 25, 37, and 50°C for 48 hours. The nutrient broth was used to prepare production media. Sterile production media was prepared by addition of 0.5g concentration of either squid pen in 100ml Erlenmeyer and inoculated with 5ml of *E.coli* suspensions and incubated at 4,8, 25, 37, 45, and 50°C for 48 hours. Control was run for all the temperatures. Fermented media was collected after incubation time of 2 days, centrifuged at 4500 rpm for 6 minutes at 4 °C, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content. The optimum temperature achieved was taken for further experiments.

3.5 Effect of Different Incubation Periods

Different incubation periods of 0 to10 days were employed to study the effect of different incubation periods on L-asparaginase production. The nutrient broth was used to prepare production media. Sterile production media was prepared in 100ml Erlenmeyer flasks. 0.5g of squid pen was added to the flask as a substrate. Production media was inoculated with 5ml of *E.coli* suspension and incubated at 30°C for 0 to 10 days. Control was also run with replicate samples. Fermented media was collected every day for 10 days, centrifuged at 4500 rpm for 6 minutes at 4 °C by using freeze centrifuge machine, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content. The optimum period achieved was taken for further experiments.

3.6 Effect of Different Inoculum Volumes

In order to know the optimal inoculum size, the flasks with the production medium were inoculated with a range of inoculum 2, 46, 8 and 10ml and incubated for 48 hours. Sterile production media was prepared with the addition of 0.5g concentration of squid pen as a substrate in 100ml Erlenmeyer flasks and inoculated with 1,2,3,4,5,6,7,8,9 &10ml of *E.coli* suspension and incubated at 30°C for 48 hours. Fermented media was collected after incubation

time of 2 days, centrifuged at 4500 rpm for 6 minutes at 4 °C, filtered through Whatman#2 filter paper and cell free filtrate was used as crude enzyme solution to estimate L-asparaginase activity and protein content.

RESULTS AND DISCUSSION

4.1 Screening of Food Waste by-Products as Carbon Source

A selection of appropriate substrate is vital in any bio process, and it should be based on nutrient value, cost and availability. The substrate provides essential nutrients required for microbial growth. We carried out series of experiments using cooked chicken bone (CCB) and Squid pen (SP) as a carbon source for L-asparaginase production by *E.coli*. Figure1 shows the experimental results with enzyme activity0.376 and 2.216 IU/mL respectively. SP showed maximum enzyme activity as compared to CCB. SP showed better results than CCB. Thus we carried out process optimization studies using SP as a substrate and sole carbon source. As a waste by product of food the use of squid pen as a substrate and as a supplementary carbon source may reduce the cost of bulk enzyme production. For the first time, these substrates were tested for production of L-asparaginase enzyme, so there is no previous report.Coconut oil cake (COC), Cottonseed oil cake (CSOC) and Groundnut oil cake (GOC) were used as carbon source for L- asparaginase production by *Serratia marcescens.* All four substrates showed positive results for production of L-asparaginase.[10]. L-asparaginase from *Pseudomonas plecoglossicida* exhibited higher *activity* in M-9 medium containing 0.8% sugar cane industry effluent (3.25 ± 0.12 IU mL^{-1})[15]

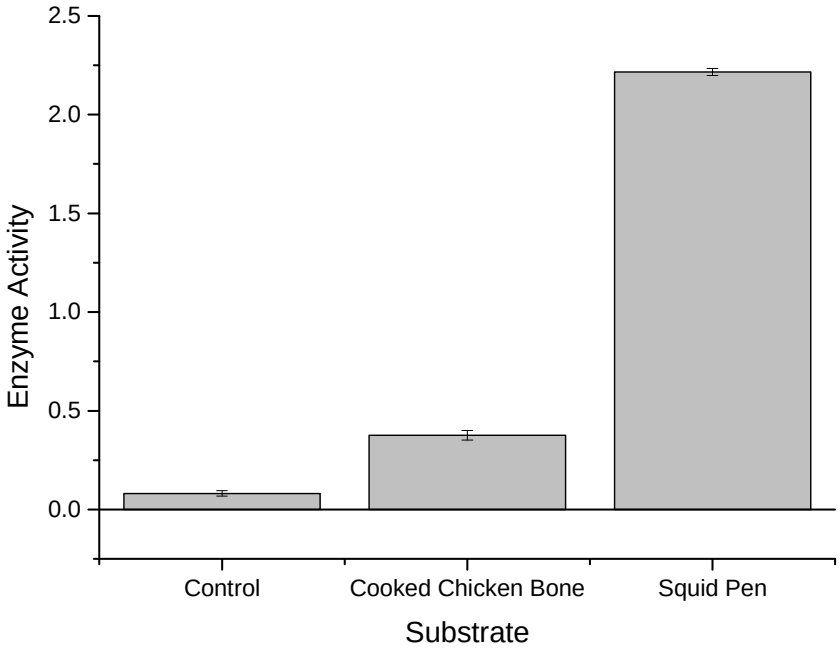

Figure1. Screening of food waste by-products for L-asparaginase production

4.2 Effect of Different Nitrogen Source on L-Asparaginase Production

The impact of supplementation of various nitrogen sources (1% w/w) such as yeast extract, malt extract, sodium nitrate, ammonium sulphate and ammonium chloride on L-asparaginase production is shown in figure 2. Each source was supplied additionally with SP. Ammonium sulphate followed by ammonium chloride was observed to improve the enzyme activity having a yield of 4.651 and 2.491 IU/mL respectively. The positive effect of supplementation of ammonium sulphate has been observed by [16] while [17] reported maximum L-asparaginase production by *Aspergillus terreus* MTCC 1782 with 2% concentration of ammonium sulphate.

4.3 Effect of Different Substrate Concentration

Substrate concentration plays crucial role in the enhancement of enzyme production. Six various initial substrate (Squid Pen) concentrations (0.1, 0.3, 0.5, 0.7, 0.9 and 1 g) were used for optimization studies. Figure 3 shows an increase in enzyme activity with an increase in the concentration of substrate with maximum enzyme activity value of 4.268 IU/mL for 1 g (2% w/v) of the substrate. An optimum substrate concentration is prerequisite for microorganisms in order to produce the required metabolites. Low levels of substrates can lead to low yield while high amount of substrate can inhibit the production of enzyme. Therefore, an optimal amount should be used in order to get the better yield [18]. In our study, the best enzyme activity was shown when 1g of the substrate was

used. These results are in agreement with those reported by[19] where gradual increase in enzyme activity was noticed with increase in substrate concentration which reached to maximum at 1.2ug/ml which reached a steady state at this concentration.[20] also described the increase in enzyme activity with an increase in the concentration of substrate.

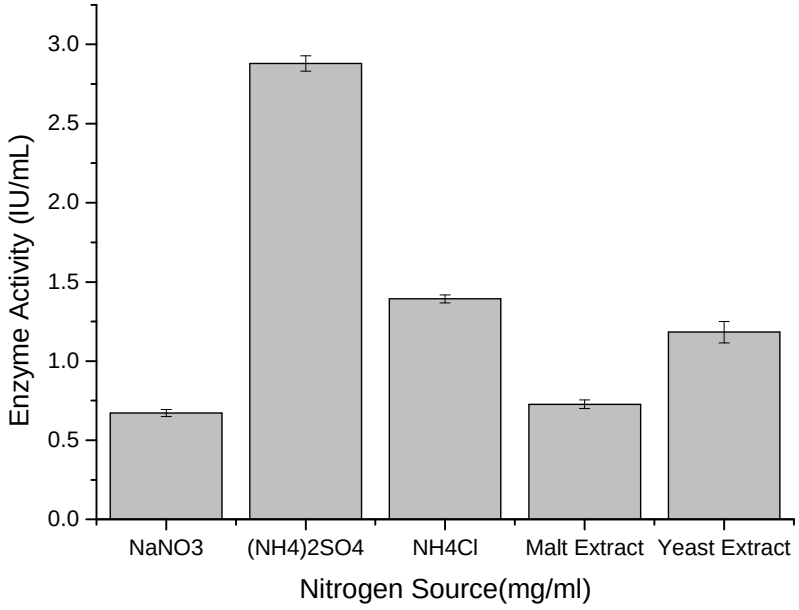

Figure2. Effect of different nitrogen sources on L-asparaginase production

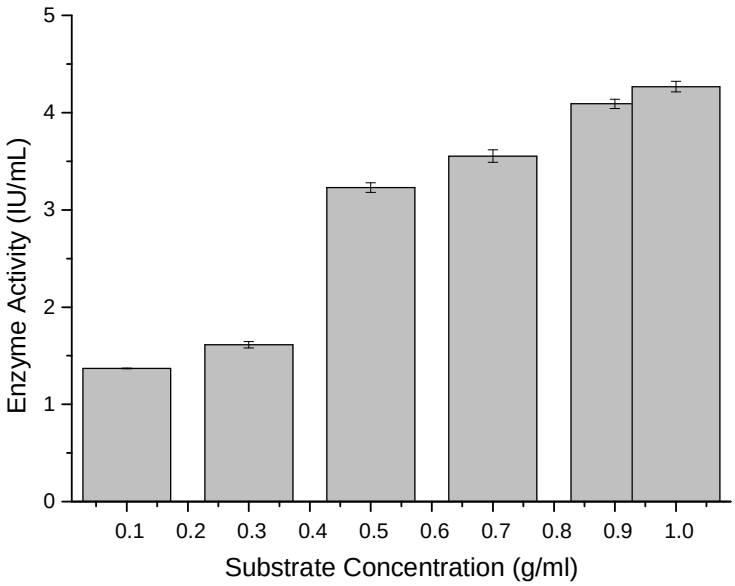

Figure3. Effect of different substrate concentrations on L-asparaginase production

4.4 Effect of Different pH Values on L-Asparaginase Production

The extra cellular pH is yet another significant production parameter and has a strong influence on the pathways of metabolism and product generation by the microorganism. To obtain the optimum pH, different pH values i.e. 3, 5, 7, and 9were taken into observation. Highest enzyme activity of 3.819 and 3.855 (IU/mL) was recorded at pH 7 and 9 respectively. Figure 4 shows the enzyme activity for the various pH values. [10, 21] obtained maximum L-asparaginase production from *Serratia marcerens* and *Erwinia carotovora* at pH 7. A similar optimal value of pH 9 was obtained for *E. coli* by[22, 23].Whereas, *[24]*reported the highest activity at pH 9 for fungal isolates of water samples.

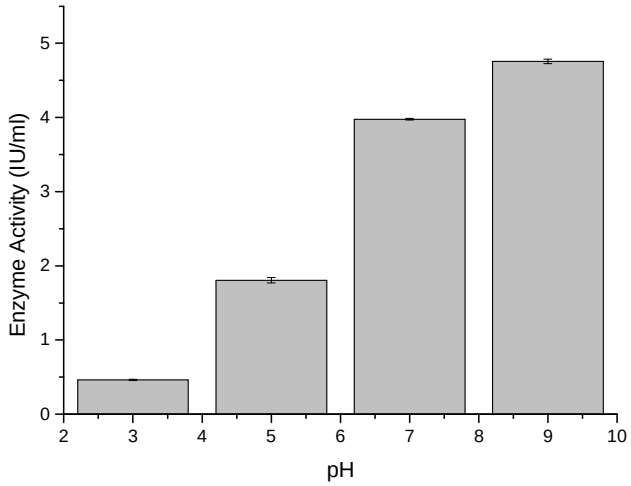

Figure4. Effect of pH values on L-asparaginase production

4.5 Effect of Different Incubation Temperature on L-Asparaginase Production

Temperature is also important as it affects the conversion efficiency of the substrate into cell mass which affect the product formation, particularly when the product is growth associated. The impact of different incubation temperatures on the production of L-asparaginase by *E. coli* is presented in Figure 5. Temperature profile depicts that maximum enzyme activity 3.417 IU/mL was obtained at 37 °C while more than 50% activity was retained even at 50°C. Similar results were observed for L-asparaginase from *Pseudomonas fluorescens*, aeuroginosa 5007, *Pseudomonas stulzeri* MB-405 and *Erwinia carotovora*[25-27] whereas [25]also showed that L-asparaginase activity reached its maximum value when incubation was done at 37°C for 30min.

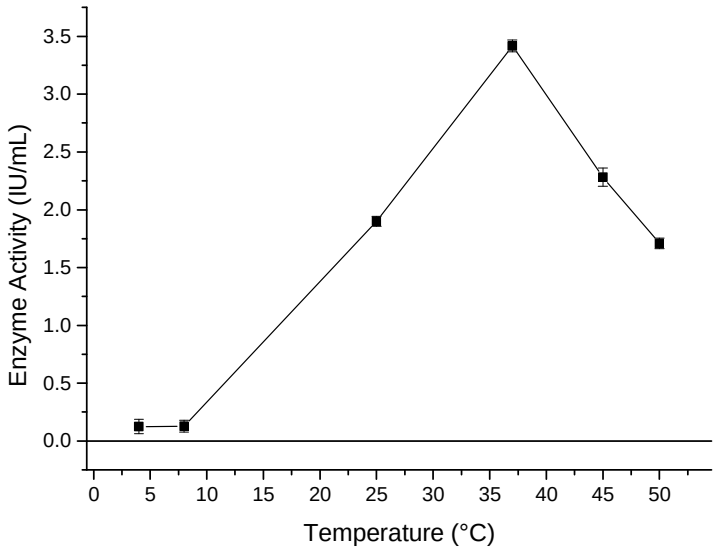

Figure5. Effect of different incubation temperature on L-asparaginase production

4.6 Effect of Incubation Period on L-Asparaginase Production

Data recorded in figure 6illustrate the variations in enzyme level produced by *E.coli* during different periods of incubation using SP as sole carbon source. The production of L-asparaginase was gradually increased with incubation time until day 5 and reached to a maximum yield of 4.218 IU/mL. Further incubation after 5th day showed slight declination in enzyme activity till 8th day reaching 1.279 IU/mL. At longer incubation periods, the enzyme activity decreased which might be due to depletion of nutrients, accumulation of toxic end products. Similar results have been reported by [28]. Besides, [29]reported the maximum enzyme activity after 96hours for *Pseudonocardia endophytica* VUK-10.

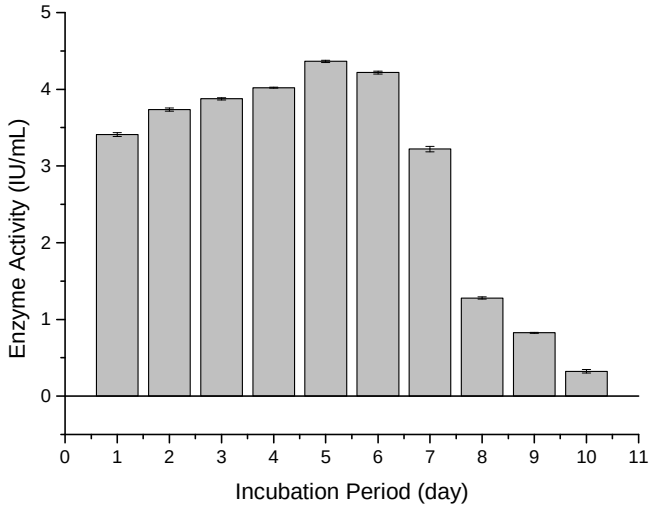

Figure 6.Effect of incubation period on L-asparaginase production

4.7Effect of Inoculum Size

Inoculum volume was also studied by inoculating set of flasks containing production media with different volumes of inoculum. Enzyme production varied with inoculum level and showed parabolic nature in the studied range. Maximum enzyme activity (4.16IU/mL) was observed with inoculum size of 6 ml as shown in figure 7. The increase of inoculum level from 6to 10 ml negatively affected the enzyme production and caused a reduction in enzyme activity. Nevertheless, a higher inoculum level increased the moisture content to a significant point and free excess liquid present in an unabsorbed form will, therefore, give rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and lead to a decrease in growth and enzyme production. Preceding studies by [30] indicated the same trend for L-asparaginase activity where a further increase in inoculum size negatively affected the enzyme production.

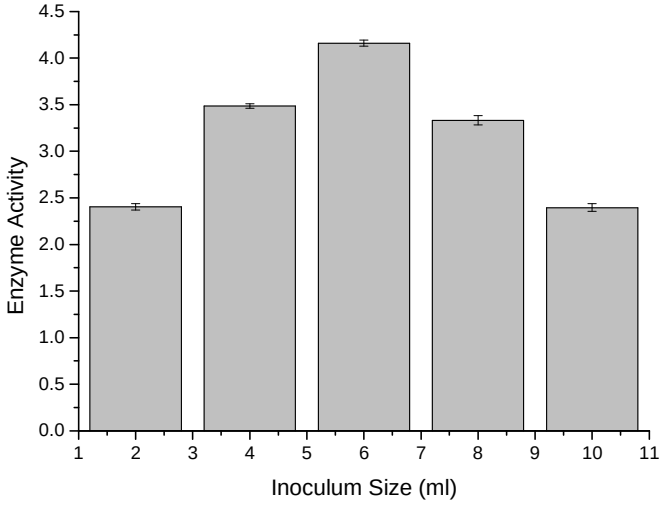

Figure7. Effect of inoculum size on L-asparaginase production

CONCLUSION

The present work indicated remarkable L-asparaginase production potential of *E. coli* ATCC 10536from squid pen, a food by-product whichwas used as carbon source. Different growth factors were successfully optimized, and 2.5 folds increase in enzyme activity was observed after optimization. Moreover, Squid pen is an efficient, economical and easily available carbon source. In conclusion, this study was designed to find out the optimal fermentation conditions for production of improved yields of L-asparaginase. However, the aptness of the enzymes for industrial applications can be investigated after purification.

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