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L-ASPARAGINASE-AN ANTI TUMOR AGENT PRODUCTION BY *FUSARIUM EQUISETI* USING SOLID STATE FERMENTATION

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Abstract- L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is a promising chemotherapeutic agent which plays a vital role in treatment of a variety of lymphoproliferative disorders, lymphosarcoma and acute lymphoblastic leukemia in particular. An attempt is made in the present study to optimize the production of L-asparaginase by *Fusarium equiseti* using soya bean meal under solid state fermentation (SSF). The maximum yield of L-asparaginase (8.51 IU) was achieved with the following optimized fermentation parameters: incubation period (48 hrs), initial moisture content (70% v/w), particle size (3 mm), inoculum volume (20%), supplemented with glucose (0.5% w/v), ammonium sulphate (0.5% w/v) and yeast extract (0.5% w/v).

Keywords: L-asparaginase, ALL, *Fusarium equiseti*, Optimization parameters, Solid state fermentation

Introduction

Asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an enzyme which converts L-asparagine to L-aspartic acid and ammonia. The therapeutic potential of this enzyme is well established, as it has remarkably induced remission in most patients suffering from acute lymphoblastic leukemia (ALL) [1]. Asparaginases are widely distributed in nature from bacteria to mammals and play a central role in the amino acid metabolism and utilization. In human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxalo acetate in the gluconeogenic pathway leading to glucose formation [2]. L-asparaginase is a unique cancer chemotherapeutic agent [3]. It has been demonstrated that this enzyme is responsible for anti lymphoma activity in guinea pig serum [4]. L-asparaginase is the first enzyme with anti leukemic activity to be intensively studied in human beings [5]. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine [6]. So, cancer cells are unable to produce L-asparagine and depend on external L-asparagine from the circulating blood plasma [7]. Since 1922, L-asparaginase has been considered as a therapeutic agent against malignant tumors [8-9].

A wide range of microorganisms such as filamentous fungi, yeasts, and bacteria have

proved to be beneficial sources of this enzyme [10-12]. Several *Streptomyces* species such as *S. karnatakensis, S. venezuelae, S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 are capable of producing detectable amount of L-asparaginase [13-15].

Most of microbial L-asparaginase is intracellular in nature except few which are secreted outside the cell [16]. Extracellular L-asparaginase is more advantageous than intracellular since they could be produced abundantly in culture under normal condition and could be purified economically. L-asparaginase production throughout the world is carried out by submerged fermentation (SmF). However, this technique has many disadvantages. For instance, it is cost intensive and has low product concentration. In addition the extracellular excessive effluents and consequently needs handling and disposable of large volumes of waste water during downstream processing. In recent years, the production of enzymes by solid state fermentation has emerged as advantageous method over submerged fermentation [17] including superior productivity, low capital investment, low energy requirement and less water output. The use of agricultural wastes source of energy makes the SSF environment friendly. The increasing importance of L-asparaginase in recent years for its anticarcinogenic applications promoted us to screen for newer L-asparaginase producing organisms. Therefore, in the present study, we have investigated the optimized fermentation parameters: incubation period, initial moisture content, particle size, inoculum volume, carbon source such as glucose, inorganic nitrogen source such as ammonium sulphate and organic nitrogen source such as yeast extract respectively for maximum

production of L-asparaginase using an isolated *Fusarium equiseti* under solid state fermentation (SSF) conditions.

Materials and methods

Chemicals

All chemicals used in this study were of analytical grade.

Microorganism and inoculum preparation

The isolated fungus *Fusarium equiseti* was used in the present study. The culture was maintained on the Czapek-dox agar medium, incubated at 28^{0}C for 7 days, stored at 4^{0}C and sub-cultured monthly. Conidial suspension was prepared from freshly raised seven day old culture of *Fusarium equiseti* on Czapek-dox agar slants by suspending in 10 ml of 0.85% sterile saline solution.

Fermentation medium

Soya bean meal obtained from the local market of Dharwad was used as the substrate for the production of the enzyme, L-asparaginase by *Fusarium equiseti.* Ten grams of soya bean substrate having the particle size of 3 mm was taken in 250 ml Erlenmeyer flasks and moistened by adding sterile distilled water to all flasks. The flasks were plugged with cotton and autoclaved at 121 ^{0}C for 20 minutes, cooled to room temperature. The contents of flasks were inoculated with 1 ml of spore suspension. The flasks were mixed thoroughly by gently beating on the palm and incubated in slanting position at room temperature for 7 days. After incubation the moldy substrate were analysed for L-asparginase production.

Optimization of fermentation parameters for L-asparaginase production under SSF

The production of L-asparaginase by *Fusarium equiseti* under Solid state fermentation conditions mainly depends on various factors like incubation period, moisture content, particle size, inoculum volume, and carbon and nitrogen source. In order to achieve optimized activity under SSF conditions each parameter is varied one at a time. All experiments were conducted in five replicates and the mean values are presented.

Effect of incubation period

Different incubation periods of 24, 48, 72, 96 and 120 hours were employed to study the effect on L-asparaginase production. The fermentation was carried out at 45 ^{0}C and other experimental conditions were kept constant. The optimum period achieved was taken for further experiments.

Effect of Moisture content

To study the effect of initial total moisture content (before autoclaving) substrate, the fermentation was carried out at 45^{o}C with varying initial moisture from 50-100% as per the method described by Sreenivasulu *et al*., [18].

Effect of particle size

To investigate the various particle size, the soy bean was ground and the particle size were obtained using standard sieves of 2 mm, 3 mm and 4 mm respectively. *Fusarium equiseti* was cultivated at 45^{o}C for 48 hours with other parameters constant.

Effect of inoculum volume

Various inoculum levels were prepared 5-50% as per the method described by Sreenivasulu *et al*., [18]. The study was carried out at 45^{0}C for 48 hours keeping other conditions at their optimum level.

Effect of carbon source

To investigate the effect of various carbon sources the substrate was supplemented with 0.25%-1.25% carbon sources such as glucose, sucrose and fructose respectively. The strain *Fusarium equiseti* was cultivated and the production was assayed by carrying out enzyme activity.

Effect of Nitrogen source

The effect of additional inorganic and organic nitrogen source on L-asparaginase production was studied using inorganic nitrogen source like ammonium chloride, ammonium sulphate and ammonium nitrate and organic nitrogen source such as peptone, yeast extract and beef extract which were varied as 0.25%, 0.5%, 0.75%, 1% and 1.25% respectively as per the method described by Ameena *et a*l., [19].

Extraction of the fermented substrate

For the extraction of the fermented substrate 1 gm of the moldy substrate were withdrawn periodically every 24 hours in aseptic conditions and mixed with 1:10 phosphate buffer. The mixture was homogenized and filtered. The filtrate was centrifuged and clear supernatant was used for enzyme assay.

Quantitative assay of L-asparaginase activity

Quantitative estimation was carried out following Nessler's reagent according to the method of Imada *et al*., [20]. The reaction mixture containing 0.5 ml of 0.04 M L-asparagine, 0.5 ml 0.5M phosphate buffer (pH 7.8), 0.5 ml of an enzyme preparation and 0.5 ml distilled water was added to make up the total volume to 2 ml. The tubes were incubated at 30ºC for 30 minutes. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. 0.1 ml from the above mixture was taken and added to 3.7 ml of distilled water and to that 0.2 ml of Nessler's reagent was added. After incubating the mixture at 20ºC for 20 minutes the OD was checked at 450 nm with Spectrophotometer [Systronics]. The enzyme activity was expressed in International unit.

International unit

One IU of L-asparaginase activity was defined as that amount of enzyme which catalyses the formation of 1µmole of NH_3 per minute per ml under the optimal assay conditions.

Results

Effect of incubation period

Incubation period during the process of Solid state fermentation is very much essential to study the optimum incubation time for maximum L-asparaginase production. The fermented samples were withdrawn periodically every 24 hours up to 120 hrs and the results are indicated in the fig.1. The organism shows maximum activity of 4.32 IU at 48 hours followed by 4.12 IU at 72 hours, 4.02 IU at 96 hours, 3.76 IU at 24 hours and 3.74 IU at 120 hours. Therefore 48 hours is considered as optimum incubation period for *Fusarium equiseti* under solid state fermentation conditions.

Effect of moisture content

The moisture content is a critical factor in SSF media that attritubutes to biosynthesis and secretion of the enzymes. The results are indicated in fig.2. High enzyme titre of 4.27 IU was attained when the initial moisture level was 70% followed by 3.91 IU at 60%, 3.63 IU at 80%, 3.53 IU at 50% 3.31 IU at 90% and 3.11 IU at 100% moisture content. In the present study 70% of moisture was consider as optimum for initial moisture content.

Effect of particle size

Particle size of the substrate has greater influence on the production under SSF process. The results are depicted in fig.3. Maximum activity of 4.76 IU was obtained with the particle size of 3 mm followed by 4 mm with the activity of 4.51 IU and least activity of 4.21 IU was obtained with 2 mm at 48 hours of incubation. L-asparaginase production increased with the increase in particle size and further decreased with the particle size.

Effect of inoculum volume

Inoculum volume is also an important factor in the production of L-asparaginase. To evaluate the effect of inoculum volume on the production of L-asparaginase different inoculum levels of 5, 10, 20, 30, 40 and 50% (v/w) were used for the study. The results are presented in fig.4. The data reveals that maximum activity of 4.2 IU was obtained at 20% (v/w) of the inoculum level and the least is 3.12 IU for 50% (v/w) inoculum volume at 48 hours. From the above result it is clear that L-asparaginase production increases readily with increase in the inoculum level until the maximum enzyme productivity and thereafter it decreases with increase in the inoculum.

Effect of carbon source

In order to find out the optimum carbon source different carbon sources such as glucose, sucrose and fructose were used for the study. The results are presented in the fig.5, 6 and 7. The data revealed that the maximum activity of 6.81 IU was obtained for 0.5% and the least activity of 4.26 IU for 1.25% glucose. Maximum activity of 6.2 IU was obtained with 0.75% sucrose and the least activity of 4.25 IU for 1.25% sucrose similarly maximum activity of 5.2 IU was obtained for 0.5% fructose and the least activity of 4.62 IU was obtained with 1.25%. From the above studies it is clear that glucose is the best carbon source for L-asparaginase production by *Fusarium equiseti.*

Effect of nitrogen source

A nitrogen source is the limiting factor and plays key role in the L-asparaginase production. Most of the microorganism utilize nitrogen source either inorganic or organic form or sometimes both. Therefore, in the present context, the L-asparaginase production was studied supplementing nitrogen both inorganic and organic forms. Inorganic forms such as ammonium sulphate, ammonium chloride and ammonium nitrate were used for the study. The results are depicted in the fig.8, 9 and 10 respectively. The data reveals that 0.5% ammonium sulphate shows the maximum activity of 7.36 IU and the least activity of 3.18 IU was obtained with 1.25%. Similarly maximum activity of 4.79 IU for 0.5% ammonium chloride and least activity of 3.23 IU for 1.25% ammonium chloride was obtained. For ammonium nitrate the maximum activity obtained was 6.27 IU for 0.25% where as the least activity of 3.61 IU was obtained for 1.25%. The results for organic nitrogen source optimization are depicted in the fig.11, 12 and 13 respectively. The highest activity of 8.51 IU was obtained with 0.5% yeast extract and least activity of 4.27 IU was obtained with 1.25% yeast extract. Maximum activity of 5.5 IU was obtained for 0.75% peptone and the least activity of 3.64 IU for 1.25% was attained. Similarly maximum activity of 4.86 IU was obtained at 0.5% beef extract and 3.81 IU was obtained for 1.25%. From the above studies it is clear that ammonium sulphate can be used as the best inorganic nitrogen source and yeast extract can be used as the best organic nitrogen source for L-asparaginase production by *Fusarium equiseti*.

Discussion

Solid state fermentation has emerged as a potential technology for the production of microbial products utilizing the cheaply available raw materials. Soya bean meal proved to be one of the best substrate for L-asparaginase production. Experimental L-asparaginase activity were analyzed to study the independent effect of incubation period, moisture content, particle size, inoculum volume, carbon source and nitrogen source on L-asparaginase production by *Fusarium equiseti* using soya bean meal as substrate under solid state fermentation.

In the present study production of L-asparaginase started at 24 hours and reached maximum at 48 hours and the decreased significantly with increase in the incubation time. Similar results have been reported by Lapmak *et al.,* [21] where the highest activity of 6.3 U/ml for 72 hours using *Bipolaris* sp.BR438 where as Venil and Lakshmanaperumaisamy [22] have reported maximum L-asparaginase production of 79.84 U/gds at 36 hours of incubation. At longer incubation periods, the enzyme activity decreased which might be due to the depletion of nutrients, accumulation of toxic end

products, and the change in pH of the medium, or loss of moisture. In the present study L-asparaginase production increased with moisture content and was maximum at 70% and the least activity was obtained at 100%. Siddalingeshwara and Lingappa [23**]** have reported 5.63 IU at 65% moisture. The present results were in good agreement with Mishra [24] who have reported 40.9 U/gds at 70% moisture content. For instance, a reduction in enzyme production at high initial moisture content may be as a result of a reduction in substrate porosity, changes in structure of substrate particles, reduction in gas volume, and reduced fungal growth [25]. On the other hand, low moisture level could lower the solubility of nutrients, reduce substrate swelling, and decrease the water retention by the substrate. All these conditions affect fungal growth and ultimately the enzyme production.

Regarding the particle size, the activity decreased with increased particle size. In the present study L-asparaginase production was maximum at the particle size of 3 mm and the least activity was obtained with the particle size of 4 mm. Similar results have been reported by Mishra [24**]** at the particle size of 1205–1405 µ. L-Asparaginase production increased with particle size may be due to increase in the interparticle porosity that could support mycelium growth and provide better aeration for SSF. However, further increase in porosity with particle size coupled with reduced saturated surface area could reduce nutrient availability for fungal growth.

Optimization of inoculum volume is necessary as high inoculum levels are inhibitory in nature. In the present study, L-asparaginase production increased readily with increase in the inoculum level and maximum enzyme activity was obtained at 20% (v/w) and least activity was obtained at 50% (v/w) inoculum level. Chanakya *et al.,* [26] have reported the maximum L-asparaginase production of 4.81 IU with the inoculum volume of 1.5 ml of 7 days old *Fusarium oxysporum*. The present results were in good agreement with Sreenivasulu *et al.,* [18] who have reported 50.2 U/gds with 20% (v/w) inoculum level. The reason may be because of higher inoculum density is inhibitory to the enzyme production as too much biomass can deplete the substrate nutrients or accumulation of some non-volatile self inhibiting substances that inhibits the product formation [27] and lower density may give insufficient biomass causing induced product formation; where as higher inoculum may produce too much biomass which is inhibitory to the product formation [28].

The influence of various carbon sources namely glucose, sucrose and fructose were studied for L-asparaginase production by *Fusarium equiseti* under solid state fermentation. Optimal glucose concentration was observed at 0.5% with maximum activity of 6.81 IU and the least activity of 4.25 IU for 1.25% sucrose. Chanakya *et al*., [26] have reported the increase in activity of 6.02 IU to 6.92 IU at 0.3% glucose for L-asparaginase production by *Fusarium oxysporum*. Sukumaran *et al*., [29] have also reported glucose as the best carbon source for L-asparaginase production by *Serratia marcescens.* The present results were in good agreement with Lapmak *et al*., [21] who has reported 0.4% glucose. It has been reported that the microbial synthesis of L-asparaginase is under catabolic repression and requires less amount of carbon source [30-31]. But the incorporation of glucose may be attributed to the positive influence of additional carbon sources on enhanced biosynthesis.

Most of the microorganism utilize nitrogen sources either inorganic or organic form or sometimes both. Therefore in the present context, the L-asparaginase production was studied supplementing the inorganic nitrogen forms such as ammonium sulphate, ammonium chloride, ammonium nitrate and organic nitrogen forms such as yeast extract, peptone and beef extract respectively. 0.5% ammonium sulphate and 0.5% yeast extract gave the optimum activity. Hence in the present study ammonium sulphate and yeast extract can be used as best nitrogen source. Narayan et al. [16] have reported 2% of yeast extract as the best nitrogen source for L-asparaginase production by *S.albidoflavus.* Ameena *et al.* [19**]** have reported 0.25% ammonium sulphate can be used as best inorganic nitrogen source for L-asparaginase production by *S.gulbargensis.* Venil *et al*., [22] have reported peptone as the best nitrogen source for L-asparaginase production using *Serratia marcescens* SB08 where as ammonium chloride was found to be the best nitrogen source for L-asparaginase production using *Aspergillus terrus* MTCC 1782 was reported by Baskar and Rangathan [32]. Gaffar [33] have showed positive effect of supplementation of 1% ammonium sulphate. Liu and Zajic [34] have studied the effect of yeast extract (0.5%). Hence from the present findings ammonium sulphate and yeast extract were the best inorganic and organic nitrogen sources that can be used for L-asparaginase production by *Fusarium equiseti.*

Conclusion

The observations made in the study hold great promise for maximum value of L-asparaginase activity obtained after optimization of fermentation parameters such as incubation period, initial moisture content, particle size, inoculum volume, carbon source such as glucose, inorganic nitrogen source such as ammonium sulphate and organic nitrogen source such as yeast extract respectively for maximum production of L-asparaginase using an isolated *Fusarium equiseti* under solid state fermentation (SSF) conditions. This clearly demonstrates the exploring the soil fungi for the production of L-asparaginase as therapeutic enzyme using cheaper substrates in solid state fermentation. The results also indicate that *Fusarium equiseti* is the potential strain for L-asparaginase production under SSF conditions.

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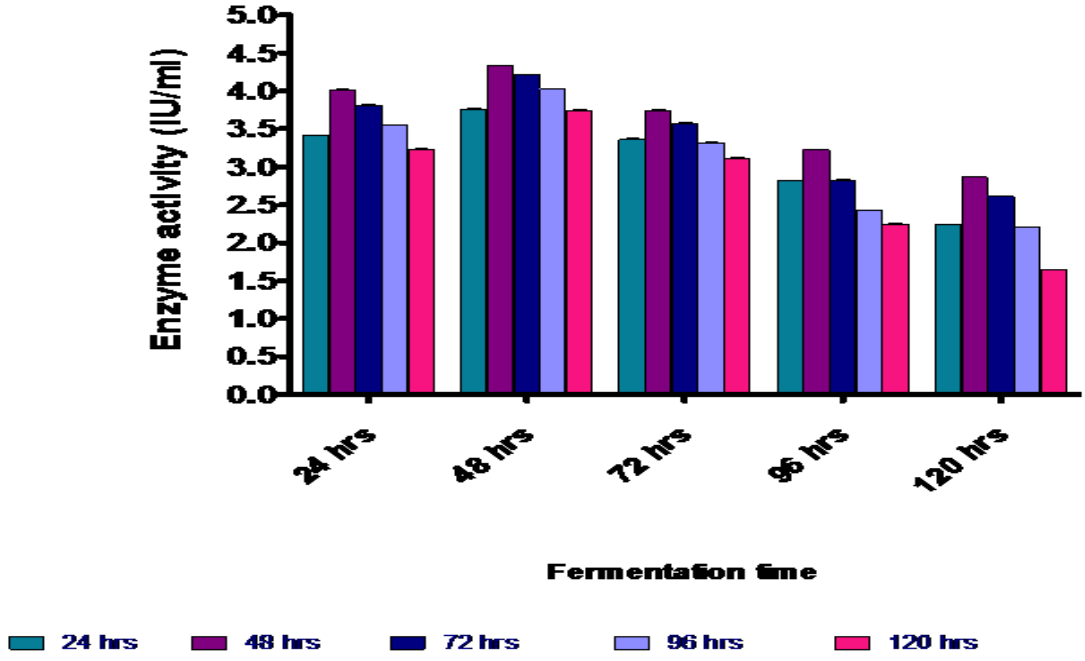

Fig.1- Effect of incubation period on the production of L-asparaginase

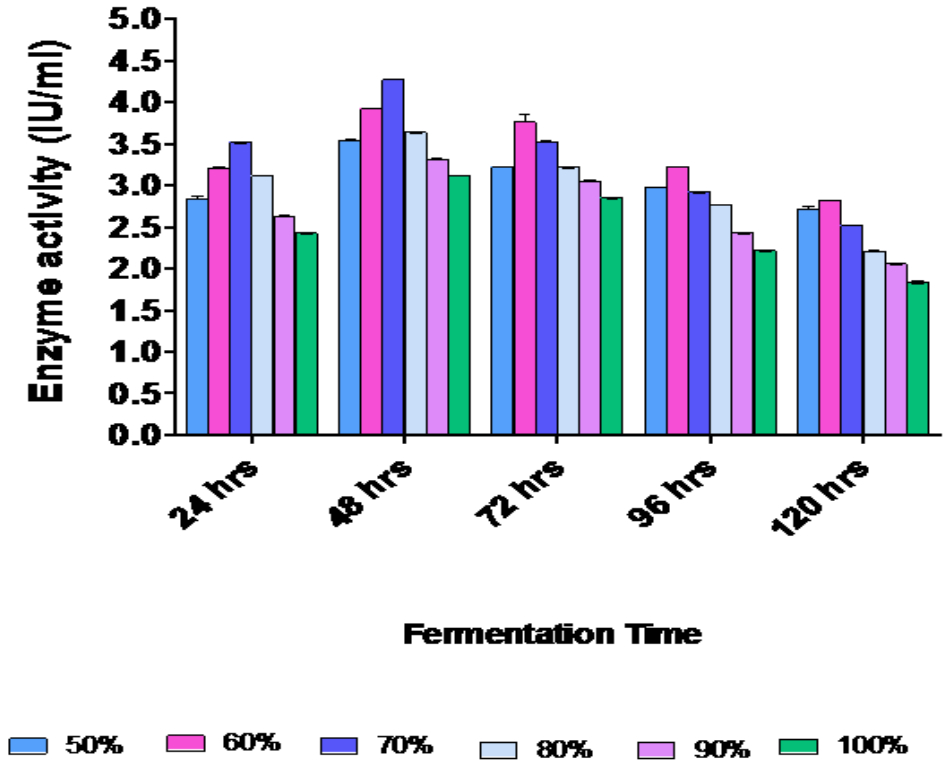

Fig.2- Effect of moisture content on the production of L-asparaginase

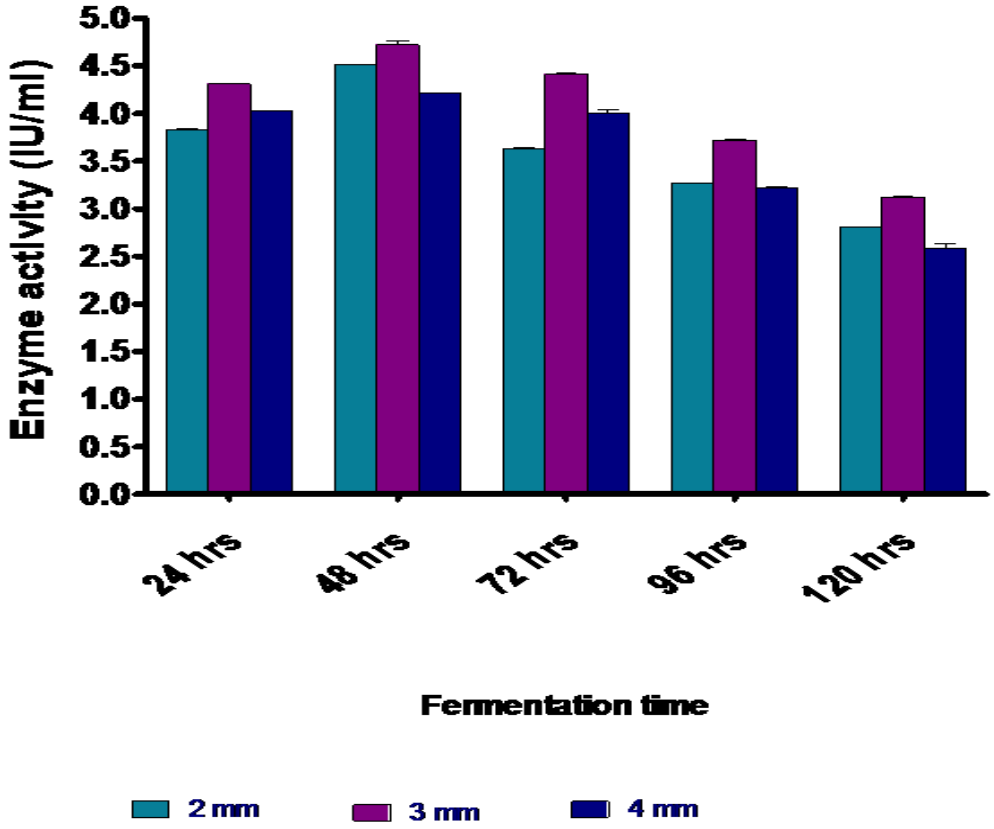

Fig.3- Effect of particle size on the production of L-asparaginase

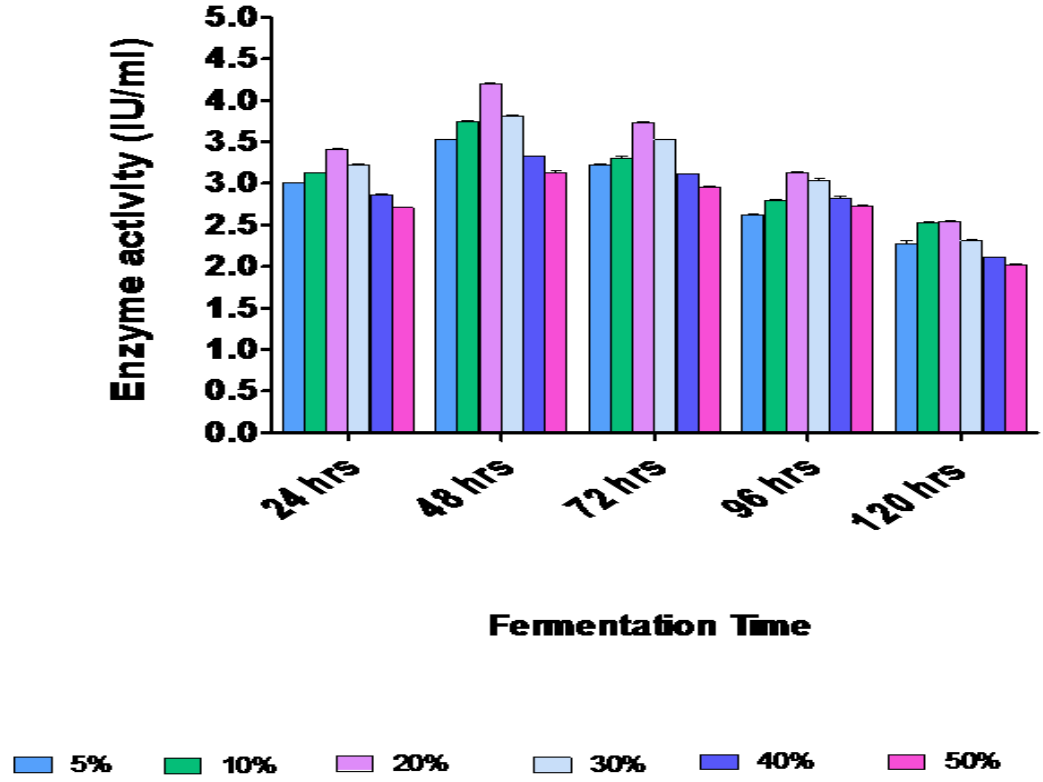

Fig.4- Effect of inoculum volume on the production of L-asparaginase

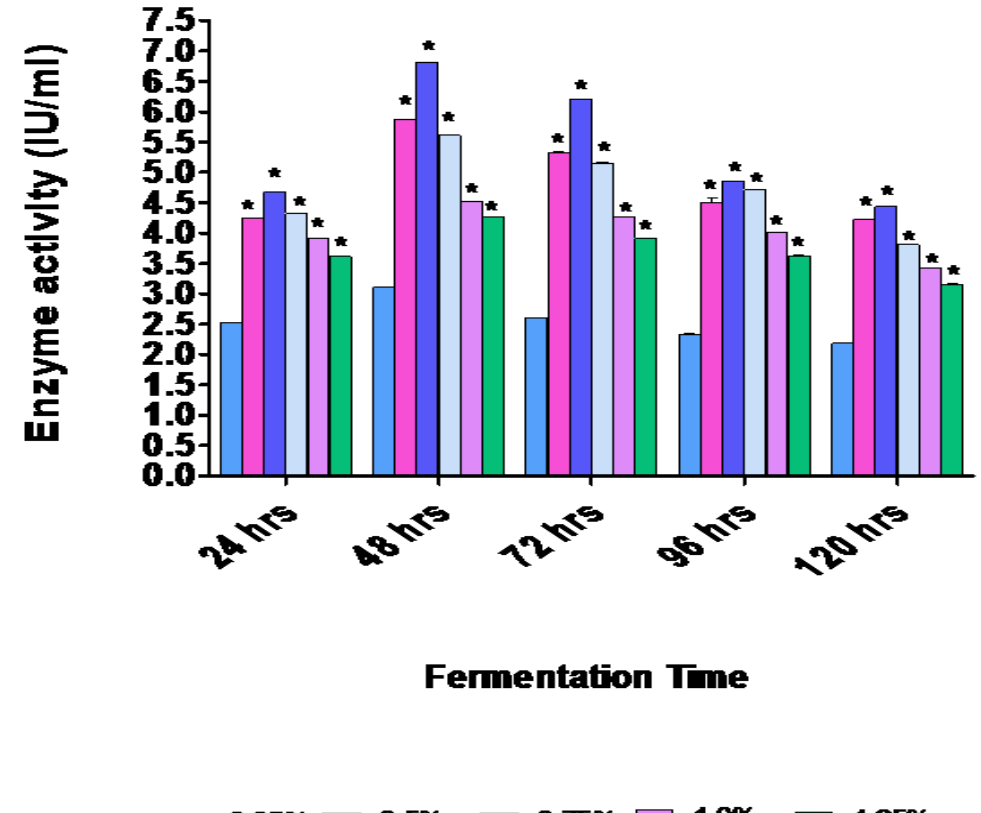

Fig.5- Effect of Glucose on the production of L-asparaginase

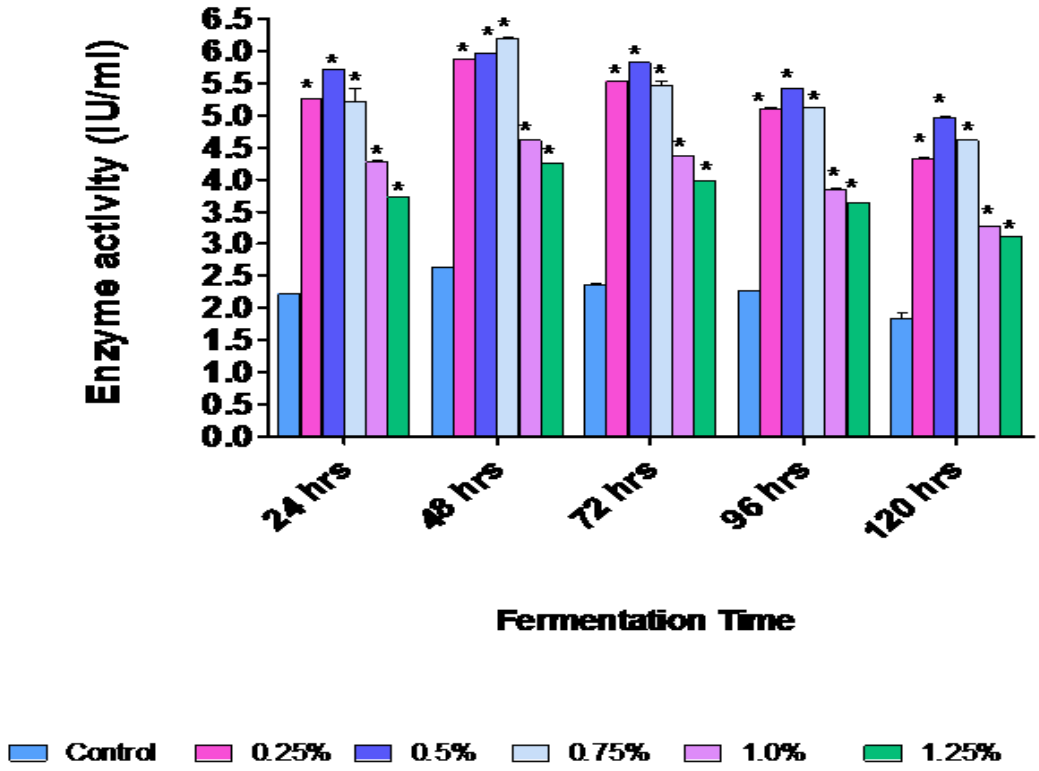

Fig.6- Effect of Sucrose on the production of L-asparaginase

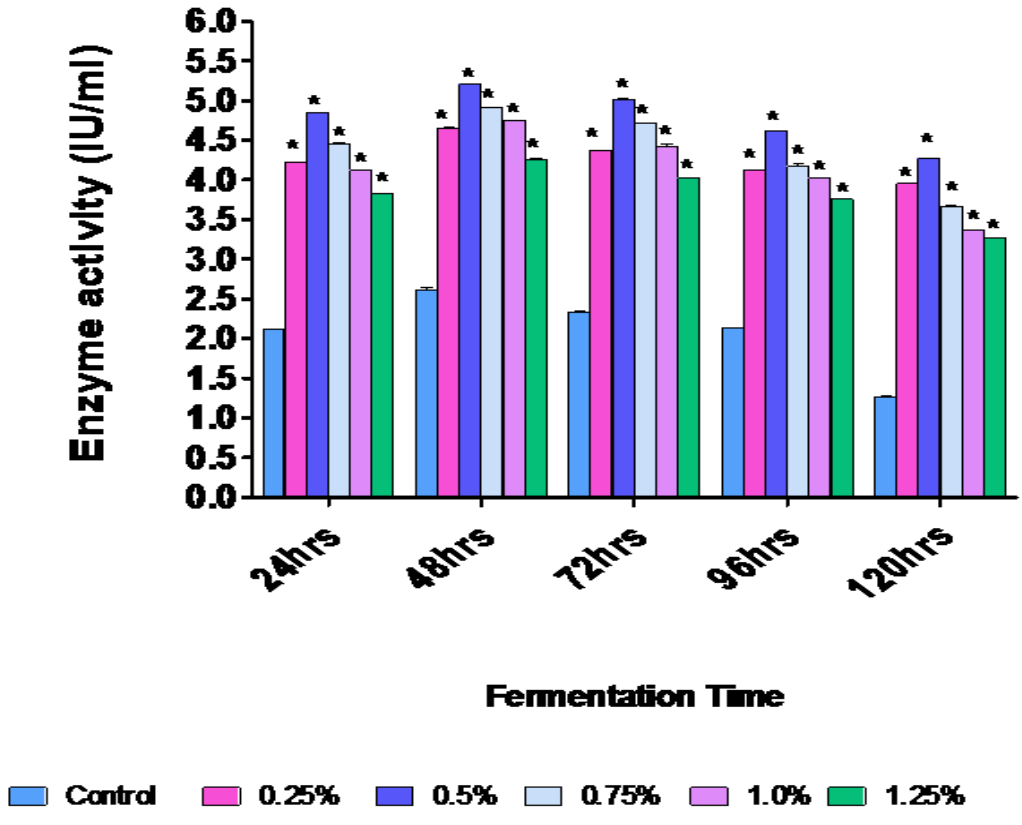

Fig.7- Effect of Fructose on the production of L-asparaginase

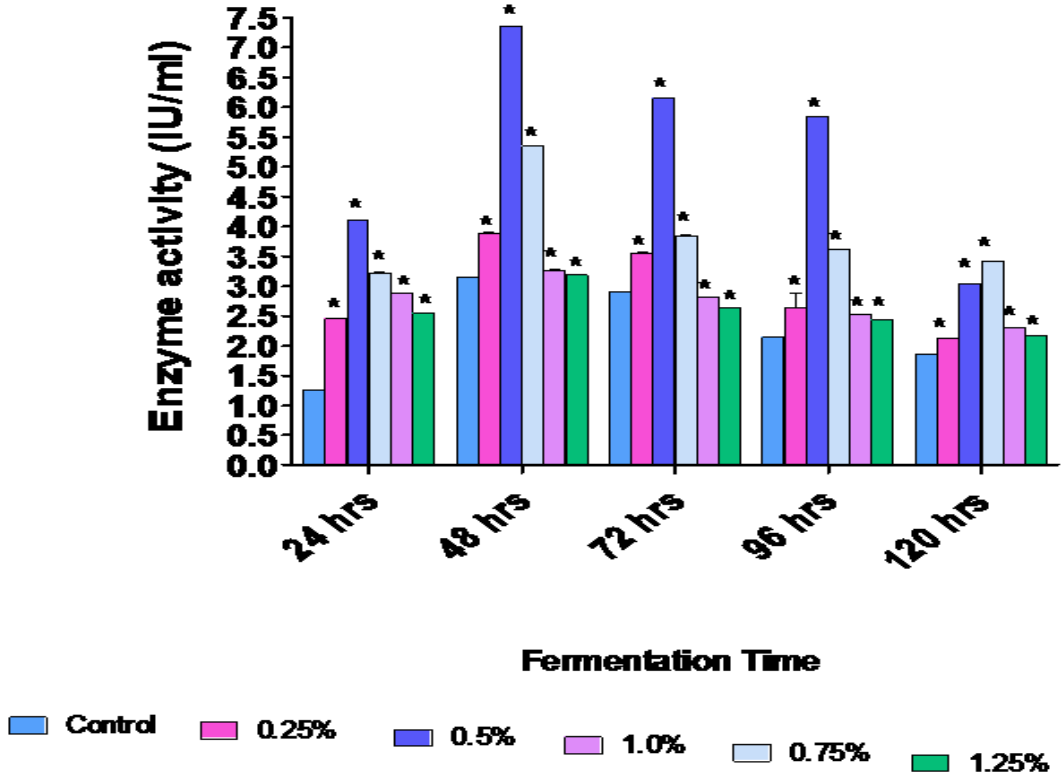

Fig.8- Effect of Ammonium sulphate on the production of L-asparaginase

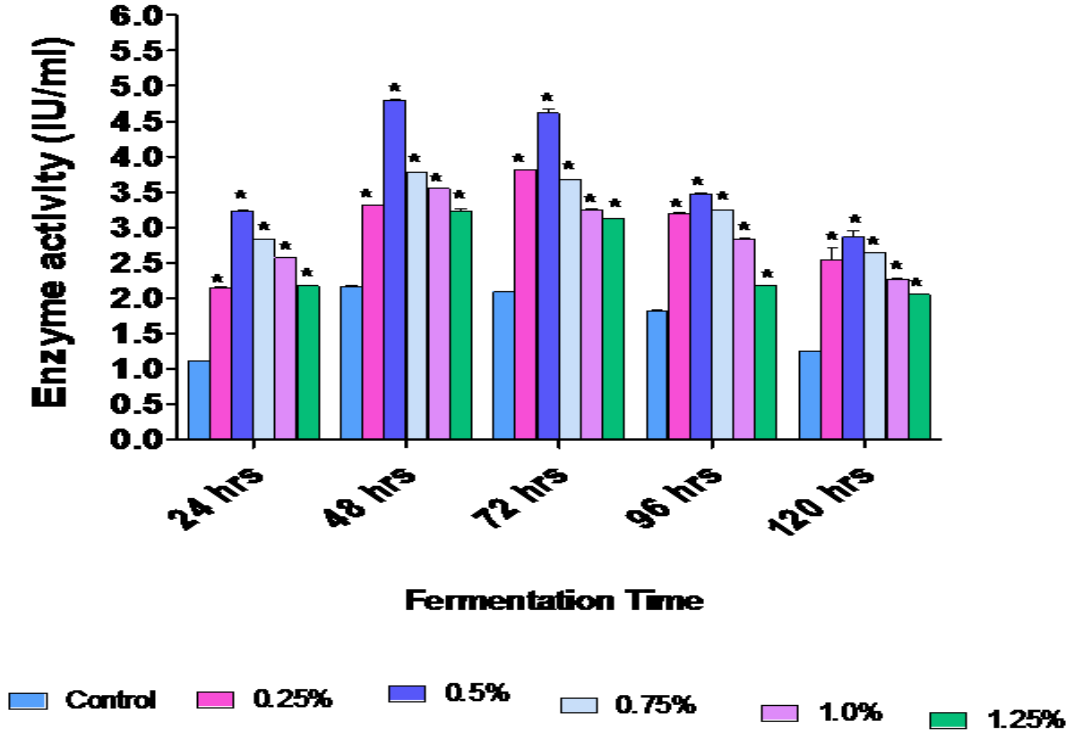

Fig.9- Effect of Ammonium chloride on the production of L-asparaginase

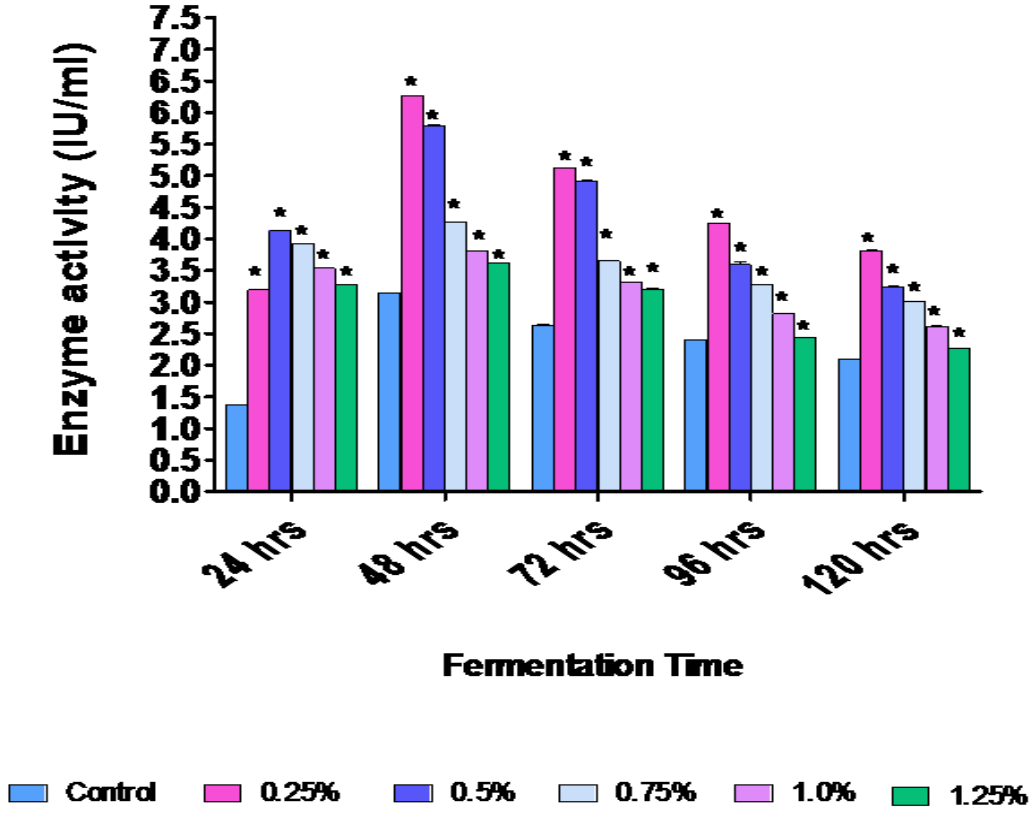

Fig.10- Effect of Ammonium nitrate on the production of L-asparaginase

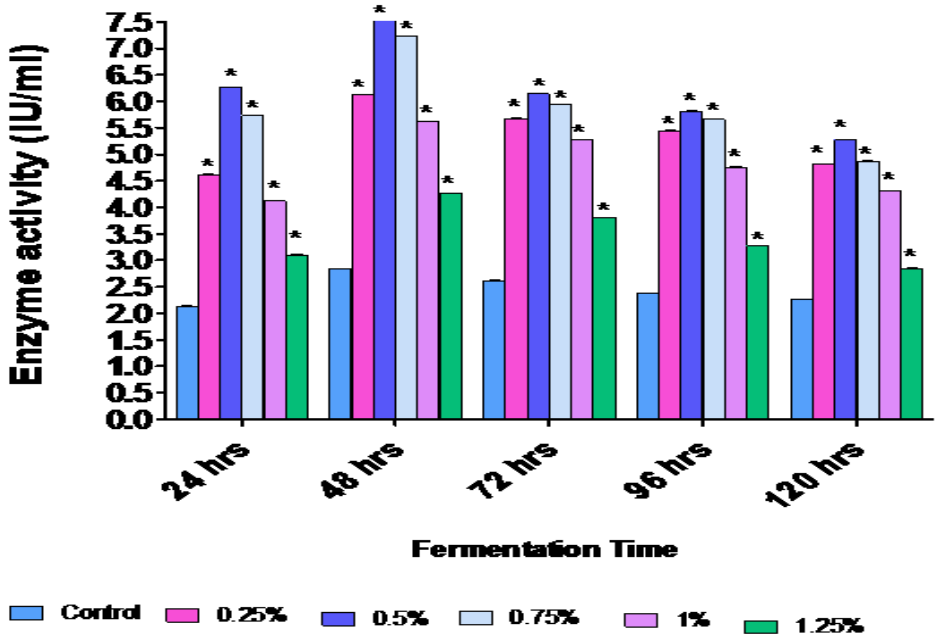

Fig.11- Effect of Yeast extract on the production of L-asparaginase

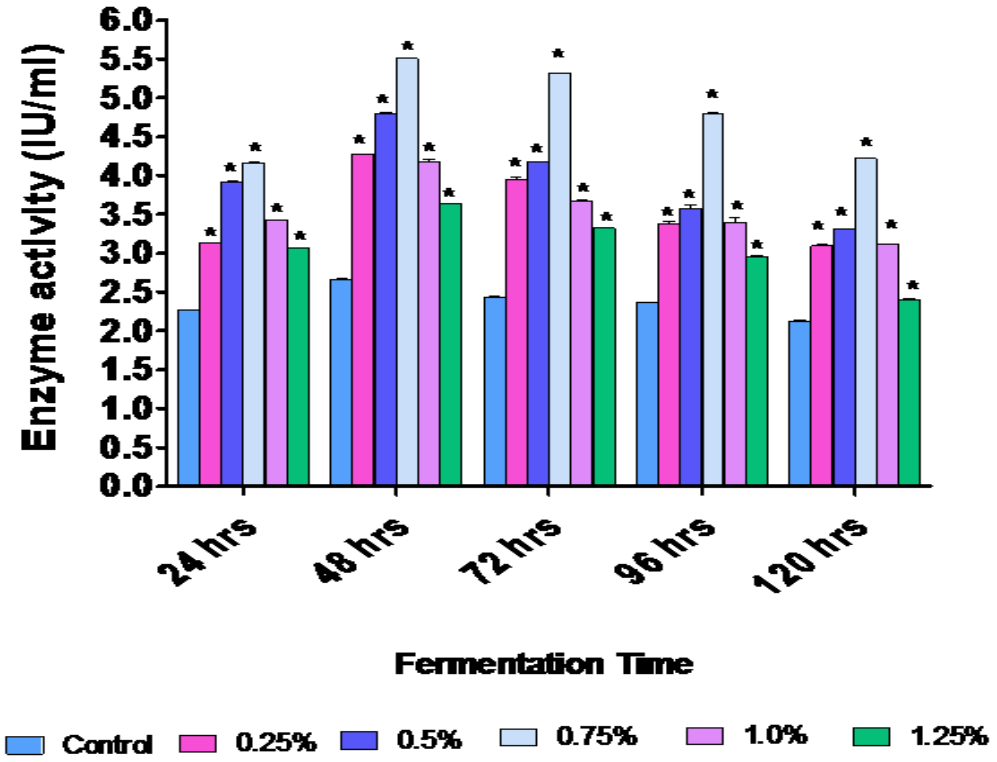

Fig.12- Effect of Peptone on the production of L-asparaginase

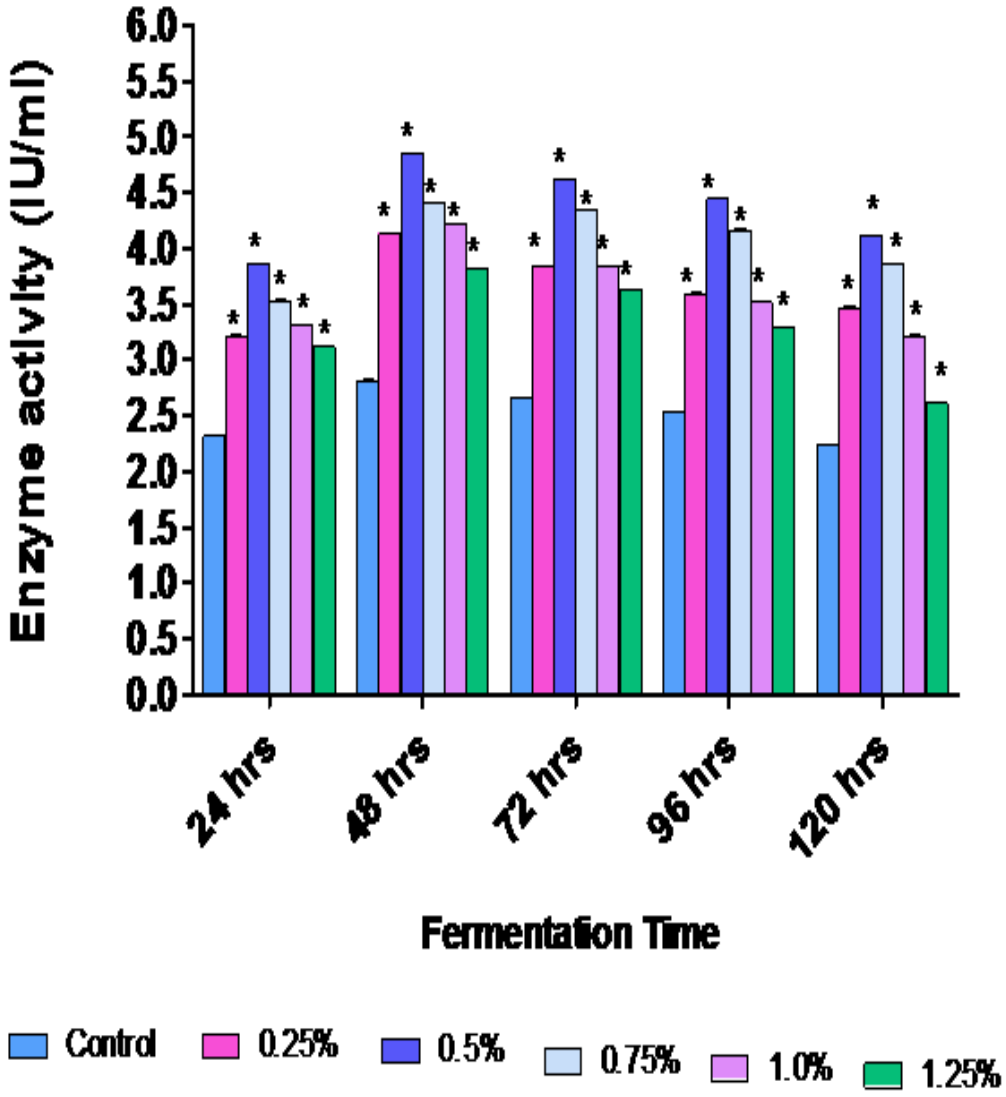

Fig.13- Effect of Beef extract on the production of L-asparaginase.