OPTIMIZATION OF PROCESS PARAMETERS FOR L-ASPARAGINASE PRODUCTION BY *Aspergillus terreus* MTCC 1782 UNDER SOLID STATE FERMENTATION USING MIXED SUBSTRATE

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Abstract

L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extra cellular enzyme that has received considerable attention since it is used as an anticancer agent. L-asparaginase belongs to an amidase group that hydrolyses the amide bond in L-asparagine to aspartic acid and ammonia. The clinical action of this enzyme as an anti-carcinogenic is attributed to the reduction of L-asparagine; tumour cells unable to synthesise this amino acid are selectively killed by L-asparagine deprivation. L-Asparaginase has its application in food industry also. It helps in reducing the content of acrylamide in baked food products by hydrolysing the L-asparagine. L-Asparaginase is majorly produced by microorganisms including bacteria, yeast and fungi. The potential of Aspergillus terreus MTCC 1782 using cauliflower stalk: corn ears (3.75: 1.25) as substrate under SSF is the purpose of the study. Solid state fermentation (SSF) is a very effective technique opposed to submerged fermentation in various aspects. Various fermentation parameters such as types of agro material, their ratios, carbon source, nitrogen source, inoculum level, moisture content, temperature, pH, fermentation time, metal salts, and L-asparagine concentration, which influence the rate of enzyme production under SSF, were optimized. The optimized production of L-asparaginase has been obtained at 35°C for 4 days with a pH of 9.0, along with 50% moisture content, and 20% inoculum volume as the optimized fermentation conditions. The optimization was done using a 'one-factor-at-a-time' approach. The highest yield was obtained with, sucrose (1%w/v), ammonium sulphate (1%w/v), NaCl (1%w/v), L-asparagine (1%w/w), added to the fermentation medium, as supplements. Use of cauliflower stalk along with corn ear as potential raw materials for enzyme production could be of great commercial significance.

Keywords: *L-asparaginase, chemotherapeutic agent, Aspergillus terreus, SSF, mixed substrate, optimization*

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1. INTRODUCTION

L-asparaginase (L-asparagine amino hydrolase, EC3.5.1.1), the enzyme which converts L-asparagine to L-aspartic acid and ammonia, by hydrolysis which, proceeds in two steps via a beta-acyl-enzyme intermediate [1], has been used as a chemotherapeutic agent [2]. Kidd (1953) [3] observed that certain transplanted murine leukaemias were suppressed by treatment with guinea-pig serum.

The clinical action of this enzyme as an anticarcinogenic[4] is attributed to the reduction of L-asparagine; tumour cells unable to synthesise this amino acid are selectively killed by L-asparagine deprivation. These leukemic cells depend on circulating asparagine for their ample nourishment and diet. This deprives the leukemic cell of circulating asparagine and prevents them from the rapid malignant growth [5].

L-Asparaginase has its application in food industry also. It helps in reducing the content of acrylamide in baked food products by hydrolysing the L-asparagine [6]. The reason it is preferred for the purpose is it is biodegradable, non- toxic and can be administered at the local site quite easily [7].

ELSPAR, ONCASPAR, KIDRPLASE, ERWINASE are the brand names of L-Asparaginase as drug.

Though several L-asparaginases of bacterial origin have been developed and their potential usage in clinical trials have been studied to prevent the progress of L-asparagine-dependent tumours, mainly lymphosarcomas, the success hitherto has been rather limited, and most of the treatments must be interrupted due to severe side effects and immunological reactions in the patients. Since the 1970s, several microbial strains like *Aspergillus tamari, Aspergillus terreus, Escherichia coli, Erwinia aroideae, Pseudomonas stutzeri, Pseudomonas aeruginosa, Serratia marcescens,* and *Staphylococcus sp*. [8] having potential for L-asparaginase production have been isolated and studied in detail. Among the actinomycetes, several *Streptomyces* species such as *S.karnatakensis, S.venezualae, S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-asparaginase production [9].

Solid state fermentation (SSF) is a very effective technique as the yield of the product is many times higher than in submerged fermentation. It offers many advantages over submerged fermentation such as lower energy requirements,

less risk of bacterial contamination, less waste water generation and less environmental concerns regarding the disposal of solid waste. Also includes, ease of product extraction that does not require complicated methods of treating the fermented residues. In comparison with SmF, SSF offers better opportunity for the biosynthesis of low volume-high cost products [10].

Mixed substrate fermentation has been more advantageous for the production of enzymes than single substrate fermentation [11].

In the present study, *Aspergillus terreus* MTCC1782 was used for the production of L-Asparaginase using agricultural wastes as substrates, which provides an edge to the research work by reducing the substrate cost as well as assisting in waste management. Effect of parameters over the production and their optimization were also studied. According to my study, cauliflower stalk has not been used as substrate for production of asparaginase, at least in this specific combination.

2. MATERIALS AND METHODS

2.1 Microorganism and Inoculum Preparation

2.1.1 Microorganism

The fungal strain *Aspergillus terreus* MTCC 1782 was procured from Institute of Microbial Technology, Chandigarh, India.

2.1.2 Growth Medium and Growth Conditions

It was maintained on Malt extract agar (MEA) medium slants having the composition (g/L): malt extract 30.0, peptone 5.0, agar-agar 15.0with pH 5.4±0.2. 50 gm of MEA medium was weighed in 1000ml distilled water and used as growth medium. The microbial strain was grown at 30°Cfor 4 days after which, it was stored at 4°C until further useand sub-cultured after every two weeks.

2.1.3 Inoculum Preparation

For preparing a spore suspension, to a well sporulated slant of *A.terreus*, 10 ml of sterilized 0.1% Tween 80 solution was added. The surfaces were scrapped with an inoculating loop to suspend the spores and the spore suspension was taken as inoculum.

2.2 Substrate

Seven different substrates namely sweet pea peel, cauliflower stalks, corn ear, Bengal gram husk, sorghum, pearl millet, and groundnut shells were collected from a local market of Visakhapatnam, India. The substrates were sun dried and ground to fine powder.

2.3 Solid State Fermentation

5g of each substrate was weighed in 250ml Erlenmeyer flasks separately. The substrates were moistened with 2ml of moistening medium (distilled water) and were autoclaved at 121°C (15 lb) for 20 min, cooled to room temperature and then inoculated with 2ml of inoculum under aseptic conditions. The inoculated flasks were incubated at 30°C in an incubator for 96h. All experiments were carried out in duplicate.

2.4 Mixed Substrate Composition

Two high yielding substrates cauliflower stalk and corn ear were selected and mixed in different compositions according to mixture design (Design Expert).

2.5 Enzyme Extraction

After the incubation period, the crude enzyme from the fermented substrate was extracted using 0.1M phosphate buffer (pH 8). After mixing the fermented substrate with 41 ml of phosphate buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was filtered and the filtrate was centrifuged at 10,000 rpm for about 10 min at 4°C in a cooling centrifuge. Supernatant was collected and used for enzyme assay.

2.6 Enzyme Assay

The activity of L-asparaginase was determined by estimating the amount of ammonia liberated from L-asparagine. The method of Imada *et al.*, 1973 [12] was followed.

2.7 Optimization of Process Parameters

The strategy adopted was to optimize one particular parameter at a time and then include it at its optimum value in the next optimization step. The parameters optimized were: temperature, pH, incubation time, inoculum volume, moisture content, carbon source, nitrogen source, metal salts and asparagine concentration.

3. RESULTS AND DISCUSSION

3.1 Screening of Substrates

In the present study, seven substrates, viz. sweet pea peel, cauliflower stalks, corn ear, Bengal gram husk, sorghum, pearl millet, groundnut shells were screened with *Aspergillus terreus* and the results were, as shown in fig.1. All the substrates promoted enzyme production with *A.terreus.*

The maximum L-asparaginase activity of 129.83 U/gds was achieved with corn ear powder as the substrate, followed by cauliflower stalk powder with a yield of 109.33 U/gds,and the lowest activity of 13.66 U/gds was observed in case of sorghum powder.

Hymavathi et al., 2009 reported asparaginase production by isolated Bacillus circulans MTCC 8752 under solid state fermentation using different agricultural materials like red gram husk, Bengal gram husk, coconut, and groundnut cake [13].

V.Varalakshmi, (2013) reported production of L-Asparaginase by *Aspergillus terreus* MTCC 1782 using Bajra seed flour under solid state fermentation [10].

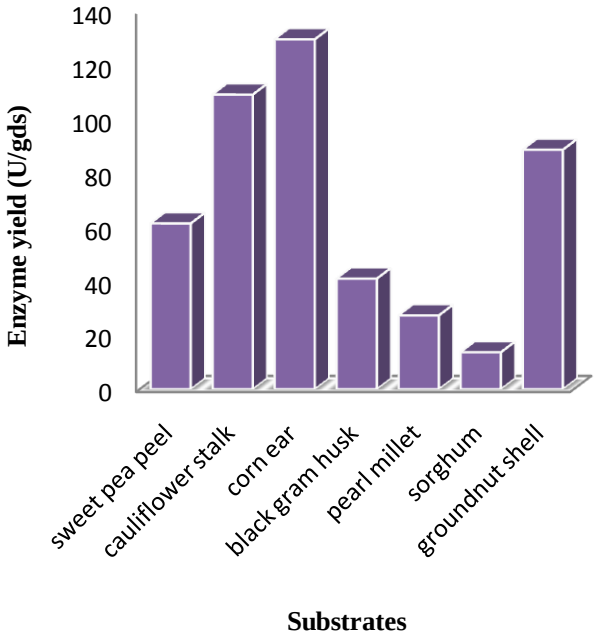

Fig-1: Production of asparaginase using different substrates

3.2 Determination of Substrate ratio with Maximum Yield

The substrates with highest yield from the set of substrates screened for L-asparaginase production were selected. Since, from fig.1, the highest yield was that of corn ear and cauliflower stalk, these two substrates were taken and mixed in different ratios. The various compositions taken were according to the mixture design. The resulting maximum yielding substrate ratio, from fig.2, was 3.75: 1.25 (CS: CE).

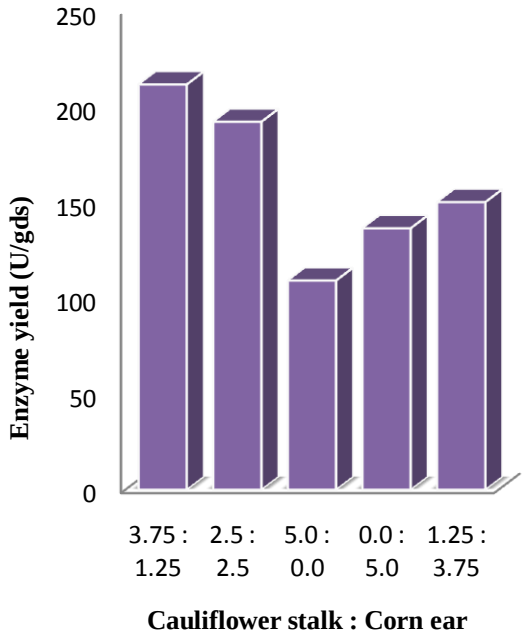

Fig-2: Screening of substrate ratio for highest yield of L-asparaginase

3.3 Optimization of Fermentation Process

Fermentation parameters that influence the L-Asparaginase production during SSF were optimized over a wide range. The parameters considered in the following study were; temperature, pH, time, inoculum volume, moisture content, carbon source, nitrogen source, metal salts, and asparagine concentration.

3.3.1 Effect of Temperature

Five different fermentation temperatures were maintained for the determination of the temperature which leads to highest yield of the enzyme. The different temperatures were: 20°C, 25°C, 30°C, 35°C, and 40°C. The resulting highest yield of 232.33 U/gds was as a result of fermentation at 35°C (as seen in fig.3). K.J.P.Narayana *et al.*., (2008), showed similar result by *Streptomyces albidoflavus* [9]. Shown as optimum for *Paenibacillus validus* and *Bacillus polymyxa*, by Sherifah M. Wakil and Adesewa A. Adelegan, (2015) [14].

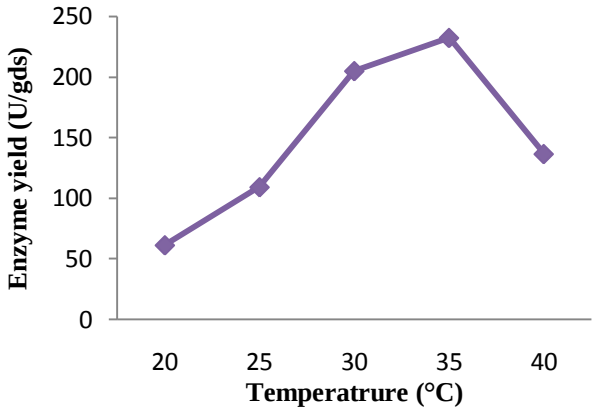

Fig-3: Effect of temperature on L-aspapraginase production

3.3.2 Effect of pH

pH of the extraction buffer plays a key role in the change in enzyme yield. To confirm this relation, pH of the buffer was varied from 6 to 10 and the maximum yield was reported at pH 9.0 which is 246.0 U/gds. Further increase in pH led to decrease in the yield of enzyme. There have been reports on similar pH, which confirm the result; Ashraf *et al.*, (2004) by *Pseudomonas aeruginosa* 50071 [15], Vaishali Dange, Swati Peshwe, (2013) from *A.niger* [16].

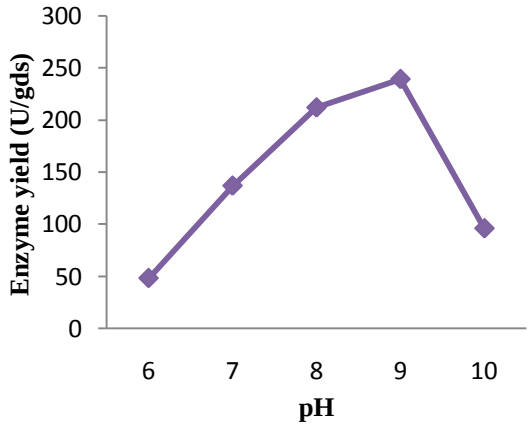

Fig.4 Effect of pH on asparaginase production

3.3.3 Effect of Fermentation Time

Optimum fermentation time for asparaginase production was determined by conducting experiments with the CS: CE substrate ratio using different time intervals from 48h to 144h with a variation of 24h. From fig.5 it can be concluded that there were variations in the enzyme yield with the period of incubation. Analysis of culture supernatant showed enzyme activity rise from an initial of 150.33 U/gds at 48h to its peak activity of 246.0 U/gds at 96h of the enzyme production. Swathi Nageswara *et al.,* (2014), also, reported the highest yield at 96h [17]. Vaishali Dange, Swati Peshwe, (2013), showed production of L-Asparaginase from *Aspergillus niger* [16].

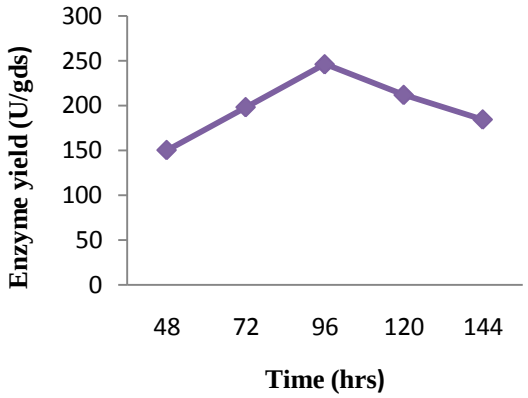

Fig-5: Effect of fermentation time on asparginase production

3.3.4 Effect of Inoculum Volume

The effect of inoculums level on L-Asparaginase production was studied by conduction of the fermentation with different inoculums levels. The substrate was inoculated with culture of 10%, 20%, 30%, 40%, and 50% of inoculum volume in different flasks. The substrate was incubated at 35°C for 4 days. 20% inoculum volume gave the maximum production of L-asparginase with 280.16 U/gds (as shown in fig 6). With the optimum inoculum concentration, there is a balance between the proliferating biomass and availability of nutrients that supports maximum enzyme production.

These results correlate with the results of Swathi nageswara et al., (2014) [17]. V.Varalakshmi, 2013 reported production of L-Asparaginase by *Aspergillus terreus* (1 ml) [10].

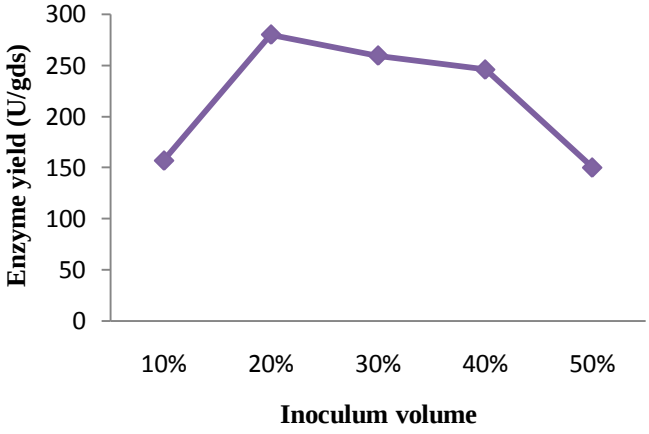

Fig-6: Effect of inoculum volume on asparginase production

3.3.5 Effect of Moisture Content

The effect of different moisture contents of fermentation medium were determined for L-Asparaginase production by maintaining the medium with moisture content range of 20 to 60 % (v/w) with a variation of 10% (v/w). In SSF, microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents (Pandey et al., 1994). The highest enzyme production of 293.83 U/gds was achieved at 50% initial

moisture content. A further increase in the initial moisture content beyond 50% resulted in a significant reduction in the enzyme production. A.R Soniyamby *et al*., (2011), produced the enzyme from *Pencillium* sp. [18]. Swathi Nageswara *et al*., (2014) gave similar optimum value [17].

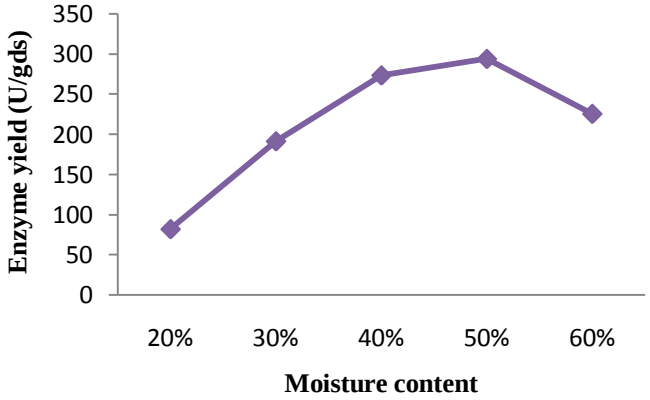

Fig-7: Effect of moisture content on asparaginase production

3.3.6 Effect of Carbon Source

Different carbon sources were taken and added to the fermentation medium, as supplements, for enhancing the production of L-asparaginase. The selected carbon sources lactose, glucose, sucrose, maltose, and fructose, were added to the fermentation medium at 1% (w/v). All carbon sources showed appreciable amounts of hike in the enzyme yield with highest at 382.66 U/gds shown by sucrose.

Chidambram.K.V *et al.,* (2009) showed supporting results [19]. Susmita.S et al., (2012) added sucrose as a supplement for asparaginase production [20].

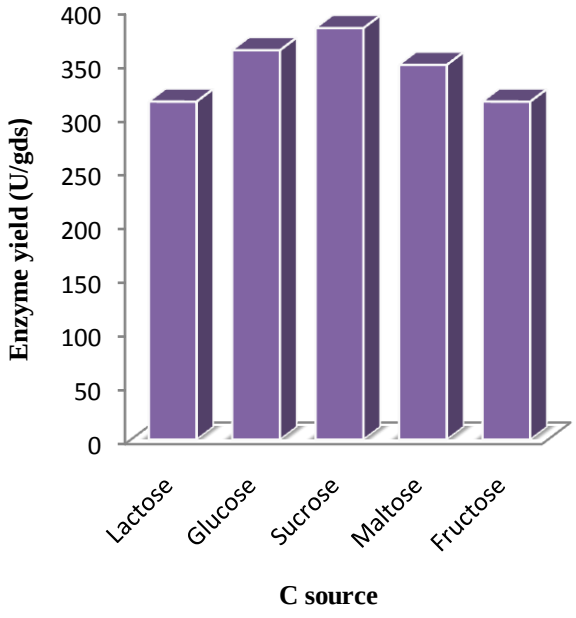

Fig-8: Effect of Carbon source on asparaginase production

3.3.7 Effect of Nitrogen Source

Peptone, yeast extract, ammonium sulphate, ammonium nitrate, and sodium nitrate were added to the substrate at 1% (w/v), to determine the change in enzyme yield. Ammonium sulphate gave the highest yield with 444.16 U/gds, followed by sodium nitrate with 393.6 U/gds.

Ammonium sulphate acts as the optimum N source; Elizebeth.T *et al.,* (2014) [21] and Indira et al., (2015) [22].

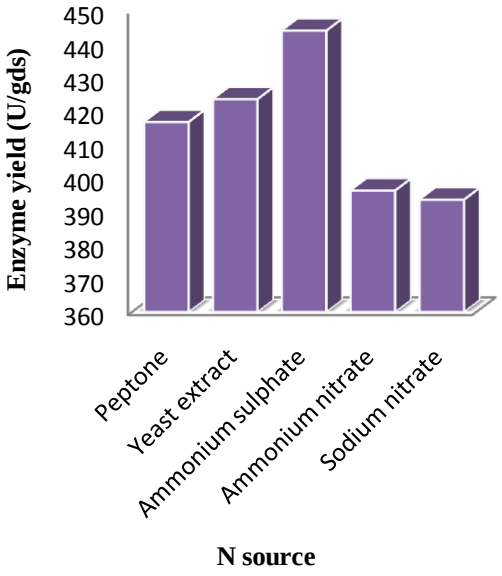

Fig-9: Effect of Nitrogen source on asparaginase production

3.3.8 Effect of Metal Salts

Metal salts act as supplements in the production of L-asparaginase. Among magnesium sulphate, zinc sulphate, sodium chloride, calcium chloride, potassium chloride, as the metal supplements, highest yield recorded was by sodium chloride with 498.83 U/gds (as shown in fig.10). Debajit borah *et al*., (2012) also screened NaCl as the optimized metal salt [23]. Thenmozhi C et al., (2011) produced by mangrove derived *Bacillus sp.* [24].

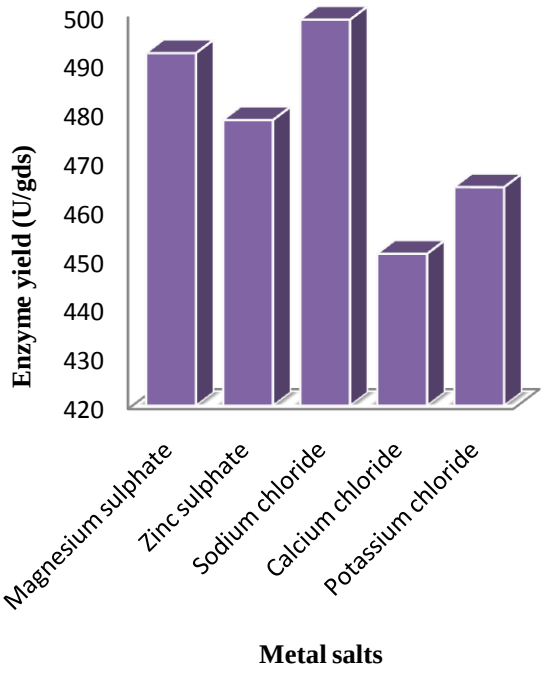

Fig-10: Effect of metal salts on asparaginase production

3.3.9 Effect of L-Asparagine Concentration

Different concentrations of asparagine were added to the fermentation medium, to determine the effect it shows on the enzyme yield, if any. 0%, 0.5%, 1.0%, 1.5%, and 2.0% (all in w/w) were added to the medium. The highest yield was obtained at 1.0% w/w of asparagine in the fermentation medium, with 567.16 U/gds. The enzyme yield, however, showed an inversely proportional relation with increase in asparagine concentration thereafter. K.J.P Narayana *et al.*, (2007) [9] and Rachna Goswami *et al.*, (2014) [25], also showed similar relation between L-asparagine concentration and L-asparaginase production.

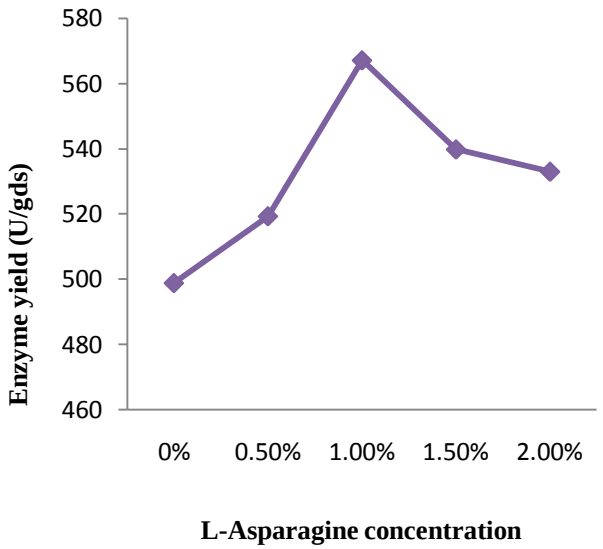

Fig-11: Effect of L-Asparagine concentration on L-asparaginase production

4. CONCLUSION

The results are encouraging for production of L-asparaginase from *A.terreus* MTCC 1782 with a mixed ratio of corn ear and cauliflower stalk .The choices of substrate stand justified by the resulting enzyme yield. The optimization was done using a 'one-factor-at-a-time' approach. The study of effect of process parameters and its optimization was helpful in raising the potential yield of the enzyme four folds, from an initial 129.83 U/gds by corn ear to an intermediate 211.83 U/gds with 3.75:1.25 substrate composition to a final yield of 567.16 U/gds. Further work, in this regard seems encouraging.

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