SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

L-Asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is an enzyme which converts L-asparagine to L-aspartic acid and ammonia. L-asparaginase belongs to an amidase group that hydrolyses amide bond in L-asparagine to aspartic acid and ammonia. L-asparaginase constitutes one of the most bio-technologically and bio-medically important group of therapeutic enzymes accounting for about 40% of total world-wide enzyme sales. Global requirements of anti-leukemic and anti-lymphoma agents are far greater than those of other therapeutic enzymes of which asparaginase contribute one third. This enzyme has been intensively investigated over the past two decades owing to its great importance as an antineoplastic agent. L-asparaginase enzymes catalyse the hydrolysis of L-asparagine to L-aspartate and ammonia, and to a lesser extent the hydrolysis of L-glutamine to L-glutamate.

Among the major human diseases, cancer is a most dangerous disease; cancer the very name alone scares most people. The cancers are smart and cagey buggers. They can hide and grow for a good spell undetected. It is the second biggest disease of the human beings. The number of deaths due to cancer in developed countries is three times greater than the number (85 per 100,000 populations) in developing countries. As per World Cancer Report, cancer rates could further increase by 50% to 15 million in 2020.

Many enzymes have been used as drugs to treat cancer, likewise L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic

leukemia and other kinds of cancer in man L-asparaginase breaks down (disassembles) the amino acid Asparagine which is needed for cell maintenance and growth. In many cases of leukemia cells are unable to make their own asparagine and must rely on outside source of asparagine for survival. By depleting free asparagine in the body, which is necessary for cancer cell, results in a depletion of cancerous cells while normal cells are more likely to be preserved. Therefore, the discovery of new L-asparaginase is utmost important. Traditionally, the L-asparaginase is being produced by means of submerged fermentation, but there are scanty reports on the production of L-asparaginase through submerged fermentation (Smf) by employing *Penicillium sp*. Therefore, in the present study attempts have been made on the suitability and utility of synthetic medium as fermentation medium for the production of L-asparaginase by employing locally isolated strain of *Penicillium Sp.*

The summary of the results obtained and the conclusions arrived at on the basis of these results presented in the present thesis are briefly outlined as below:

1. **The Chapter-1** includes a brief introduction to the research undertaken, wherin the necessity of undertaking the work is justified and the aims and objectives of the study are specified.
2. **In chapter-2** an exhaustive review of the relevant aspects of L-asparaginase chemistry and historical aspects, the characteristics of the L-asparaginase and medium, etc., are presented and also an attempt has been made to evaluate the different medium were used in fermentation for production of L-asparaginase.

3. **The chapter-3** deals with the materials used and methodology adopted in the present study. The aspects covered are mainly-Isolation, Screening, Confirmation by thin layer chromatography, Submerged fermentation procedure and methodology adopted to optimize the fermentation parameters, purification of L-asparaginase as well as process economization through nutrient supplementation, mutation of *Penicillium sp* KGSM05 for maximum production of L-asparaginase and assay of anti-tumor activity.
4. **In the chapter-4** the results obtained during the study are presented. The results are indicated in brief as below:
   i. The isolates of *Penicillium* were initially subjected for L-asparaginase production through paid plate assay by evaluating their pink zone of clearance around the colony. All thirty (30) isolates exhibited pink zone. Out of these 30 isolates, *Penicillium sp* KGSM05 exhibited pink zone of diameter 0.98cm and hence the same was considered as the promising strain for L-asparaginase production.
   ii. The results on the confirmation of L-asparaginase production from the isolates were using TLC method are presented. On the basis of Rf values of aspartic acid, confirmed the L-asparaginase production by *Penicilium sp*.
   iii. The success and direction of fermentation depends on obtaining a proper balance between the components of the synthetic medium, the process itself and the fermenting organisms. Hence, optimization of submerged fermentation parameters like intial pH of the substrate, the ambient temperature and the inoculum's size, on L-asparaginase production was

carried out. Once a parameter was optimized, the optimum level of the parameter was continued in the next step of experiment:

a) The studies reveal that the optimum fermentation period required for production for production of maximum L-asparaginase from synthetic medium was 72hrs by employing *Penicillium sp* KGSM05.

b) The maximum production of L-asparaginase by the organism was observed at pH of 6.0, temperature of 35^0c, inoculum size of 1.0ml ($1X10^7$spores/ml) were observed as optimum for maximum production of L-asparaginase using synthetic medium.

c) In the studies dealing with the process economization, attempts have been made to improve the production of L-asparaginase during submerged fermentation by using synthetic medium through supplementation of various nutrients and also by mutation of the fermenting organism.

   i. In the studies involving nutrient supplementations, the synthetic medium was amended with varying concentrations of different carbon sources, organic and inorganic nitrogen, and metal ions individually as below:

   ii. Amongst the carbon sources tested for L-asparaginase production glucose proved to be beneficial. The optimum concentration of glucose needed to be supplemented to the synthetic medium to yield maximum L-asparaginase production by *Penicillium sp* KGSM05 was observed to be 1.0%. At this level of glucose supplementation, *Penicillium sp*

KGSM05 produced 177 IU of L-asparaginase at 72 hrs of fermentation.

iii. The optimum concentration of organic nitrogen needed to be supplemented to the fermenting medium to cause maximum L-asparagnase production by *Penicillium sp* KGSM05 was observed to be 1.0%. At this level of yeast extract supplementation, *Penicillium sp* KGSM05 produced 215 IU 72 hrs of fermentation.

iv. Amongst the inorganic nitrogen sources tested for L-asparaginase production ammonium sulphate proved to be beneficial. The optimum concentration of ammonium sulphatet needed to be supplemented to the synthetic medium to effect maximum production of L-asparaginase by *Penicillium sp* KGSM05 was observed to be 1.0%. At this level of ammonium sulphate supplementation, *Penicillium sp* KGSM05 produced 220 IU at 72 hrs of fermentation.

v. Amongst all the metal ions tested for maximum L-asparaginase production zinc sulphate proved to be beneficial. The optimum concentration of zinc sulphate needed to be supplemented to the carob pod substrate to effect maximum L-asparaginase production by *Penicillium sp* KGSM05 was observed to be 0.01%. At this level of zinc sulphate supplementation, *Penicillium sp* KGSM05 produced 225 IU at 72hrs of fermentation.

vi. Induction of mutation was attempted by UV rays treatment at distance of 15cm for 10min. The mutant strain exhibited zone of clearance of

0.95cm on rapid plate assay. Based on the performance of the strain obtained on rapid plate assay, the strain *Penicillium sp* KGSM05 *mu* was selected for further studies. The results obtained after solid state fermentation revealed that the mutant strains yielded 231 IU of L-asparaginase when compared to the parent strain *Penicillium sp* KGSM05 which has yielded 167 IU in synthetic medium for 72 hrs fermentation.

vii. The results revealed that the crude enzyme extract showed highest specific enzyme activity of 3.77 U/mg proteins, whereas ammonium sulphate precipitation, Sephadex G-75 (gel filtration) and CM-cellulose (Ion-exchange) chromatography showed highest specific enzyme activities of 4.75 U/mg proteins, 7.4 U/mg proteins and 50 U/mg proteins respectively. In the ammonium sulphate precipitation method about 1.25 fold purity with 20.24% recovery of proteins. The CM-cellulose (Ion-exchange) and Sephadex G-75 gel filtration chromatography showed 13.23 and 1.89 fold purity with 22.22% and 77.77% recovery of proteins respectively.

viii. The molecular weight of L-asparaginase was determined to be 29kDa.

ix. The enzyme characteristics indicated that the enzyme is optimum at alkaline pH of 6.0 and the optimum temperature was found to be 37^0C. The purified enzyme activity was induced up to 2200% by $CuSO_4$, and completely inhibited by Mg^{2+}, Zn^{2+}, Cd^{2+}, Hg^{+} and Fe^{+}. The enzyme was possibly confirmed as metallo-enzyme because of the inhibition of

its activity in presence of 1, 10-phenanthroline and EDTA. The LB-plot developed by plotting 1/[S] against 1/[V] displayed a Vmax of 4.2 IU/ml with a Km of 1.44M.

x. MTT assay for anti-tumor activity showed that purified sample of L-asparaginase is toxic to MCF-7 cell line and IC_{50} for the given sample of L-asparaginase is 318.190µg/ml.

5. **In Chapter-5** a detailed discussion of the findings of the present work is made with reference to the works done by the earlier workers in this field. The present study indicated that the synthetic medium (production medium) is suitable for the production of L-asparaginase by employing *Penicillium sp* KGSM05 strain under submerged fermentation.
6. The references referred in the present thesis are included in the Bibliography section at the end of the thesis.