Project: Studies on production, process optimization, kinetics and clinical application of fungal asparaginases

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Executive Summary :

L-asparaginase has been used as anti-tumour agent for the effective treatment of acute lymphoblastic leukemia. At present, microbial asparaginases are recognized as one of basic drugs for improvement of remission induction mainly in infant acute lymphoblastic leukemia and are being positively incorporated in the treatment of high risk diseases such as intractable acute lymphoblastic leukemia and the myelogenous recurrence.

In the present studies the main objective was to explore new source of the therapeutic L-asparaginase producing microorganism. In the present investigation, primary and secondary screening of bacteria, fungi and actinomycete for asparaginase production has been carried out. 26 different bacterial species, 17 fungal cultures and 10 actinomycete cultures were screened for L-asparaginase production. Enzyme assay was carried out for selection of maximum L-asparaginase producers. The isolates exhibited extracellular enzyme activity. The bacterial culture SVB1 (288.3506 IU), actinomycete culture Act1 (275.3172 IU) and fungal culture SVF4 (304.4983 IU) were screened as maximum L-asparaginase producers and identified as *Alcaligenes fecalis, Streptomyces Sp*. and *Aspergillus niger* using 16S rDNA, Slide culture technique and 28S rDNA technique respectively.

The studies of nutritional parameters on the growth of the selected L-asparaginase producing isolates revealed that maximum L-asparaginase production with the three isolates was obtained in stationary condition as compared to shaking condition. The optimum pH for all the three isolates was found to be pH 9.0 while the optimum temperature was found to be 30^0C. Among these isolates fungal strain *Aspergillus niger* was found to be the maximum L-asparaginase producing isolate.

Extensive literature survey carried out during the studies revealed that various side effects are associated with the administration of therapeutic bacterial L-asparaginase, therefore the present studies were further confined to fungal L-asparaginase. Hence the optimization of fermentation parameters was carried out for production of L-asparaginase production using the fungal isolate *Aspergillus niger*.

An economic culture medium for *Aspergillus niger* was formulated by using MINITAB 14 software and using Plackett Burman Statistical design. The most effective nutrient from each category was identified based on the result obtained after subjecting the data to statistical analysis. The

optimization studies showed more than threefold improvement in L-asparaginase production using SSF. The enzyme activity was slightly higher when dextrin & maltose was used rather than mannitol, sucrose, fructose & cellulose. Among the different salts, $CaCo_3$ was found to be the best one, among the inorganic nitrogen sources NH_4Cl, among the amino acids L-asparagine were found to be best among different nutrients.

L-asparaginase was partially purified by ammonium sulfate precipitation. Desalting was carried out by Dialysis and further purification was achieved by gel filtration using Sephadex G-150 and Sephadex G-25. In the final step, the purity of enzyme precipitation was found to be 158.46-fold pure and the final recovery of protein was 43.57 per cent.

Molecular weight of purified L-asparaginase was determined by SDS-PAGE and it was found to be approximately 136 kDa.The characterization of the enzyme revealed an optimum at pH 9.0.. The optimum temperature for L-asparaginase activity was found to be 37^0C which is the physiological temperature. This property of enzyme is most suitable for complete elimination of asparagines from the body when tumor patient is treated with L-asparaginase *in-vivo*. The effect of metal ions revealed that the enzyme contains free –SH group at the active site because of its sensitivity to mercury. EDTA did not affect the enzyme activity suggesting that the enzyme is not a metalloprotein. The enzyme was found to be stable in presence of human serum *in-vitro*. The studies of enzyme activity in presence of the product revealed that it is not inhibited by feedback mechanism.

The Km value of the enzyme was found to be 0.002×10^{-5} M. The immobilization studies using various carriers revealed sodium alginate as a support for the production of L-asparaginase by *A.niger*. However the immobilization does not improve its Km or alter the temperature and pH optima.

Any therapeutic drug should be pyrogen free. Therefore in order to evaluate the endotoxin limit the LAL test was performed. It was found to be less than 0.125 EU/ml, which is well within the prescribed limit hence it is concluded that the preparation is pyrogen free.

In order to study the anti-leukemic effect of L-asparaginase, the MTT and SRB assay was carried out using A431 tumor cells, human leukemia cell line Molt-4 and human breast cancer cell line MCF7.
In vitro testing indicated that fungal L-asparaginase along with its crude extract was safe; therefore this fungal strain has potential for use in therapeutic applications. The results clearly showed that the enzyme had strong antioxidant activity. The results showed that L-asparaginase has anti-proliferative activity in different cell lines growth *in vitro* (antitumor activity against A431 tumor cells, human leukemia cell line Molt-4 and human breast cancer cell line MCF7).

From the above results it would be possible to suggest that L-asparaginase prepared from *Aspergillus niger* is superior to the commercially available drug, and hence can be recommended to replace this drug by *Aspergillus niger* L-asparaginase for the treatment of leukemia.