Chapter 7

Summary and Conclusions

The L-asparaginases are the amidohydrolases that have broad biotechnological significance due to their diverse applications in the food and medical sectors. But, the current L-asparaginase preparations available in the market suffer from several drawbacks, including immunological issues in terms of usage and the high price in terms of cost. The enzyme can be produced in both the solid-state and submerged production processes. The present research aimed to address and overcome the pitfalls associated with the current L-asparaginases.

Considering the cost challenge, the first part of the present research was focused on the reduction of the production costs of the L-asparaginase enzyme. The production of the L-asparaginase enzyme involves the utilization of expensive carbon (glucose) and nitrogen sources (yeast extract, peptone, and tryptone) that can significantly increase the production cost during the production process. So, there is certainly a need to lower the expensive production costs of the L-asparaginase enzyme preparations by utilizing cheaper and readily available substrates. The production of L-asparaginase was performed in both the submerged (SmF) and solid-state fermentation (SSF) processes. In the submerged production, corn steep liquor was utilized as a cheaper nitrogen source, and in the solid-state production process; a waste of the food processing industry in the form of niger de-oiled cake (*Guizotia abyssinica*) was utilized for growth and L-asparaginase production. In the SSF study, the agro-substrate Niger de-oiled cake exhibited a lower C/N content on elemental (CHNS) analysis when compared with different agro-substrates and, thus resulted in the higher production of L-asparaginase enzyme. The C/N content was found to be lower than commonly utilized brans (wheat bran, maize bran), which has been reported for L-asparaginase production in other

studies. The present study is the first to experimentally report through CHNS analysis that the L-asparaginase production is positively influenced by the lower C/N content of the agro-substrate. Moreover, the substrate can be utilized as a sole source (without the addition of supplements) for the L-asparaginase production. Enzyme activity of 34.65 ± 2.18 IU/gds was achieved at an autoclaving period of 30.3 min, moisture content of 62%, temperature 30 ^{o}C and 6.2 pH using machine-learning based artificial neural network (ANN) technique. In comparison to the response surface methodology approach (RSM), the ANN approach performed better in predictive capabilities even with limited number of experiments. Considering the extremely low cost of niger de-oiled cake in comparison to the costly semi-synthetic medium requirements, enormous amounts, easy availability and inherent nutritional constituents, utilization of niger (*Guizotia abyssinica*) de-oiled cake for the production of L-asparaginase offers a promising and reliable process to cater to the demands of L-asparaginase application in pharmaceutical and food industries.

Considering the usage-associated challenges that resulted from the multifarious side-effects of commercial L-asparaginase formulations, there are existing requirements for new alternative L-asparaginase preparations that possess the desired properties and must carry out the apoptotis of the cancer cells. The search for new L-asparaginase preparations has sparked the interest to explore new microbial sources that produce L-asparaginase enzyme with significant anti-cancer potential against leukemia cells and also possess low/negligible L-glutaminase activities. The present study reports a new and safe L-asparaginase preparation from *Bacillus indicus* that causes potent cytotoxicity and apoptotis in leukemia cells. The purified L-asparaginase from *Bacillus indicus* MTCC 4374 showed excellent cytotoxicity against acute lymphoblastic leukemia cells (MOLT-4 cell line) with an IC_{50} of 1.21 μM. The cytotoxicity shown by the purified L-asparaginase was found to be dose-dependent and confirmed by carrying out live/dead cell assay using acridine orange/propidium iodide dual

staining. The standard anti-leukemic drug doxorubicin showed an IC_{50} of 6.4 µM. The purified L-asparaginase was able to induce the apoptotic cell death mechanism in MOLT-4 leukemia as confirmed using Annexin V/propidium iodide in flow cytometry studies. The purified L-asparaginase treatment resulted in the apoptotic cells percentage of 21.4%, while the standard drug doxorubicin resulted in an apoptotic cell percentage of 14.84%. The higher cytotoxicity of leukemia cells was achieved by the purified L-asparaginase in comparison to the standard doxorubicin. Moreover, the preparation is highly specific towards the substrate L-asparagine by possessing significant L-asparaginase activity with a very low K_m value. The K_m and V_{max} kinetic parameters were 0.287 mM and 2.178 IU/µg respectively. The turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) were evaluated to be 1263.24 s^{-1} and 4.40×10^3 mM^{-1} s^{-1}. This low value of K_m obtained with the *Bacillus indicus* MTCC 4374 L-asparaginase is a favorable aspect as it signifies the high binding affinity of the enzyme to the substrate L-asparagine and is also required for the specific removal of L-asparagine to target the leukemia cells. The study reports the bench-scale production process using cost-effective substrates, viz. sucrose as a carbon source instead of glucose and corn steep liquor as a nitrogen source in place of expensive peptones and yeast extract. The production was carried out using a combination of Plackett Burman design for screening of significant medium components followed by the central composite experimental design for the optimization of the medium concentration and enzyme activity of 15.34 ± 0.24 IU/mL was observed. The scale-up of the production in a 3.7 L stirred bioreactor resulted in 16.42 ± 0.17 IU/mL of L-asparaginase enzyme activity. The outcomes obtained are beneficial for the industrial production of the enzyme at a low-cost. The crude extracellular enzyme was purified to homogeneity using anion exchange chromatography followed by gel filtration chromatography. A single band of approximate 35 kDa molecular weight was obtained on SDS-PAGE, while native PAGE analysis confirmed it to be a tetramer of 4 identical subunits.

The circular dichroism (CD) spectra demonstrated that the purified enzyme belongs to the mixed (α + β) class of proteins as it contains 38.7 % alpha helix region and 27.4 % beta sheets region. The current study evidently revealed the tremendous anti-leukemic potential of L-asparaginase from *Bacillus indicus* MTCC 4374 for utilization as a potent anti-leukemic agent. Conclusively, the L-asparaginase of *Bacillus indicus* is a highly promising candidate that can be introduced as a new enzyme therapeutic against various leukemia disorders.