Chapter 1

Introduction and Objectives

1.1 Introduction

Enzymes as therapeutic agents have immense potential due to their important attributes of specificity and high level of activity. Specific binding between the enzyme and target molecules make them potent drugs having the ability to perform diverse biochemical reactions that other drug molecules cannot. Among the innumerable therapeutic drugs, L-asparaginase is an indispensable enzyme that plays versatile roles ranging from the therapeutic drug to the food processing agent. L-asparaginase (EC 3.5.1.1) is an amidohydrolase enzyme that catalyzes the hydrolysis of the amide bond in the L-asparagine amino acid to form L-aspartic acid and ammonia. The threonine residues in the active site play a critical role in the catalytic reaction mediated by the L-asparaginase enzyme. In the characteristic L-asparaginase reaction mechanism, these threonine residues can act as a primary nucleophile by attacking the C atom of the amide substrate, resulting in the formation of the product L-aspartate and liberation of ammonia (Aghaiypour *et al.*, 2001; Sanches *et al.*, 2007). These active site threonine residues are found to be highly conserved among the L-asparaginase family (Gesto *et al.*, 2013; Sharma *et al.*, 2019). The enzyme is present in many genera, including bacteria, fungi, plants and animals. Yet the reported studies are primarily centred on the L-asparaginase production from bacterial sources owing to the higher growth rates and higher productivities in a short duration of time. Also, the research studies on L-asparaginases from different sources have reported that the enzyme can be produced under both solid-state and submerged production conditions. Structurally, the bacterial asparaginases are tetrameric in the molecular weight range of 120-160 kDa and consist of 4 similar polypeptide subunits while the L-asparaginase from fungal sources are trimeric in the molecular weight range of 75-120 kDa. Each of the monomeric chains consists

of a larger N-terminus domain and a smaller C-terminus domain connected by the flexible loop (Ran *et al.*, 2021). The presence of a highly flexible loop region is a classical feature of L-asparaginases that acts as a cap to the active site (Lubkowski *et al.*, 1996; Maggi *et al.*, 2017). This flexible loop was deeply studied in the L-asparaginases of *Escherichia coli* and *Erwinia chrysanthemi*. A presence of a flexible loop makes the size of the active site quite large and provides no restriction for the movement of substrate/product (Aung *et al.*, 2000).

The exploration of the anti-cancer potential of L-asparaginase started with studies on the lymphoma bearing mice. The mice, when administered with guinea pig serum, showed complete regression of lymphomas. Serum from other animal species was devoid of anti-lymphoma activity (Kidd, 1953). The earlier studies proved that the substance accountable for the anti-lymphoma effects in guinea pig serum was the enzyme L-asparaginase (Broome, 1961; Broome, 1963). Later, research showing the anti-cancer potential against diverse leukemia types was reported (Capizzi *et al.*, 1970; Tallal *et al.*, 1970). Thus, the enzyme showed significant anti-cancer potential against leukemia and lymphomas. The mechanism by which the cancer cells were being targeted by the L-asparaginase enzyme was reported in some studies. Malignant lymphoblasts in comparison to normal cells have a nearly complete absence of L-asparagine synthetase activity resulting in diminished endogenous production of L-asparagine amino acid (Haskell and Canellos, 1969). Thus, these leukemic lymphoblast cells become dependent on extracellular asparagine from blood serum for their viability and proliferation (Stams *et al.*, 2003). Administration of L-asparaginase hydrolyzes extracellular asparagine leading to asparagine starvation of leukemic lymphoblast cells. This asparagine depletion results in suppression of protein synthesis or the inhibition of mechanisms by which aspartate is accumulated (Broome, 1981). The utility of L-asparaginase was not limited to the medical sector, and the enzyme also finds acceptance in the food industry as a food

processing aid. The enzyme showed acrylamide mitigation potential and has been given GRAS (Generally regarded as Safe) status by the FDA (Jia *et al.*, 2021).

Despite the tremendous potential shown by the L-asparaginase enzyme, the current L-asparaginase preparations suffer from several drawbacks including immunological issues in terms of usage and the high price in terms of cost. Considering the expenses incurred in the enzyme production processes, the initially required substrates or raw materials account for up to 30 percent of the total production costs. A similar nature is also seen in the production process of L-asparaginase enzyme in which expensive carbon (glucose) and nitrogen sources (yeast extract, peptone, and tryptone) are utilized for the production resulting in the extremely high cost of L-asparaginase preparations in the market. So, there is undoubtedly a requirement to lower the expensive production costs of the L-asparaginase enzyme preparations. Agro-industrial residues and their by-products are nutrient-rich raw materials that have a tremendous potential for the enzyme production processes. Moreover, they are inexpensive and inexhaustible sources available in huge quantities throughout the year. Their utilization for different biotechnological production processes also solves the agro-waste disposal problem that would otherwise be dumped in landfills or burnt in the open fields leading to pollution of diverse nature.

Secondly in terms of usage, the utilization of current therapeutic formulations leads to several immunological side-effects including hepatotoxicity, nephrotoxicity, pancreatitis, coagulative abnormalities and many others. (a) All these immunological side issues arise due to the high intrinsic L-glutaminase co-activities associated with current L-asparaginases. The same L-asparaginase enzyme in addition to L-asparagine hydrolysis also breakdown L-glutamine and up to 10% intrinsic L-glutaminase activities have been reported. (b) The treatment regimen requires multiple different types of L-asparaginases as the enzyme preparation from the single microorganism will be rapidly cleared during the subsequent doses by the host immune

system. All these associated side effects indicate the urgent requirement of new L-asparaginase preparations that show anti-leukemic properties and also show the desired kinetic properties with minimal or no side effects. The therapeutic efficacy of the L-asparaginase preparation must be checked against leukemia cells and other related types of cancer as L-asparaginase is specifically used for the treatment of acute lymphoblastic leukemia (ALL).

The aim of the present research was to address and overcome the pitfalls associated with the L-asparaginase production processes and its utilization. In the present study, the L-asparaginase enzyme production was carried out through both the submerged (SmF) and solid-state fermentation (SSF) routes. In both production processes, low-cost nutrient sources are explored in place to expensive nitrogen sources with the aim to lower down the productions costs. Secondly, owing to the side-effects shown by the current L-asparaginase preparations, there is a need for new L-asparaginase preparations that must demonstrate the anti-cancer effects. In view of this challenge, a new and safe microbial source was explored for L-asparaginase production. The new purified L-asparaginase preparation demonstrated excellent therapeutic potential against acute lymphoblastic leukemia cells in-vitro and exhibited desired kinetic properties.

1.2 Objectives:

- Exploration of cheaper agro-industrial nutrient sources for the L-asparaginase production process.
- Exploration of a new microbial source for the production and purification of L-asparaginase preparation with desired kinetic properties.
- Evaluation of the anticancer potential against leukemia cells by the purified L-asparaginase.