MANGLICOLOUS ENDOPHYTIC FUNGI AS A SOURCE OF L-ASPARAGINASE

Abstract

Mangroves are a fluctuating intertidal zone between land and marine habitats. They exhibit high levels of biodiversity along tropical and subtropical coastlines as they embrace both these dynamic environments. Since mangrove forests remains the second largest nesting ground for marine fungal community, they are always regarded as biodiversity "hotspots." The mycotic inhabitants of mangroves, especially endophytes, are adapted to changing environmental conditions and can withstand a variety of physical and biological stressors, making them promising sources for the recovery of novel bioactive chemicals. Additionally, several mangrove species are useful sources of secondary metabolites with therapeutic benefits. As a result, the pharmaceutical, food, and agricultural industries have recognized extensive potential use for secondary metabolites generated from mangrove endophytic fungus. L-asparaginase is a renowned chemotherapeutic drug that is widely used in the treatment of acute lymphoblastic leukaemia (ALL), the most common pediatric cancer. This enzymes selectively differentiates normal cells and promotes apoptosis of only cancerous cells due to their mode of action. Fungal asparaginases being eukaryotic appears more promising in light of the increased similarity to human beings, thereby minimalizing the immunological side reactions. Another advantage of L-asparaginase is its application in food and processing industry for reducing acrylamide production in fried and processed food meals with high starch contents. To identify asparaginase production, extensive screening and quantification approaches have been developed, however, the majority of them have shown to be more complicated and deficient in established pharmaceutical quality control standards. To get around

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these restrictions, this review provides information on straightforward screening techniques using modified Czapek Dox medium incorporated with phenol red and bromothymol blue as dye detectors, a well reasonable, simple and accurate colorimetric techniques for enzyme quantification (Nessler method, L-aspartic acid - hydroxamate (AHA), and indo-oxine). It is also worth noting that this method is better suited for assuring accurate and representative asparaginase activity in research. Finding innovative remedies is critical in today's world, where cancer and other terrible diseases are so prevalent. Despite the fact that asparaginase production from medicinal plants has been extensively studied, this review will look at the potential benefits of using marine fungi associated with mangroves as a tool for biotechnological, therapeutic, and industrial applications.

Key Words: Endophytic fungi;Mangroves; lymphoblastic leukaemia; L-asparaginase

I. INTRODUCTION

Marine fungi form an ecological group that is either obligate or facultative. Among them, Strict obligates mutiply and sporulate solely in marine environment, whereas facultative fungi thrive in freshwater or terrestrial surroundings and migrate to marine environment for sporulation under varying physiological conditions (Raghukumar 2008). Out of the expected fungal diversity of 1.5 million species, only a very minor percentage of about 74,000 fungal species was identified so far. This is mainly due to lack of exploration from unexplored and underexplored habitats, for example, marine fungi from sediments and other related associations (Rateb and Ebel 2011).

These marine fungi are mainly important in coastal systems, as decomposers of vascular plants and are also found among the “symbionts”, the “parasites” and the “super parasites”. (Sieburth 1979). The fluctuating environmental parameters in marine ecosystems, such as a change in pressure, temperatures, salinity, and relative oxygen amount (Raghukumar 2008) induce great challenges. The endophytic organisms that are inhabitants of these extreme habitats got adapted and display metabolic plasticity thereby producing novel biometabolites that can be a promising drug lead. Therefore, many researchers are interested in bioprospecting the newly emerging group of association, endophytic fungi that are less investigated in the marine field.

Because of their peculiar environment, plant endophytic fungi associated with mangrove plants are of particular interest. Mangroves are halophytes that grow in the intertidal zone of tropical or subtropical climates. Mangrove plants exhibit significant biodiversity along the tropical and subtropical coastlines because they embrace both marine and terrestrial habitats. Furthermore, mangrove forests are regarded as biodiversity "hotspots," and dwelling place for the second biggest fungal assemblage among marine fungi (Thatoi et al. 2012). The tremendous richness seen in mangrove species emphasizes the relevance of studying these plants as part of a series of studies on fungi connected with marine plants (Kohlmeyer and Volkmann-Kohlmeyer 1991).

Various natural metabolites are reported from mangrove associated fungi including antimicrobial, antiphlogistic, antitumor, antituberculosis, and antimalarial activities (Gunatilaka 2006; Hazalin et al. 2009; Gordien et al. 2010; Elfita et al. 2011; Ahmed et al. 2011; Mohana et al. 2012; Praptiwi et al. 2013; Zhang et al. 2014). Moreover, endophytes are valuable source of various extracellular enzymes with potential biotechnological applications (Fiáková et al. 2007; Pimentel et al. 2011). This includes numerous hydrolytic and oxidative enzymes; such as amylase, cellulase, lipase, chitinase, pectinase, tyrosinase, protease, ligninase and DNAases (Bonugli-Santos et al. 2015). These extracellular hydrolytic enzymes actively contribute in litter degradation of mangrove soil bed (Kumaresan and Suryanarayanan 2002). This knowledge paves way for the role of the enzymes and promote the significance of mangroves are one of the most productive natural ecosystems and breeding ground for marine organisms (Kohlmeyer and Volkmann-Kohlmeyer 1998).

Exploration for enzymes, like asparaginase and tyrosinase has recently augmented due to their medicinal value in cancer therapy, and in treatment of neurodegenerative and myocardial diseases. Among them, L-asparaginase is the pioneer and largest group of anticancer agent widely employed in the treatment of leukemias especially Acute

Lymphoblastic Leukaemia (ALL) and in related non-Hodgkin's lymphomas (Kumar and Sobha 2012). Yet another exciting application of L-asparaginase is its role in food processing industry to lower acrylamide in fried and oven-cooked meals. This enzyme is produced by diverse source of organisms including plants and animals, to bacteria, and fungi. As extraction of L-asparaginase from eukaryotic cells is very challenging and expensive, the best-known source was from bacterial origin chiefly *E. coli* and *Erwinia carotovora.* But, in most cases, it ends up with solemn medical complications like sensitized immunologic responses, cytoxicity, and even death. Additionally, bacterial asparaginases are often associated with glutaminase, and triggers blood sugar level, disrupts the homeostatic balance, and lipid metabolism, and create nervous disorders (Geuenich et al. 1998; Duval et al. 2002; Kumar et al. 2011b). Moreover, another concomitant urease leads to hydrolysis of urea in blood and causes toxic reactions, also demands additional purification steps needed to maintain the drug efficacy.

Due to this hypersensitive allergic side effects linked with the currently used bacterial sources mainly *E. coli* and *Erwinia carotovora*, there is a requisite to switch to new candidates with with comparatively less sideeffects and high yield of L-asparaginases. Nowadays, endophytic fungi gains much attention because of their efficacy in producing diverse metabolites including enzymes. The main reason for studying endophytic fungi is that they are phylogenetically related to the host and are non-immunogenic. Being eukaryotic their post-translational modifications are the same and considered an unexplored source of novel drugs. As fungal endophytes reside in the host as a mutualistic partner, they may share some metabolic pathways and mimics the host. This might have assisted the inhabitant fungi a consistent and promising supplier of L-asparaginase. It was also found that several species of this ecological group i.e., halophilic mangrove endophytes are an excellent source of L-asparaginase and are considered comparatively safe for the treatment of hypersensitive patients. Thus, in this present scenario, where cancer-like dreadful diseases are so prevalent, the discovery of novel therapeutics is of utmost importance for the treatment. As a result, this review focuses on the enzyme L asparaginase, various preliminary screening methods, quantitative estimation, testing the efficacy and biocompatibility of the enzyme in different cancer cell lines, the possibility of cloning, and its future applications in industrial and medical fields. The potential of a fungal endophyte generated from mangroves to create the novel anticancer medication L-asparaginase is a starting point for further research into its metabolic activity.

II. L-ASPARAGINASE AS AN ANTICANCER AGENT

L-asparaginase (E.C.3.5.1.1, L-asparagine amidohydrolase) are hydrolases that convert L-asparagine into L-aspartic acid and ammonia (Goodsell 2005; Verma et al. 2007). Normally, L-asparagine is synthesized from aspartate and glutamine using ATP by asparagine synthetase a gene located on chromosome no. 7q 21.3. But, in certain tumour conditions, cells solely depend on external source of asparagine for their development and progression, as they are defective or devoid of asparagine synthetase. Therefore, starvage or shortage of asparagine by L-asparaginase cause the death of tumour cells alone (Broome 1968). L-asparaginase breakdown the serum asparagine, and the tumor cells without asparagine synthetase will undergo cell cycle hault at the G0/G1 stage (Kumar and Sobha 2012; Offman et al. 2011). Purified L-asparaginase is administed intravenously to lower the level of serum asparagine thereby, terminating the cancer cell development and

multiplication. The developmental deficiency induced by asparaginase damage the central dogma of various cancers like Acute Lymphoblastic Leukaemia (ALL), acute myeloblastic leukemia (AML), and related tumor conditions consequential in programmed cell death (Fig. 1).

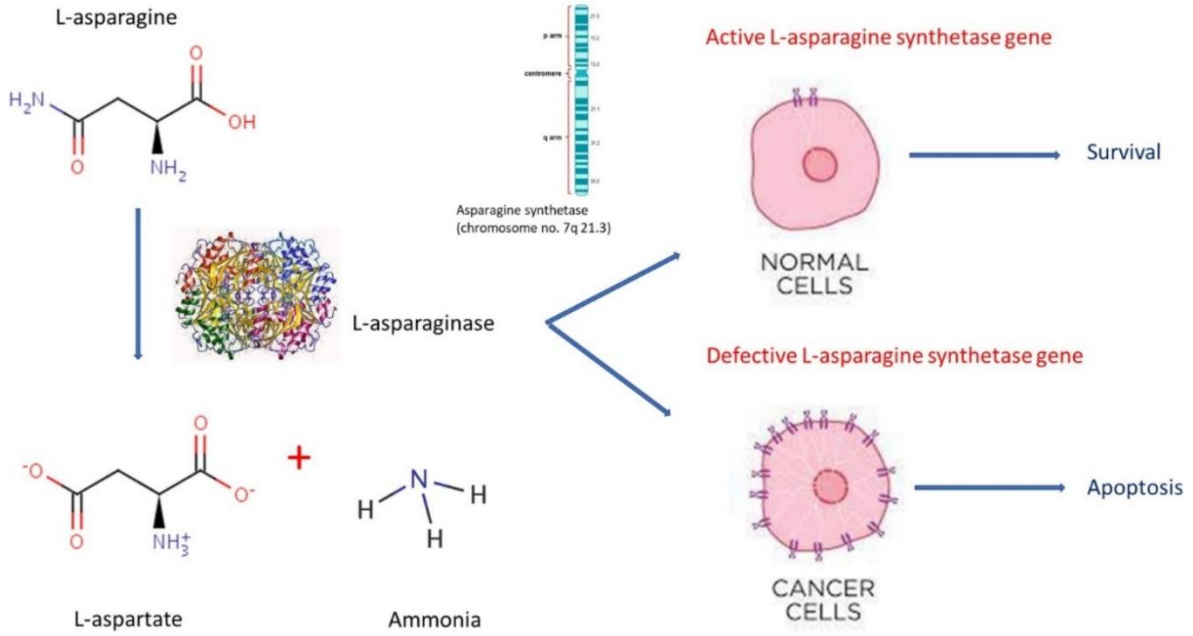

Figure 1: Functional mode of action of L-asparaginase in healthy and leukemic cells

III. L-ASPARAGINASE AS AN ACRYLAMIDE NUERATILIZING AGENT

Enhancing flavour and appearance by browning and crispening of bread and carbohydrate containing foods at high temperatures of about 120 °C (Maillard reaction), may result in the production of a neurotoxin, called acrylamide. At high temperatures, alpha-amino group of asparagine and carbonyl group of glucose in bread, potato, and other starch foods, reacted to form this carcinogenic acrylamide. As L-asparaginase has the ability to breakdown L-asparagine, this opens the way to minimize the precursor availability and thus preventing the formation of acrylamide in baked and fried foods. Moreover, being tasteless and odourless, they can be readily incorporated in bread doughs and other baked products to avoid acrylamide formation (Fig. 2). Nowadays fungal asparaginases particularly extracted from *A.oryzae* and *A niger* are widely used to serve this purpose (Mottram et al. 2002; Pedreschi et al. 2008; Morales et al. 2008).

IV. GENERIC L-ASPARAGINASE DRUGS

Prospect of using L-asparaginase as an anticancer agent was first effectively confirmed by Oettgen et al. (1967). *E.coli* asparaginases are marketed with commercial brands Kidrolase, Elspar (US), Crasnitin (Germany), Leunase (France), and many more. A PEGylated enzyme, Oncaspar1 (Maryland), is widely used for pediatric ALL and is widely available in market (Kumar and Sobha 2012). Without hindering the antitumor activity, PEGylation successfully modifies the chemical nature of an enzyme with reduced

immunogenicity. Asparaginase and PEGylated forms of asparaginase are effective adjuncts for standard chemotherapy (Blain et al. 2002). Recombinant *Erwinia* L-asparaginase was marketed as Erwinase (UK) (Allas et al. 2009).

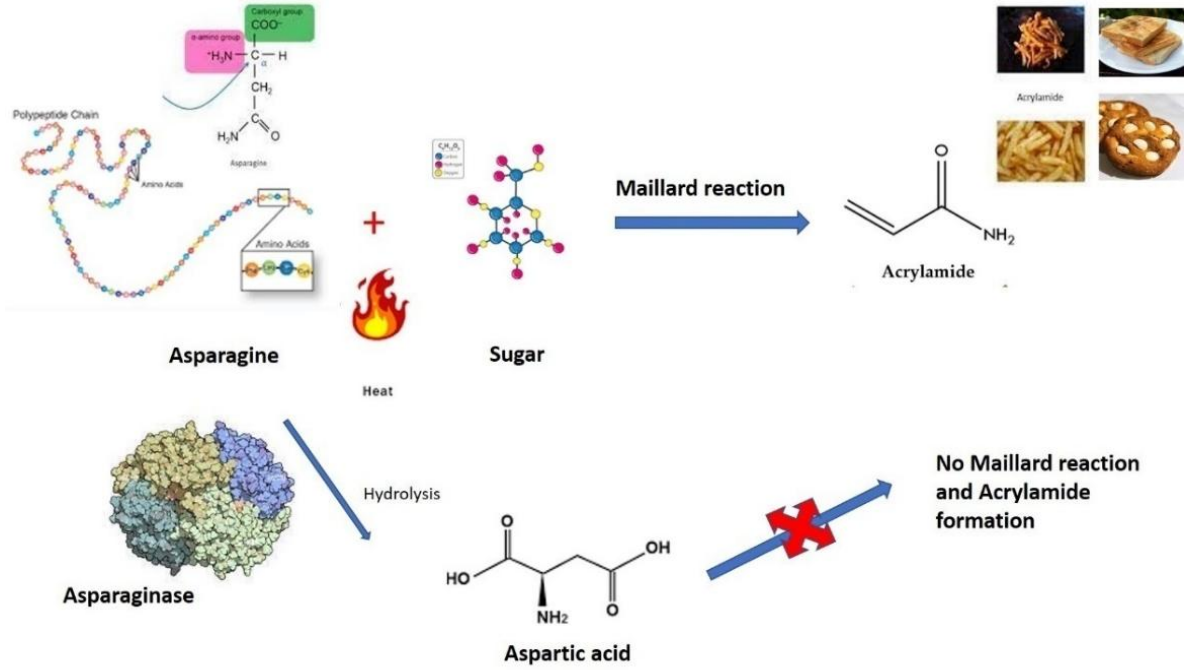

Figure 2: Mode of Action of L-Asparaginase in Baked Food Products

V. NATURAL SOURCES

During 1920's, there was an initial attempt to extract L-asparaginase from blood serum of an animal source like guinea pig (Clementi 1922). Later Kidd (1953) described the antitumor activity of this serum against lymphosarcoma in mice and rats. Identification of L-asparaginase as the key constituent in inhibition of tumor was reported by Broome (1963). Then onwards, L-asparaginase holds a pivotal role in the cancer treatment (Boyse et al. 1967; Alpar and Lewis 1985). Afterwards, there were several reports on L-asparaginase from animal tissues of rodents, fishes, mammals and even from aves (Adamson and Fabro 1968; Cooney and Handschumbcher 1970). Similarly, plant species like *Pisum sativum*, *Glycine max*, *Oryza sativa*, *Hordenum vulgare*roots, and *Lupinus*, were extensively studied for extraction of L-aparaginases (Konrad et al. 1988; Sieciechowicz and Ireland 1989; Borek and Jaskolski 2001). In the case of microbial origin, the major bacterial source reported were *Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum, Erwinia chrysanthemi, Rhodosporidium toruloides, Serratia marcescens, Thermus thermophiles, Vibrio proteus* and *Zymomonas mobilis*(Cedar and Schwartz 1967; Stern et al. 1976; Mesas et al. 1990; Sinha et al. 1991; Ramakrishnan and Joseph 1996; Aghaiypour et al. 2001; Prista and Kyridio 2001; Pinheiro et al. 2001; Kelo et al. 2002; Fisher and Wray 2002; Kotzia and Labrou 2007). Two major forms of L-asparaginases was found in *E. coli*, among which Asn I was considered to be cytosolic and Asn II to be periplasmic in origin. The amido-hydrolytic activity was observed only for ECAII (*E.coli* asparaginase II). Numerous *Streptomyces* species were also identified with active asparaginase production

(Narayana et al. 2008). Similarly several microscopic filamentous fungal species, molds and yeasts were identified as asparaginase producers. Mitosporic fungal species such as *Penicillium, Mucor, Aspergillus,* and *Fusarium* sp. and the yeast variety *Candida utilis*, were also acknowledged to yield L-asparaginase that is nontoxic and ensure myelosuppressive and immunosuppressive activity (Ali et al. 1994; Patil et al. 2012).

Correspondingly attempts were also made to purify L-asparaginase from marine microalgae, *Chlamydomonas* sp. (Paul 1982), a pioneer study of this sort with restricted antitumor activity. Asparaginase was even reported to be isolated from protozoans like *Tetrahymena pyriformis.* Even if there are several reports of L-asparaginase from varying souces, only limited candidate displayed promising yield and antitumor property to be considered for further scale up processes. Among the bacterial sources, *Escherichia coli, Erwinia carotovora,*and *Serratia marcescens* showed the prevalence of both periplasmic and cytoplasmic forms of enzymes with notable antineoplastic property (Schwartz et al. 1966). Thus these findings suggest the importance of further research in localization of an enzyme and its significant role in development of a suitable bioprocess (Moorthy et al. 2010).

VI. MARINE ENDOPHYTIC FUNGI AS A SOURCE OF L-ASPARAGINASE

Hidden endomyconts live symptomless inside the host tissue and the share communication signals with their host leading to the production of a cascade of bioactive metabolites. Fungal endophytes were screened for L-asparaginase on modified Czapek Dox agar with L-asparagine as sole nitrogen supplier (Jain et al. 2012). Similarly, endomyconts inhabitant of Thai medicinal plants were also evaluated to produce asparaginase (Theantana et al. 2007). Several fungal residents in soil and sediment samples, medicinal plants, and tropical pitcher plants also showed the presence of L-asparaginase (Patil et al. 2012; Lee et al. 2014). Microorganisms living in harsh conditions like marine environments are expected to yield halophilic proteins and enzymes with modified structures and diverse immunological properties. During long term coexistence, the endophytic partner might have progressively adapted to the microenvironment through genetic variation, by uptaking certain host DNA into their own genomes, and vice versa, that might have resulted in biochemical metabolic diversity, having distinct medicinal and industrial applications (Debashish et al. 2005; Zhang and Kim 2012). Studies by Thirunavukkarasu et al. in 2011 revealed that apart from the fungal genera like *Aspergillus, Penicillium,* and *Fusarium,* several other endophytic fungal species *Plectosphaerella, Stemphylium, Alternaria, Phoma*, and *Cladosporium* are also good sources of L-asparaginase. The absorptive mode of nutrition in marine endophytic fungi results in the secretion of this enzyme.

An interesting fact is that fungal asparaginases are not always allied with glutaminase as seen in the case of bacterial sources. The association of glutaminase activity with L-asparaginase enhances the toxic side effects, by converting blood glutamine to glutamate and ammonia. The glutamate thus formed combine with sodium in the blood, resulting in the formation of monosodium glutamate (Kurtzberg et al. 2003). Glutaminase activity of the asparaginase cumulatively activates side effects in patients (Kravchenko et al. 2008; Ramya et al. 2011). Hence, the exploration for L-asparaginase enzyme devoid of glutaminase activity is still a genuine scientific mission. Similarly, in addition to all the parameters, L-asparaginase production vary on culture media nitrogen and carbon composition, aeration conditions, pH and temperature of the culture medium, inoculum size, and fermentation time

and also alter considerably with source microbe (Bascomb et al. 1975). Eventhough both solid-state fermentation (SSF) and submerged fermentation (SmF) are exclusively employed for fungal asparaginase production, submerged fermentation conditions were considered more ideal. This was mainly due to the easefulness in opmitimization of individual components to achieve desired enzyme activity (Warangkar et al. 2009). Production, purification and characterization of asparaginase is also a very crucial part for the evaluation of the toxicity of the drug (Kumar and Sobha 2012).

VII. ASSAY FOR SCREENING AND QUANTIFYING L-ASPARAGINASE

Considering the pharmacological and food significance of L-asparaginase, there should be a simple, sensitive, reliable and cost effective estimation method to determine L-asparaginase activity. Although basic function of L-asparaginase is to deaminate asparagine to aspartate and ammonia (NH_3), it also undergoes a reversible aspartate hydroxymate to asparagine, and hydroxylamine interconversion (DeGroot and Lichtenstein 1960 a, b; Ehrman et al. 1971).

Primary analysis can be done using Czapek Dox (MCD) medium with asparagine as sole nitrogen source and incorporating phenol red and bromothymol blue as pH indicators. Individual control can be kept with $NaNO_3$, urea, L-asparagine and glutamine as the sole nitrogen source. Modified Czapek Dox medium with L-asparagine as an individual nitrogen source were inoculated with test organisms and their colony diameter and zone diameter can be estimated by measuring fungal mycelium and enzyme zone respectively after 7-8 days of incubation. The zone index was calculated as

$$\text{Zone Index} = \frac{\text{External zone diameter}}{\text{Inner mycelium diameter}}$$

L-asparaginase quantification is done by measuring the amount of ammonia released during the conversion of asparagine, with a distinctive yellowish orange colour detected spectrophotometrically (Meister 1955). The quantity of ammonia liberated was estimated with ammonium sulphate $(NH_4)_2SO_4$. One Unit (IU) of L-asparaginase is represented as the quantity of enzyme required to catalyse the formation of 1µ mole of NH_3 from L-asparagine per minute at ambient temperature and pH. Even though this method exhibits good reproducibility, Nessler's reagent can give false positive results, when used in complex media, and with contaminants thereby interfering in the actual quantification and estimation of enzyme (Miller and Miller 1948). To overcome the limitations of the Nesslerization process, another approach was used, based on the AHA method (L-aspartate β-hydroxamate). In the presence of hydroxylamine, L-asparaginase can also convert asparagine into L-aspartic acid β-hydroxamate (AHA), when reacted with ferric chloride ($FeCl_3$) yielded a brown colouration determined spectrophotometrically (Grossowicz et al. 1950). Yet alternative echnique uses the hydrolysis of L-aspartate β-hydroxamate to release hydroxylamine, which when reacted with oxine at basic pH, to form an deep green-coloured, indooxine spectrophotometrically detectable at 705-710 nm (Lanvers et al. 2002; Wehner et al. 1992). The enzyme activity was defined as nmol substrate formed per minute per milligram of protein. Aspartyl transferase activity can be interrelated with the NH_3 production determined using nessler's method (Imada et al. 1973).

Latest methods employing high-performance liquid chromatography (HPLC), helps in direct quantification of depleting asparagine using an isocratic HPLC fluorescence, thereby overcoming the drawbacks of colorimetric methods (Nath et al. 2009; Gentili et al. 1994). Direct amino acid quantification can also be done using circular dichroism (CD), and electrophoresis assays (Kudryashova and Sukhoverkov 2016; Broome 1968). Further detection invoves the estimation of indo-8-hydroxyquinoline, L-Aspartic acid b-benzyl ester 7-amido-4-methylcoumarin (a fluorogenics substrate), NADH, asparagine analogue- 5-diazo-4-oxo-L-norvaline, and the use of conductometry to detect elevation of conductivity in response to the production of L-aspartic acid and ammonia (Magri et al. 2018; Lanvers et al. 2002; Ylikangas and Monone 2000; Cooney and Handschumacher 1970; Handschumacher et al. 1968; Drainas and Drainas 1985). Protein quantification can be carried out using the Bradford's or Lowry's method. With the most predictability and accuracy, our aim was to analyse, compare, and highlight convention colourimetric assays with the latest methods to estimate and quantify the enzyme activity.

VIII. PRODUCTION, OPTIMIZATION, AND PURIFICATION OF L-ASPARAGINASE

Production of L-asparaginase may vary considerably depending on the species and is controlled by the media composition, and numerous physical parameters. Optimization of culture medium and cultivation parameters were critical aspects, that ensure the efficient growth of organisms and thus production of L-asparaginase. Statistical experimental design and data analysis tools like Design of Experiments (DoE) can be used to systematically optimize medium and process parameters, and to scale up the production process. Plackett-Burman design was widely used and found successful in evaluating and optimising enzyme production (Baskar and Renganathan 2009). Similarly, optimization using Response Surface methodology (RSM) and Computational Model Networking in association with Genetic Algorithms can also be employed to solve complex optimization process and to elevate asparaginase production (Baskar and Renganathan 2011, 2012). Furthermore, statistical softwares such as Simplex mixture design, Latin square design (LSD), Central composite rotatable design, and Box-Behnken design (Dias et al. 2015; Baskar and Renganathan 2009a; Baskar et al. 2010; Mohan Kumar and Manonmani 2013; Uppuluri et al. 2013) can be successfully employed in bioprocess optimization, allowing researchers to systematically explore and identify the best conditions for maximizing asparaginase production. Even though most of the studies often reported submerged liquid fermentation and solid-state fermentation (Dias et al. 2015; Mohan Kumar and Manonmani 2013), usage of agricultural wastes such as wheat bran, groundnut oil, corn flour, oat flour, and sesame oil cake as substrates enhanced L-asparaginase production (Dias et al. 2015; Baskar and Renganathan 2009, 2011; Gurunathan and Sahadevan 2011; Baskar et al. 2010; Huang et al. 2014; Uppuluri et al. 2013)

All the investigated L-asparaginase displayed a wide range of molecular size from 35-133 kDa. The enzyme was found as tetramers with identical subunits ranging from 20–70 kDa (Eisele et al. 2011; Huang et al. 2014). A major aspect involved in this is the elevated affinity of the enzyme towards the substrate asparagine (reduced Km). Among the selected studies that evaluated enzyme purification, the major steps used for asparaginase purification were acetone or ammonium sulphate precipitation, dialysis, ion chromatography, gel

permeation chromatography, and affinity chromatography (Dutta et al. 2015; Mohan Kumar and Manonmani 2013; Patro et al. 2014; Huang et al. 2014).

IX. ENZYME KINETICS

L-asparaginase enzyme is a conjugative homotetramer with 4 identical subunits, having carbohydrate moiety. Various studies reported that metallozyme inhibitors Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and Ni^{2+} possibly degrdade the enzyme activity, while metallic cofactors like Na^{+}, diphosphates, Li^{+}, Mg^{+}, CN^{-}, cysteine, etc., improved the activity. Its activity was also enhanced in the presence of reduced glutathione and the catalytic activity was found to be dependent on K^{+} (Bruneau et al. 2006). Glucose and maltose were found to activate L-asparaginase production and were elevated by $MgCl_2$ and repressed by EDTA. A detailed dynamic study involving thermal inactivation for the L-asparaginases at different temperatures can be performed along with other kinetic parameters using the experimental design having different concentraton of asparagine, chelators, and metal ions. The stability of this enzyme at optimum pH, temperature, and with specific substrates helps us to utilize it in medicinal and therapeutic industries (Dias et al. 2019).

X. RECOMBINANT CLONING AND EXPRESSION OF ASN GENE

Eventhough most of the Asn genes are cloned from bacteria, very few information have been reported in fungal L-asparaginases. As fungal asparaginases are widely accepted in cancer treatment with less toxic side reactions, an effective consolidation of information about fungal Asn gene cloning and expression is the need of the hour. Here we will be discussing a general strategy for cloning the L-asparaginase gene, its heterologous expression, along with its purification, and characterization.

Based on the conserved sequences of already reported L-asparaginase genes, a known sequence will be used for amplification with degenerate forward and reverse primers using genomic DNA from fungal isolates. The 5'and 3' regions of the fragment can be deduced by rapid amplification of cDNA ends. Thus it can be assembled with main central region to create a putative full-length Open Reading Frame. This region can be sequenced and can be subjected to MEGA BLAST analysis in NCBI. For amplification of this L-asparaginase region from the fungal DNA, specific primers can be designed for the amplification of the gene and the PCR product will be purified, restricted and ligated with a suitable high-efficiency TA cloning vector and transformed into competent cells for sequencing. Deduced nucleotide and thus amino acid sequences can be be analyzed using different software packages like Swiss-Prot or TrEMBL.

Later the purified PCR product after digestion with suitable restriction enzymes will be subcloned into the suitable expression vector, and transformed into competent cells for protein expression (Fig. 3). The recombinant L-asparaginase can be purified using any specialized tagged column like histidine or Ni-NTA nickel-nitrilotriacetic acid. The purified L-asparaginase can be electrophoresed on the SDS-polyacrylamide gel to check the molecular range.

XI. STRUCTURE ANALYSIS

Understanding the structure is essential to study the function and property of L asparaginase. Gene sequence of L asparaginase gives an insight into the primary structureof the protein. Secondary and tertiary structure elucidation can be aquired from aminoacid sequences and structure prediction softwares. As this enzyme can form complexes or multimeric structures further confirmation of tertiary structure can be accomplished by experimental techniques like X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Knowledge of active site of the enzyme can identify the residues and motifs involved in binding and catalysis. These functional awareness helps in designing the L-asparaginase drug to specifically target cancer cells. In summary it is a multidisciplinary approach that combines bioinformatics, molecular and structural biology to understand the function and application in cancer treatment.

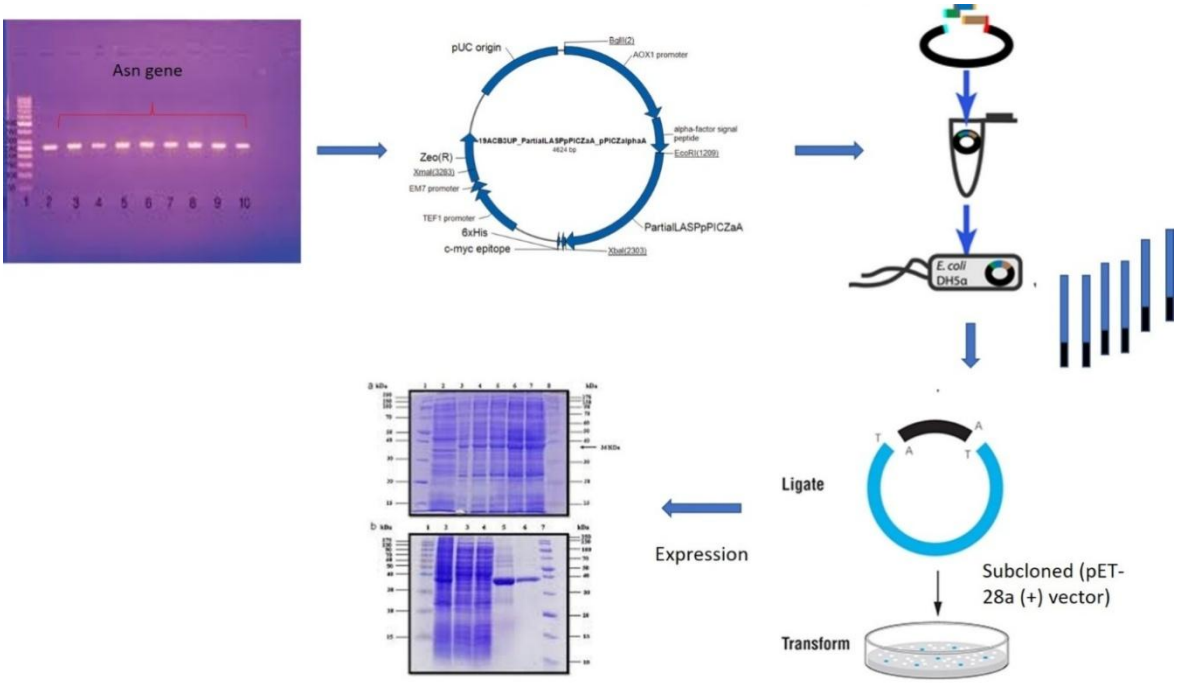

Figure 3: General overview of cloning and expression of fungal asparaginase gene

XII. ANALYSIS OF BIOCOMPATIBILITY AND ANTICANCER POTENTIAL OF L-ASPARAGINASE

A toxicological evaluation of asparaginase can be performed to investigate the erythrolytic effect on human blood. For testing the antitumor property of a drug, the US National Cancer Institute (NCI) has suggested an *in vitro* sreening cell line cluster involving about sixty tumor cell line of 9 cancer origin. This includes cancer of Bronchus, instestine, Melanocytes, Kidney, Ovary, Central nervous system, blood, breast, and endocrine glands (Boyd and Paull 1995). Cytotoxicity evaluation can be performed using different cell viability assays. Cell viability were analysed using tetrazolium reduction assay (MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), XTT methoxynitrosulfophenyl-tetrazolium carboxanilide assay, Sulforhodamine B assay), DNA

fragmentation, Comet assay, Necrosis assays, enzyme assays, proteomics assays, expression array assays etc.

Furthermore, purified asparaginases can be tested for their antineoplastic activity against leukaemia (U937, and K562 cell lines) and carcinomas of kidney, breast and prostrate origin (Hep-G2, MCF-7, PC3) (Huang et al. 2014; Shafei et al. 2015). The relation between the amount of viable cells and asparagine was plotted to get the viability curve of each cancer cell line after treatment and the Inhibitory Concentration (IC_{50}) will be noted. It is the concentration required to inhibit 50% of intact cells, and can be estimated for dose-dependent curve, using Probit analysis. Cell morphology and DNA fragmentation pattern can be used as an initial confirmation for the apoptotic action of L-asparaginase on tumour cells. Cancer regulating gene profiling by RT-PCR and protein analysis (Western blotting) validate the potential of L-asparaginase enzyme to undergo proapoptotic activity (Fig. 4).

Similarly specific *in vitro* cell-based assays like flow cytometric cell cycle analysis, topoisomerase inhibition assay, Hoechst 33342 staining, dUTP Nick End Labelling assay, Annexin V– PI double staining, Caspase activation analysis, Immunocytochemistry staining methods can be employed to establish the cytotoxic ability of the enzyme. Cytotoxicity may be due to sudden cell damage or necrosis, and it is essential to prove that the mode of cell lysis is by apoptosis accompanied by cell shrinkage, Karyorrhexis, activation of caspases etc. RT- PCR is an essential tool to analyze the expression pattern of cancer-controlling genes, cell cycle-associated genes, non-caspase protease genes, interferon-induced immune genes, and cytokine-associated immune genes in compound-treated cell lines, which will be compared with untreated control.

Table 1: NCI 60 cell line panel (Boyd and Paull 1995)

Tissue of Origin	**Cell Lines**
Alveolar & Bronchial	NCI-H23, NCI-H522, A549-ATCC, EKVX, NCI-H226, NCI-H332M, H460, H0P62, HOP9
Instestinal	HT29, HCC-2998, HCT116, SW620, COLO205, HCT15, KM12
Breast	MCF7, MCF7ADRr, MDAMB231, HS578T, MDAMB435, MDN, BT549, T47D
Ovary	OVCAR3, OVCAR4, OVCAR5, OVCAR8, IGROV1, SKOV3
Blood	CCRFCEM, K562, MOLT4, HL60, RPMI8266, SR
Kidney	UO31, SN12C, A498, CAKI1, RXF393, 7860, ACHN, TK10
Melanocytes	LOXIMVI, MALME3M, SKMEL2, SKMEL5, SKMEL28, M14, UACC62, UACC257
Endocrine	PC3, DU145
Brain	SNB19, SNB75, U251, SF268, SF295, SM539

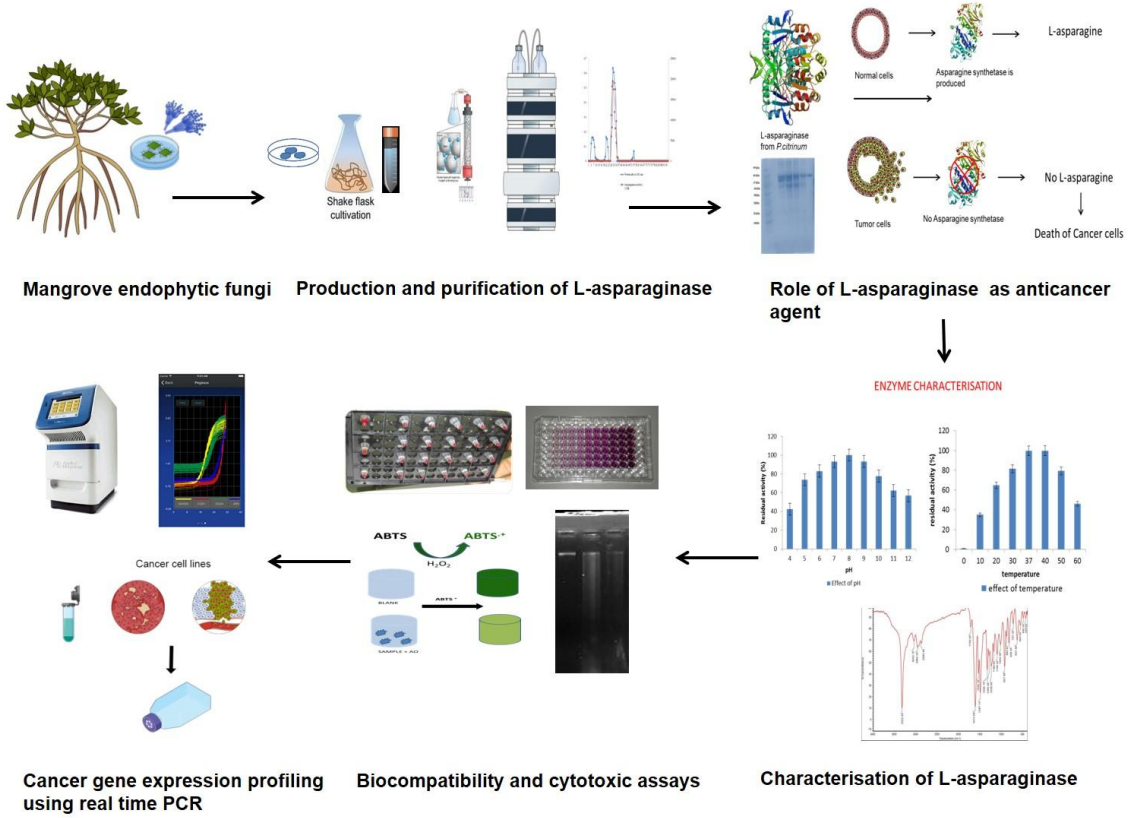

Figure 4: A general approach for screening and downstream processing of L-asparaginase from mangrove endophytic fungi.

XIII. CONCLUSION

In this review, we have presented a general method to screen and characterize the economically relevant enzyme L-asparaginase that found its application in the clinical and food processing industry. Due to the deamination property of L-asparaginase, a lot of research has been conducted to show their role in cancer therapy. Besides, this enzyme also benefits the food sector in the neutralization of carcinogenic acrylamide. Though these enzymes are widely studied in bacteria, their production in manglicolous endophytic fungi is considered significant as they reduce several side effects and the short life of enzymes in association with their applications. As we are using different screening procedures to select a potent strain that solely produces L-asparaginase enzyme, various undesirable allergic reactions such as hypersensitive effects, and immunogenic complications, of the drug, can be eliminated. L-asparaginase is often found associated with glutaminase, urease, and other contaminants, hence it induces different side effects that can be fatal. This review will give a comprehensive insight and will be a benchmark study that describes the preliminary screening of manglicolous endophytic mycobionts living in extreme harsh environment as a source of L-asparaginase, its quantification protocols, media optimization, enzyme kinetics, and purification parameters. To increase the yields of L-asparaginase, genetic engineering and cloning strategies can be made possible to increase the expression rate of this enzyme. Towards improving the knowledge about the mode of action of this enzyme as an anticancer source, biocompatibility tests, cell cytotoxicity assays, and various anticancer assays along

with the importance of in vitro gene expression analysis of cancer-controlling genes were discussed. Currently, the available strategy was collected and summarized here to initiate a study on L-asparaginase to widen the scope of non-immunogenic L-asparaginase production and downstream processing.

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