Chapter

Manglicolous Endophytic Fungi as a Source of L-asparaginase

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Abstract

Mangroves are considered as a dynamic transition zone between terrestrial and marine habitats. They exhibit high levels of biodiversity along tropical and subtropical coastlines because they coexist with both marine and terrestrial habitats. Since mangrove forests are home to the second-largest group of the marine fungal group, 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 significant enzyme that is commonly used as an antitumor agent in the treatment of acute lymphoblastic leukaemia (ALL), the most common pediatric cancer. The use of these enzymes as therapeutics selectively differentiates normal cells and promotes apoptosis of only cancerous cells due to their mode of action. The production of L-asparaginase by fungus appears more promising in light of the increased similarity of fungi to humans, which may reduce the likelihood of immunological reactions. In the food industry, asparaginase is also successfully utilized to lower the development of acrylamide in processed 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 these restrictions, this review provides information on straightforward screening techniques using a dye-based plate assay on modified Czapek Dox medium with phenol red and bromothymol blue indicators, as well as reasonably 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

Introduction

Marine fungi form an ecological group that is either obligate or facultative. Among them, obligate marine fungi are those that grow and sporulate solely in marine water, while facultative marine fungi are those that grow from freshwater or terrestrial milieus and can sporulate in the marine environment under certain physiological variations (Raghukumar 2008). However, about 74,000 fungal species have been estimated so far, with an overall expected global fungal diversity of 1.5 million species. It is expected that the fungal diversity in individual habitats has been considerably underestimated, for example, marine fungi from sediments avoid microscopic detection due to their tendency to form aggregates (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 extreme conditions in marine environments, such as a change in pressure, temperatures, salinity, and relative oxygen amount (Raghukumar 2008) induce great challenges. The organisms which live and thrive despite those stresses are expected to produce metabolites that might be of interest to drug prospectors. 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," as they are home to the second biggest fungal group 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 have been characterized from manglicolous endophytic fungi including antimicrobial, anti-inflammatory, anticancer, 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). In addition to these, endophytes have been identified as a promising source of valuable enzymes with various biotechnological applications (Firáková et al. 2007; Pimentel et al. 2011). They are reported to be producers of hydrolytic and oxidative enzymes; with amylase, cellulase, lipase, chitinase, pectinase, tyrosinase, protease, and ligninase activity (Bonugli-Santos et al. 2015). The role of these extracellular hydrolytic enzymes in litter degradation of tropical plant communities, such as a mangrove community, was studied by Kumaresan and Suryanarayanan (2002). Such knowledge is pertinent, as mangroves are one of the most productive natural ecosystems (Kohlmeyer and Volkmann-Kohlmeyer 1998).

Exploration for enzymes, like asparaginase and tyrosinase has recently augmented due to their medicinal value in the treatment of cancer, Parkinson's disease, and myocardial disease. Among them, L-asparaginase is the pioneer and largest group of therapeutic enzymes used in cancer treatment especially Acute Lymphoblastic Leukaemia (ALL) and other non-Hodgkin lymphomas (Kumar and Sobha 2012). Currently, L-asparaginase is also used in food manufacturing to reduce the formation of acrylamide in fried and oven-cooked foods. This enzyme is produced by a diverse source of organisms such as plants, animals, and microorganisms including bacteria, fungi, and actinomycetes. As extraction of L-asparaginase from mammalian cells is very challenging and expensive, the best-known source was from bacterial origin chiefly *E. coli* and *Erwinia carotovora*. But, in 60% of patients, it ends up with

serious clinical complications like hypersensitivity reactions, toxicity, and even death. Moreover, bacterial asparaginases are contaminated with glutaminase, which adds to side effects like hyperglycemia, abnormalities of homeostasis, lipid metabolism, and neurological disorders (Geuenich et al. 1998; Duval et al. 2002; Kumar et al. 2011b). In addition to the presence of glutaminase, 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 immunogenic complications associated with the currently used microbial sources (*E. coli* and *Erwinia carotovora*), there is a requisite to switch to novel natural sources to serve as non-immunogenic and better production sources of L-asparaginase. Recently, endophytic fungi have received much attention because of their ability to produce diverse bioactive 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 inside the plant body, the stability issues are also tackled and they have been recognized as a repository of novel secondary metabolites. These benefits made endophytic fungi the most reliable and potent source for screening 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.

L-asparaginase in cancer therapy

L-asparaginase (E.C.3.5.1.1, L-asparagine amidohydrolase) is an enzyme that hydrolyses the free amino acid L-asparagine to L-aspartic acid and ammonia (Goodsell 2005; Verma et al. 2007). Asparagine is a non-essential amino acid in normal healthy cells and has asparagine synthetase located on chromosome no. 7q 21.3 for the synthesis of asparagine from aspartate and glutamine using ATP.

However, certain tumour cells depend on an exogenic source of asparagine for protein synthesis, because they lack or express very low levels of asparagine synthetase. Therefore, exhaustion of asparagine by L-asparaginase leads to the selective death of tumour cells (Broome 1968). L-asparaginase selectively depletes the serum asparagine, which devoid the tumour cells of their only source of asparagine leading to their cell cycle arrest in the G0/G1 phase (Kumar and Sobha 2012; Offman et al. 2011). The free enzyme is intravenously injected to reduce the L-asparagine concentration in blood and thereby, selectively kill the cancer cells. The nutritional stress induced by asparaginase leads to DNA, RNA, and protein biosynthesis inhibition in Acute Lymphoblastic Leukaemia (ALL), acute myeloblastic leukemia (AML), and other asparagine-dependent tumor cells resulting in subsequent apoptosis (Fig. 1).
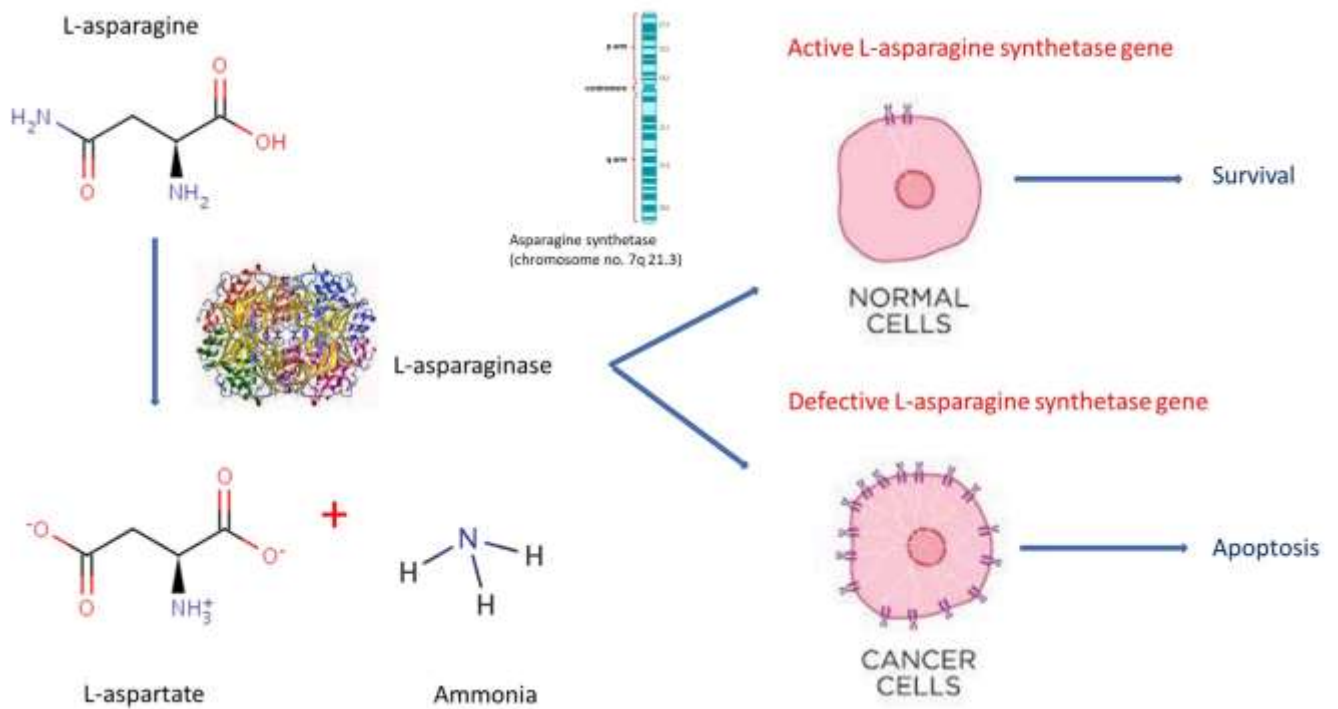
Fig. 1 Functional role of L-asparaginase in normal healthy cells and leukemic cells

L-asparaginase in the Food industry

In the food industry, during baking and frying of starchy foods at high temperatures of about 120 °C, acrylamide, a neurotoxin, is produced as a result of the Maillard reaction. At high temperatures, the alpha-amino group of amino acid asparagine and the carbonyl group of glucose in bread, potato, and other starch foods, reacted to form this carcinogenic acrylamide. As L-asparaginase could convert L-asparagine to aspartate, its ability opens the way to reduce the precursor and reduce the risk of acrylamide formation. Moreover, L-asparaginases are tasteless and odourless, they can be readily incorporated in bread doughs and other baked products to avoid acrylamide formation (Fig. 2). Nowadays L-asparaginases from *Aspergillus oryzae* and *Aspergillus niger* are used in the food industry for this purpose (Mottram et al. 2002; Pedreschi et al. 2008; Morales et al. 2008).
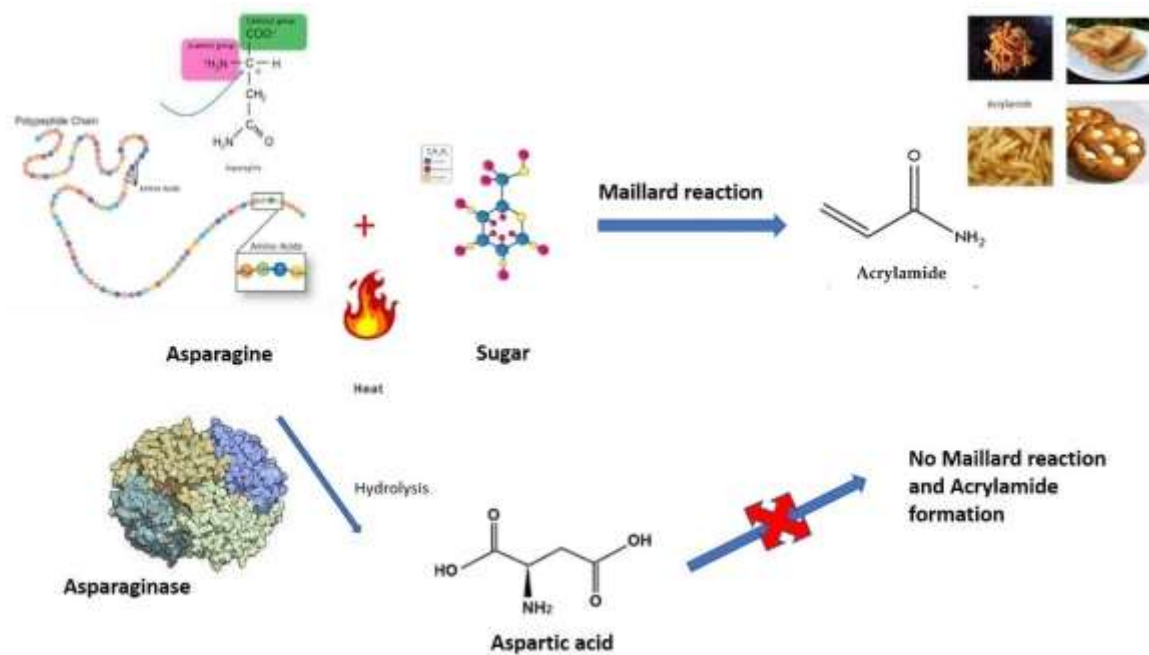
Fig. 2 Mode of action of L-asparaginase in Food industry

Clinically available L-asparaginase

The efficiency of L-asparaginase in the treatment of human leukemic subjects was first successfully validated by Oettgen et al. (1967). Mainly *E.coli* asparaginases are marketed under commercial names Kidrolase, Elspar, Crasnitin, Leunase, and Asparaginase Medac. A PEGylated enzyme, Oncaspar1 (pegaspargase), is widely used for the treatment of pediatric acute lymphoblastic leukemia and is already in use in the clinic (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* asparaginase was also available as Erwinase (Allas et al. 2009).

Sources of L-asparaginase

The presence of L-asparaginase from an animal source like the blood of guinea pig serum was first reported by Clementi (1922). Later Kidd (1953) described the antitumor activity of guinea pig serum against the growth of lymphosarcoma in mice and rats. Broome (1963) was the pioneer in recognising the role of L-asparaginase as an effective constituent for the inhibition of a tumour. Then onwards, this enzyme holds a key role in the treatment of Acute Lymphoblastic Leukaemia (ALL) (Boyse et al. 1967; Alpar and Lewis 1985). This enzyme was also found in tissues of several animals like the liver of rats, tissues of fish, and internal organs of many mammals and birds (Adamson and Fabro 1968; Cooney and Handschumbcher 1970). Similarly, in plants, it is found in *Pisum sativum*, *Glycine max*, *Oryza sativa*, *Hordenum vulgare*roots, *Lupinus,* and other species (Konrad et al. 1988; Sieciechowicz and Ireland 1989; Borek and Jaskolski 2001). In the case of microbial origin, the major bacterial source reported were *Bacillus subtilis, Corynebacterium glutamicum, Erwinia chrysanthemi, Escherichia coli, 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). In *E. coli* there are two types of L-asparaginase (L-Asn I and L-Asn II) of which Asn I is cytoplasmic and Asn II is periplasmic. The amido-hydrolytic activity is observed only in ECAII (*E.coli*asparaginase II). Among actinomycetes, several *Streptomyces* species (Narayana et al. 2008) were identified with active asparaginase production. Several eukaryotic microorganisms like yeast and filamentous fungi were identified as capable of asparaginase production. Mitosporic fungal species such as *Aspergillus terreus, Aspergillus nidulans, Penicillium, Mucor,* and *Fusarium* sp. and the yeast, *Candida utilis*, also have been reported to produce L-asparaginase that is nontoxic and ensure myelosuppressive and immunosuppressive activity (Ali et al. 1994; Patil et al. 2012).

Correspondingly L-asparaginase isolated from purified *Chlamydomonas* sp. (Paul 1982), was also identified as the first enzyme to be purified from marine microalgae, with restricted antitumor activity. Asparaginase was even isolated from protozoa, *Tetrahymena pyriformis.* Though the enzyme is widely distributed, only some of these L-asparaginases possess the anti-neoplastic activity and among the microbial sources, the most commercially notable ones are *Escherichia coli, Erwinia carotovora,*and *Serratia marcescens*. In most microorganisms, L- asparaginase is an intracellular enzyme; the prevalence of both periplasmic and cytoplasmic enzymes has been reported (Schwartz et al. 1966). The study on the localization of any enzyme plays a vital role in the development of a suitable bioprocess (Moorthy et al. 2010).

L-asparaginase production by marine endophytic fungi

Endophytic fungi live symptomless in the host plant and the interactions of endophytic fungi with their host leads to the production of a battery of secondary metabolites. L-asparaginase-producing fungal endophytes have been reported on the modified Czapek Dox medium with L-asparagine as a nitrogen source (Jain et al. 2012). Similarly, endophytic fungi from Thai medicinal plants were also evaluated to produce asparaginase (Theantana et al. 2007). Asparaginase-producing endophytic fungi have been isolated from various sources such as soil samples, medicinal plants, and tropical pitcher plants (Patil et al. 2012; Lee et al. 2014). Microorganisms in extreme living conditions like marine environments are expected to yield halophilic proteins and enzymes with modified structures and diverse immunological properties. Due to their immense genetic and biochemical diversity, marine microorganisms are viewed as a novel promising source of enzymes with immense technological applications (Debashish et al. 2005; Zhang and Kim 2012). Studies byThirunavukkarasu et al. in 2011 revealed that apart from the fungal genera like *Aspergillus, Penicillium,* and *Fusarium*, several other endophytic fungal species are also good sources of L-asparaginase. The absorptive mode of nutrition in marine endophytic fungi results in the secretion of this enzyme.

Moreover, the L-asparaginase from fungal sources is not always accompanied by glutaminase activity as seen in the case of bacterial sources. The glutaminase activity of asparaginase promotes the irreversible conversion of blood glutamine into glutamate and ammonia. This glutamate reacts with sodium in the blood and results 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). Therefore, the search for glutaminase-free asparaginase from native microorganisms is still a genuine scientific task. Similarly, in addition to all the parameters, the extracellular L-asparaginase secretion also depends on the medium components such as nitrogen and carbon sources and cultural parameters like the initial pH of the culture medium, incubation temperature, inoculum size, and fermentation time. All these factors vary from one organism to another (Bascomb et

al. 1975). Solid-state fermentation (SSF) and submerged fermentation (SmF) are in use for the extracellular production of fungal L-asparaginase. Although SSF uses cheap substrates like agricultural wastes, the SmF is preferred since the medium composition is known and can be easily altered to get a considerable yield of quality asparaginase (Warangkar et al. 2009). Biochemical characterization and purification of asparaginase is also a very crucial part of the evaluation of the toxicity of the drug (Kumar and Sobha 2012).

Assay for screening and quantifying L-asparaginase activity in endophytic fungi

Considering the pharmacological and food significance of L-asparaginase, there should be an efficient, sensitive, and reliable quantification method to determine L-asparaginase activity. Although the normal function of asparaginase is to deaminate asparagine into L-aspartic acid (L-Asp) and ammonia (NH_3), it can also catalyze the conversion of aspartic hydroxymateto asparagine, and hydroxylamine, and vice versa (DeGroot and Lichtenstein 1960 a, b; Ehrman et al. 1971).

Primary analysis can be done using Modified Czapek Dox (MCD) medium amended with two different dyes such as 0.009% phenol red and 0.007% bromothymol blue as indicators separately. Different control plates were prepared with $NaNO_3$, urea, and glutamine as the sole nitrogen source. MCD plates with L-asparagine as an individual nitrogen source were inoculated with test organisms and their colony diameter and zone diameter can be calculated by measuring the diameter of the fungal growth and enzyme production respectively after 7-8 days of incubation. The zone index was calculated as

Zone Index = <u>Outer zone diameter</u>
$\qquad\qquad$ Inner colony diameter

The most common method for L-asparaginase quantification is by measuring the amount of ammonia released during the conversion of asparagine, with a characteristic yellow reaction mixture quantified spectrophotometrically (Meister 1955). The amount of ammonia liberated was calculated using a$(NH_4)_2SO_4$ as standard. One unit (IU) of L-asparaginase is defined as the amount of enzyme required to catalyse the formation of 1μ mole of ammonia 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 like polymers, esters, salts, surfactants, alcohols, amines, and aldehydes, thereby interfering in the exact quantification and estimation of activity (Miller and Miller 1948). To overcome some of the limitations of the Nessler method, another approach was used, based on the AHA method (L-aspartic acid 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 another common technique uses the hydrolysis of AHA to liberate hydroxylamine, which when reacted with 8-hydroxyquinoline at alkaline pH, resulting in an intensely green-coloured, oxindole dye (indooxine) easily detectable between 705 and 710 nm (Lanvers et al. 2002; Wehner et al. 1992). The enzyme activity was expressed as a nmol substrate formed min-l (mg protein)-l. This aspartyl transferase activity of asparaginase was correlated with the ammonium production determined by nesslerization (Imada et al. 1973).

Other methods include the use of high-performance liquid chromatography (HPLC) that quantify directly the depletion of L-asparagine using an isocratic HPLC fluorescence, thereby overcoming the drawbacks of colourimetric 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). Additional assays include the measurement of different substrate degradation, like indooxine, L-aspartic acid β-(7-amido-4- methylcoumarin), NADH, 5-diazo-4-oxo-L-norvaline, and the use of conductometry to detect elevation of conductivity in response to the production of L-aspartate 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). All protein determinations can be carried out by using the procedure of Bradfords or Lowry method. With the most predictability and accuracy, our goal was to critically highlight different traditionally applied colourimetric assays (Nessler, AHA, and indooxine methods) and the latest methods to estimate and quantify the L-asparaginase activity.

Production optimization and purification

Production of asparaginase may vary considerably depending on the species and is controlled by the constituents of the culture medium (primarily the carbon and nitrogen source), and physical parameters (temperature, pH, agitation, inoculum size, and fermentation period). Each organism varies accordingly in L-asparaginase production; so, optimization of medium components and cultivation parameters is essential in the biological process. Some statistical experimental design helps us to analyse the role of individual factors, establish an interaction between individual factors, and finally calculate performance at the optimum levels obtained. Furthermore, statistical designs are acknowledged to be better than the traditional one-variable-at-a-time method. Plackett-Burman design was widely used and found successful in evaluating and optimising enzyme production (Baskar and Renganathan 2009). Similarly, optimization using Central Composite Design (CCD) and Artificial Neural Network (ANN) associated with genetic algorithms was also employed to optimize the model for maximum asparaginase production (Baskar and Renganathan 2011, 2012). In addition, other types of statistical methods were also employed by the selected studies, to optimize the asparaginase production, such as Simplex mixture design (Dias et al. 2015), Latin square design (LSD) (Baskar and Renganathan 2009a; Baskar et al. 2010), Central composite rotatable design (Mohan Kumar and Manonmani 2013) and Box-Behnken design (Uppuluri et al. 2013). Even though most of the studies often reported induced L-asparaginase production by submerged fermentation (SmF), solid-state fermentation (SSF) was also employed successfully (Dias et al. 2015; Mohan Kumar and Manonmani 2013). Furthermore, different agro-industrial residues such as wheat bran, soybean meal, cottonseed meal, orange peel, groundnut oil, corn flour, oat flour, and sesame oil cake were used as a substrate for 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 enzymes displayed a wide molecular weight ranging from 35 to 133 kDa. The enzyme was found as tetramers with identical subunits ranging from 25–65 kDa (Eisele et al. 2011; Huang et al. 2014). Besides the molecular weight, one of the key factors for the successful use of the enzyme includes its high affinity for its substrate asparagine (with a low Km value). Among the selected studies that evaluated enzyme purification, the major steps used for asparaginase purification were solvent precipitation, ion exchange chromatography, size exclusion chromatography, and affinity chromatography (Dutta et al. 2015; Mohan Kumar and Manonmani 2013; Patro et al. 2014; Huang et al. 2014).

Enzyme Kinetics

L-asparaginase enzyme is a tetramer of four identical subunits, a conjugate protein with 37.3% carbohydrate. Various studies reported that metal ions, such as Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and

Ni^{2+} potentially inhibited the enzyme activity, while metal chelators like EDTA, CN-, cysteine, etc., enhanced the activity indicating that the enzyme was not a metalloprotein. Its activity was also enhanced in the presence of reduced glutathione but not with dithiothreitol and 2-mercaptoethanol. The catalytic activity of L-asparaginase subtypes is found to be dependent on K$^+$ (Bruneau et al. 2006). Various other elements including Cu^{2+}, diphosphate, EDTA, Li$^+$, and Mg^{2+} have been reported to influence the activity of L-asparaginase, isolated from different sources, directly or indirectly. Carbon sources like glucose and maltose were generally used for enzyme production and were activated by MgCl$_2$ and repressed by EDTA. A detailed kinetic study involving thermal inactivation for the L-asparaginases at different temperatures can be performed along with other kinetic parameters using the experimental system having varying amounts of the substrate, chelators, and metal ions. The stability of this enzyme at physiological pH, temperature, and with specific substrates helps us to utilize it in future investigations in the food industry and for pharmaceutical applications (Dias et al. 2019).

Improving the production by Cloning and expression of the L-asparaginase gene

L-asparaginase is governed by different molecular elements in different organisms. Though many L-asparaginases have been cloned from bacteria, very few studies have focused on fungal L-asparaginases, which may have fewer adverse side effects when used for the treatment of leukaemia or lymphosarcoma. Here we will be discussing a general strategy for cloning the L-asparaginase gene, its heterologous expression, along with purification, and characterization of the recombinant L-asparaginase.

Based on the conserved amino acid sequences of known 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' flanking regions of the fragment can be obtained by rapid amplification of cDNA ends (RACE). The two flanking regions can be then assembled with the core fragment to generate a putative full-length ORF. This L-asparaginase cDNA sequence will be subjected to BLAST analysis. To amplify this region from the genomic DNA, specific primers will be used for the amplification of the gene and the PCR product will be purified and will be ligated with the pMD18-T vector and transformed into E. coli DH5α cells for sequencing. Nucleotide and deduced amino acid sequences will be analyzed using different software packages.

Later the purified PCR product after digestion with suitable restriction enzymes will be subcloned into the pET-28a (+) vector, and transformed into competent E. coli BL21(DE3) cells for protein expression (Fig. 3). The recombinant L-asparaginase will be purified using any specialized tagged column like Ni-IDA (nickel-iminodiacetic acid)/ Ni-NTA nickel-nitrilotriacetic acid) column. The purified enzyme can be migrated on the SDS-polyacrylamide gel to confirm the molecular weight.
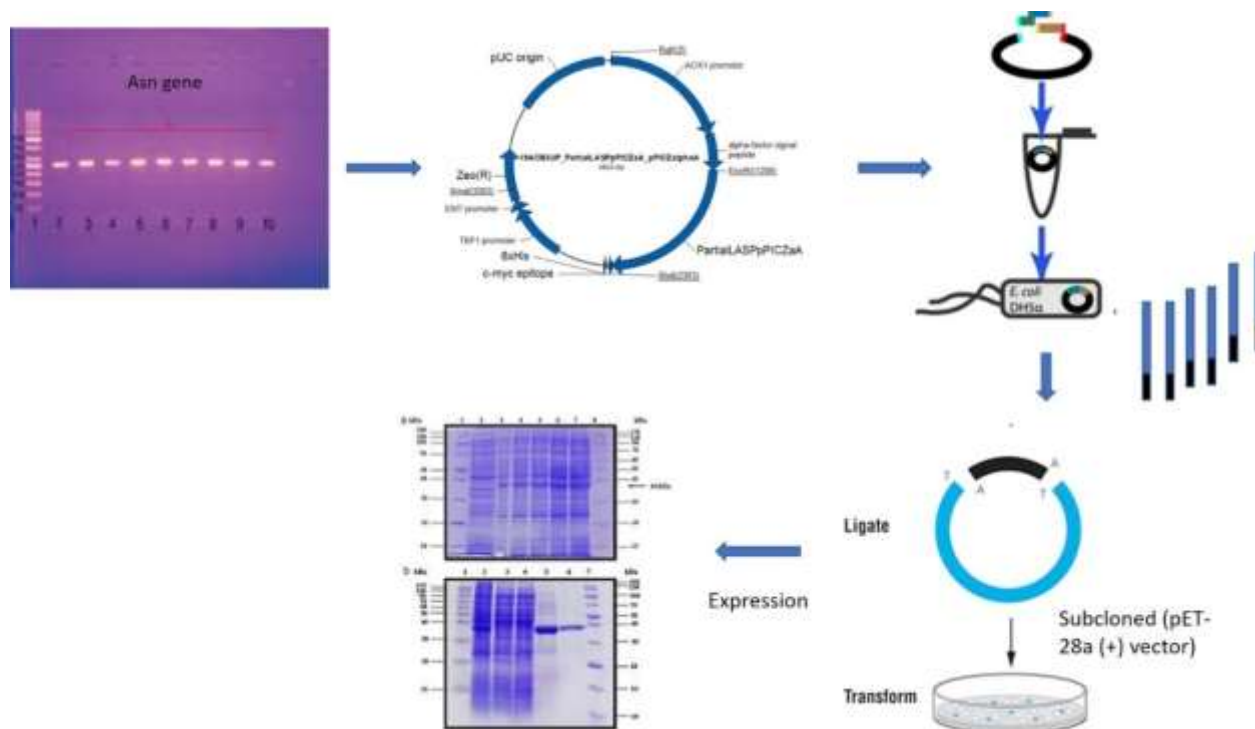
Fig. 3 General overview of cloning and expression of fungal asparaginase gene

Biocompatibility and anticancer properties of L-asparaginase

A toxicological evaluation of asparaginase was performed to investigate the hemolytic effect of purified asparaginase on human blood (erythrocytes). Similarly, the US National Cancer Institute (NCI) started anti-cancer screening support for researchers worldwide in 1955. To establish a new *in vitro* screen panel, a total of 60 different human tumour cell lines derived from nine cancer types: Lung, Colon, Melanoma, Renal, Ovarian, Brain (CNS), Leukemia, Breast, and Prostate (Boyd and Paull 1995) was used. Cytotoxicity evaluation can be performed using different cell viability assays. Three assays for cell viability were extensively evaluated, which include MTT, XTT, and Sulforhodamine B (SRB) assays.

Furthermore, the purified asparaginases can be tested for their antiproliferative activity against different types of human tumour cells, such as human leukaemia (Huang et al. 2014), hepatocellular carcinoma, breast cancer, and prostate cell lines (Shafei et al. 2015). The relation between the surviving cells and the asparaginase-used concentrations was plotted to get the survival curve of each tumor cell line after treatment with the purified asparaginase. The cytotoxic concentration (MCC50), the concentration required to cause a toxic effect in 50% of intact cells, can be estimated from graphic plots of the dose-response curve for each concentration, using prism software (San Diego, CA, USA). Cell morphology and DNA fragmentation pattern can be used as an initial confirmation for the apoptotic action of L-asparaginase on tumour cells. Gene expression analysis using real-time polymerase chain reaction (RT-PCR) and protein analysis (Western blotting), we can confirm the regulation of proapoptotic and antiapoptotic genes (Fig. 4).

Similarly specific *in vitro* cell-based assays like flow cytometric cell cycle analysis, topoisomerase inhibition assay, Hoechst 33342 staining, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) assay, Annexin V – Propidium iodide (PI) double staining, Caspase activation analysis, and Immunocytochemistry staining methods can be performed to establish the potential anticancer nature of the enzyme, as cytotoxicity can also

be due to necrosis, and it is imperative to prove that the mode of cell lysis is not due to necrosis. Quantitative real-time PCR (qPCR) will be used 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
Lung	NCI-H23, NCI-H522, A549-ATCC, EKVX, NCI-H226, NCI-H332M, H460, H0P62, HOP9
Colon	HT29, HCC-2998, HCT116, SW620, COLO205, HCT15, KM12
Breast	MCF7, MCF7ADRr, MDAMB231, HS578T, MDAMB435, MDN, BT549, T47D
Ovarian	OVCAR3, OVCAR4, OVCAR5, OVCAR8, IGROV1, SKOV3
Leukemia	CCRFCEM, K562, MOLT4, HL60, RPMI8266, SR
Renal	UO31, SN12C, A498, CAKI1, RXF393, 7860, ACHN, TK10
Melanoma	LOXIMVI, MALME3M, SKMEL2, SKMEL5, SKMEL28, M14, UACC62, UACC257
Prostate	PC3, DU145
CNS	SNB19, SNB75, U251, SF268, SF295, SM539
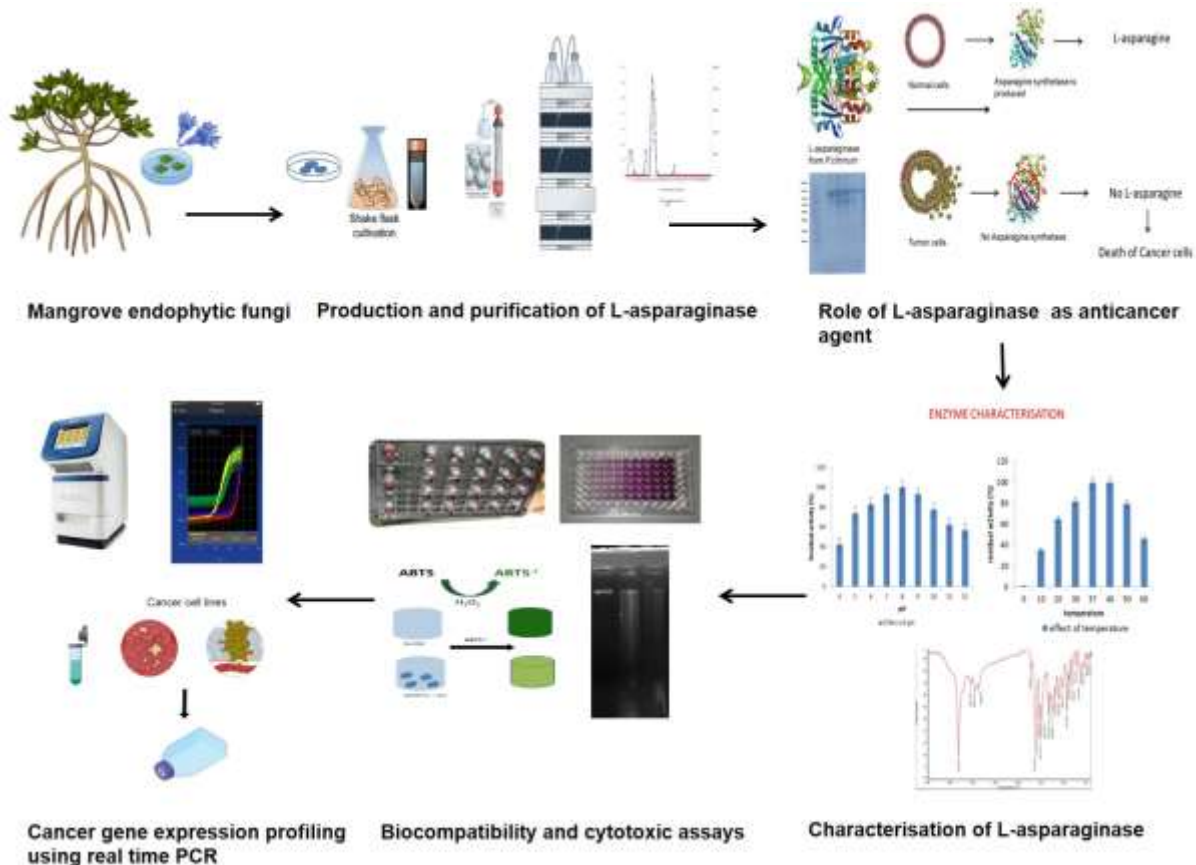
Fig. 4 A general approach for screening, production, purification, and characterization of L-asparaginase from manglicolous endophytic fungi.

Conclusion

In this review, we have presented a general method to screen and characterize the economically relevant enzyme L-asparaginase used both in the clinical and food 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 industry in the elimination 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 environmental conditions as a source of L-asparaginase, its different 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. To better understand 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 broaden the scope of cost-effective, non-immunogenic production and application.

Acknowledgment

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