Chapter

PURIFICATION, CHARACTERIZATION AND CLINICAL APPLICATIONS OF THERAPEUTIC FUNGAL ENZYMES

Mafalda R. Almeida, Ana M. Ferreira,
João C. F. Nunes and Ana P. M. Tavares*
CICECO - Aveiro Institute of Materials, Department of Chemistry,
University of Aveiro, Aveiro, Portugal

ABSTRACT

This book chapter presents an overview of therapeutic fungal enzymes and their developments in biopharmaceuticals for the treatment of several diseases, clinical applications and investigation. Enzymes are biocatalysts of many reactions with widespread use in the pharmaceutical industry and medicine. Due to their high specificity, greater affinity, and high catalytic efficiency, enzymes have been widely used for therapeutic purposes. More specifically, therapeutic enzymes are being used in the treatment of several diseases, such as leukemia, cancer, pancreatic disorders, etc. For instance, L-asparaginase, which presents antineoplastic properties, has been used for the treatment of leukemia, namely acute lymphoblastic leukemia. Nowadays, more than 50% of the enzymes are produced by fungal sources, including the therapeutic enzymes, due to the advantages of being an economically feasible and consistent process, since it has high yield and is easy for modification and optimization of new therapeutic products. In this book chapter, readers from academies, research institutes and industries will gain useful information and in-deep knowledge on the emerging therapeutic fungal enzymes, their purification processes, characterization and medical applications.

* Corresponding Author's Email: aptavares@ua.pt.

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INTRODUCTION

In this book chapter, a review about the therapeutic use of fungal enzymes over the past decades is explored. Enzymes as biopharmaceuticals have unique characteristics, such as selectivity to their substrates, that distinguish them from other types of drugs (Mane and Tale 2015). These properties make enzymes specific and potent biological with a therapeutic potential. These features have resulted in the development of many therapeutic enzymes for a wide range of diseases (Gurung et al. 2013). In recent years, the potential use of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of therapeutic fungal enzymes. Fungal enzymes including, glucose oxidase, L-asparaginase, proteases, amylases, cytosine deaminases, laccases, lipases, and chitinases, which are involved in pharmaceutical applications, have gain more attention. The potential applications of these enzymes are determined by the ability to screen new and improved enzymes, their fermentation and purification steps in large scale, and the formulations of enzymes. In this scenario, different methods have been established for enzyme purification. For specific pharmaceutical industrial applications, chromatography is still widely preferred due to its robustness, selectivity (high level of the enzyme purity), high clearance of impurities and most importantly, easy validation compared to other purification processes (Aehle 2007). Overall, traditional purification strategies are considered time-consuming with lower yields, and the trends are moving towards precipitation, crystallization and aqueous two-phase systems (Gurung et al. 2013). Furthermore, the characterization of purified fungal therapeutic enzymes has been addressed. Investigations on the pH, temperature and metal ions effect on the enzyme activity have been performed by several authors and described in this book chapter.

THERAPEUTIC FUNGAL ENZYMES

Enzymes are biological macromolecules, produced by a living organism, which acts as a highly selective biocatalyst in a specific biochemical reaction required to sustain life (Smith 1997). Enzymes are known to catalyse about 4,000 biochemical reactions accelerating both the rate and specificity of these metabolic reactions (Bairoch 2000). Each enzyme is constituted by a long and linear chain of amino acids that fold to produce

a specific and unique three-dimensional structure with specific properties (Gurung et al. 2013). During the last decades, due to the intensive research in enzymology, the development of fermentation processes, recombinant DNA technology and protein engineering for enzymes production with specific strains allowed its large-scale production and their introduction into the industrial field (Gurung et al. 2013), with many significant and vital roles in the pharmaceutical industries (Mane and Tale 2015).

Table 1. Some examples of therapeutic applications of fungal enzymes

Enzyme	Therapeutic applications	Fungus	References
α-Amylase	Digestive disorders Pancreatic insufficiency	*Aspergillus* sp.	(Gupta et al. 2003; Somaraju and Solis-Moya 2014)
Chitinases	Treatment of infections Anti-cancer	*Saccharomyces cerevisiae* *Candida albicans*	(Nagpure, Choudhary and Gupta 2014; Roopavathi, Vigneshwari and Jayapradha 2015; Karthik et al. 2014)
L-Asparaginase	Acute lymphocytic leukemia	*Aspergillus terreus*	(De-Angeli et al. 1970)
Cytosine deaminases	Tumour therapy Antimicrobial drug design Gene therapy applications	*Bacillus subtilis* *Yeast**	(Gaded and Anand 2018; Ko et al. 2003; Kievit et al. 1999)
Proteases	Acne or psoriasis Human callus Dermatophytosis Scar removal Epithelia regeneration Acceleration of healing processes	*Trichoderma pseudokoningii* *Meloidogyne incognita* *Metarhizium anisopliae* *Beauveria bassiana*	(Brandelli, Daroit and Riffel 2010; Vignardet et al. 2001; Chao et al. 2007; Souza et al. 2015; Yike 2011)
Lipases	Reduction of cholesterol Tumour therapy Pancreatic insufficiency	*Candida rugosa*	(Yang et al. 1997; Gurung et al. 2013; Takasu et al. 2012)
Glucose oxidase	Tumour therapy	*Penicillium notatum*	(Fu et al. 2018; Zhao, Hu, and Gao 2017; Sabir et al. 2007; Javed et al. 2013; Bhatti, Haq Nawaz Saleem 2009)
Laccases	Deactivation of HIV-1 reverse transcriptase Hepatitis C inhibition	*Pleurotus cornucopiae* *P. ostreatus*	(Wong et al. 2010; El-Fakharany et al. 2010).

*Name of the species is not available.

Enzymes with therapeutic properties are proteins that themselves are the therapeutic agent. They have many advantages over non-enzymatic drug products due to the highly specificity towards a target, reduced immunogenicity (most common cause for drug failure), which improve the clinical efficacy (Lutz, Williams and Muthu 2017). Therapeutic enzymes can be used, either alone or in combination with other therapies, for treating a variety of diseases. In general, enzyme as biopharmaceutical are usually used by injection due to their size and sensitivity to denaturation (Vellard 2003). However, the delivery of this biotherapeutic depends on the type of disease and the location of the enzyme target. For example, enzymes for digestive aids have been used as an oral

formulation (Vellard 2003). Therapeutic enzymes are being employed in diagnosis, biochemical investigation, monitoring and treatment of several diseases, such as leukemia, skin ulcers, Pompe's disease, cardiovascular diseases, celiac disease, Parkinson's disease, Fabry's disease, inflammation, digestive disorders, pancreatic disorders (Mane and Tale 2015). In specific, enzymes act as oncolytics, anticoagulants, thrombolytics, and replacements for metabolic deficiencies (digestive aids and metabolic storage disorders, among others).

Microbial enzymes display many advantages, such as stability, great yields, financial viability, easy product optimization, steady supply, and fast microbes growth on low-cost media (Gurung et al. 2013). In fact, many medically important enzymes are obtained from a limited number of fungi, yeast and bacteria. These organisms are also considered when a new enzyme is required (Teal 1991). Medically important enzymes are required in very less quantity as compared to the industrially important enzymes, but with a high degree of purity and specificity. The sources of these kinds of enzymes should be selected with great care and precautions to prevent any possibility of undesirable contamination by incompatible material and also to enable ready purification. A summary of different applications of therapeutic fungal enzymes for diverse health problems is presented in Table 1.

α-Amylases

α-Amylases (EC 3.2.1.1) are glycoside hydrolase enzymes that catalyse starch into low molecular weight sugars and dextrins, being present in the digestion of carbohydrates. Different species of fungi are able to produce α-amylases, being *Aspergillus* the most common microorganism (Saranraj and Stella 2013). The commercialization of amylases started in 1984, as a pharmaceutical support for the treatment of digestive disorders. Moreover, amylases find applications in the pharmaceutical and fine chemical industries, and in medical diagnosis (Gupta et al. 2003). For instance, blood serum amylase may be measured, and a normal concentration is between 23-85 IU/L (Hardwicke et al. 2010). A higher concentration indicates medical abnormal conditions, including acute inflammation of the pancreas, perforated peptic ulcer, torsion of an ovarian cyst, among others. In fact, *α*-amylase activity levels in human body fluids are extremely important in pancreatitis, diabetes and cancer research (Das et al. 2011; Gurung et al. 2013). As a therapeutic, α-amylases can be applied in the treatment of cancer, infection, and wound healing, some being approved by the US Food and Drug Administration (FDA), and others are in advanced stages of development (Azzopardi et al. 2016). These α-amylases offer promising solutions for drug delivery and combined diagnostic-therapeutic applications (Azzopardi et al. 2016). As an example, α-amylase can be used as a component in several pharmaceutical enzyme-

replacement preparations for the treatment of pancreatic insufficiency (Somaraju and Solis-Moya 2014). Furthermore, glucose, the product of α-amylase catalysis, has been shown to inhibit the production of the toxins responsible for the onset and progression of gangrene, lending some antibacterial efficacy (Méndez et al. 2012).

Chitinases

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that hydrolyse the β-1,4-glycosidic bonds of chitin (Bhattacharya, Nagpure and Gupta 2007). Fungal chitinases belong to the glycoside hydrolases family presenting a similar amino acid sequence. Chitinases can be divided into two main classes: i) endo-chitinases, which cleave chitin randomly at internal sites, generating soluble low molecular oligomers of N-acetylglucosamine, and ii) exo-chitinases, which catalyse the progressive release of di-acetylchitobiose and cleave the oligomeric products of endochitinases and chitobiosidases generating monomers of N-acetylglucosamine (Novotná, Fliegerová and Šimůnek 2008). Chitin is the component of cell wall of many pathogenic organisms, including fungi, protozoa, and helminths and is a good target for antimicrobials (Fusetti et al. 2002). These enzymes have antimicrobial properties and can be used in the treatment of several infections and also show activity against new drug-resistant bacterial strains (Nagpure, Choudhary and Gupta 2014). Recently, it has also been proved that mammalian chitinase can be used against dermatopathogenic fungi and against *Trichomonas vaginalis*, a protozoan parasite (Chen, Shen and Wu 2009; Loiseau, Bories and Sanon 2002). In the same way, amino oligosaccharide hydrolysates, the product of the hydrolysis of chitin, has an important role in regulating the life metabolism, presenting anti-inflammatory actions and has therapeutic effects on intestine and gastrointestinal ulcers besides improving immunity and anti-tumour activity (Nagpure, Choudhary and Gupta 2014). In summary, the results from the literature indicate that chitinase enzymes can be applied as new drug therapies for human healthcare.

L-Asparaginase

L-asparaginase (LA), (EC3.5.1.1; l-asparagine aminohydrolase), is widely distributed in nature, being found not only in plants and tissues, but also in fungi. In fact, microorganisms are a better source of LA than animals or plants, due to their easy fermentation production since they grow in simple and inexpensive substrates (Lopes et al. 2017). Different fungi can produce LA with potential in cancer treatment, more specifically for leukaemia, acute lymphocytic leukemia, with improved therapeutic results (Souza et al. 2017). The tumour cells lack aspartate-ammonia ligase activity,

responsible for the non-essential amino acid LA synthesis. Normal cells remain unaffected, since they are able to synthesize LA for their need, while generating a free exogenous LA concentration decline, which triggers, in the tumor cells, a state of fatal starvation. Nevertheless, LA intravenous administration effectiveness depend if the blood levels of asparagine are extremely low (Gurung et al. 2013; Mane and Tale 2015). There are two different types of LA, type I and type II, which differ in their affinities for L-asparagine substrate. The type I is a cytoplasmatic enzyme that shows low affinity to asparagine, while type II is in the periplasmic space with a high affinity to substrate. Thus, only type II can be applied as therapeutic drug, due to the enzyme's antitumor activity (Yun et al. 2007). LA is mainly produced by bacteria (Bacelar et al. 2016). However, the production process is very expensive beside the side effects of LA from bacteria. For instance, LA from fungi such as *Penicillium* sp. and *fusarium* sp. are an alternative since extracellular activity is easier to purify than the intracellular LA produced by bacteria (Bacelar et al. 2016). LA from *Aspergillus terreus* exhibited a better anti-tumour effect then LA from bacteria (De-Angeli et al. 1970). Polyethylene glycol modified LA from *A. terreus* showed effectiveness against proliferation of two leukemic cell lines (Battiston Loureiro et al. 2012). Beside this fact, marketable LA is not being produced by fungi, LA is already industrialized, being commercialized as: Crastinin®, Elspar®, Ki-drolase®, Leunase®, Asparaginase medac™, Erwinase®, Spectrila® (Souza et al. 2017).

Cytosine Deaminases

Cytosine deaminases (EC 3.5.4.1) are nucleoside-metabolizing enzymes catalysing the hydrolytic deamination of cytosine to uracil and ammonia. Originally, these enzymes are only found in fungi and prokaryotes. In addition to cytosine, cytosine deaminases convert the 5-fluorocytosine (enzyme substrate) to the chemotherapeutic drug 5-fluorouracil. This compound is a very potent inhibitor of thymidine synthase, disrupting de novo production of thymidine monophosphate, which makes this enzyme a highly promising antitumor biological. The cytosine deaminase/5-fluorocytosine method is the most studied suicide gene (gene-directed enzyme prodrug) therapy approach (Asadi-Moghaddam and Chiocca 2006). Due to the promising therapeutic action of cytosine deaminases mediated 5-fluorouracil deamination in cancer cells, a hard effort has been carried out to develop new approaches for advanced tumour therapy. For instance, cytosine deaminases have been studied for the treatment of different types of cancer such as endometrial, colon, prostate, breast and gliomas (Yi et al. 2011; Nyati et al. 2002; Miyagi et al. 2003; Li et al. 1997; Wang et al. 1998; Kievit et al. 1999; Kievit et al. 2000). 5-fluorouracil can also be used as an antifungal drug, which is generally used to treat fungal infections in humans (Waldorf and Polak 1983).

Proteases

Proteases (EC3.4.21-24, peptidases or proteolytic enzymes) hydrolyse the peptide bonds of proteins into other proteins, peptides and amino acids, being found in all living organisms (Souza et al. 2015). There are eight types of proteases which are based on their enzymatic catalysis and on the nature of the functional group at the active site: asparagine, aspartic, cysteine, glutamic, metallo, serine, threonine (Yike 2011). Proteases can be obtained by many fungal cultures since they are extracellular enzymes (Monod et al. 2002). These enzymes can be produced by fungi such as *Trichoderma pseudokoningii*, *Meloidogyne incognita*, *M. anisopliae* and *Beauveria bassiana* (Yike 2011). In the therapeutic field, proteases are a promising and well-recognized growing class of biologics due to improved clinical applications such as keratin elimination in acne or psoriasis, human callus elimination and keratinized skin degradation, vaccine preparation for dermatophytosis therapy, ungual drug delivery increase, scar removal and epithelia regeneration, and acceleration of healing processes (Brandelli, Daroit and Riffel 2010; Vignardet et al. 2001; Chao et al. 2007; Souza et al. 2015). FDA has approved twelve proteases, and other new proteases are in clinical development (Craik, Page and Madison 2011). The first protease approved by the FDA in 1978 is the drug u-PA (urokinase) used for thrombolytic therapy, which provides an alternative to the surgical removal of emboli (Craik, Page and Madison 2011). Proteases, marketed as Activase® (Genentech), are used to treat heart attacks (myocardial infarction) (Bode et al. 1996). This enzyme was the first haemophilia drug used for an efficient blood clotting and maintenance of normal haemostasis (Howard et al. 2007). Another application includes its use as surgical sealant (thrombin), a constituent of the coagulation cascade, converts fibrinogen into fibrin monomers that then multimerize to form stable blood clots. Plasma serine protease has been studied as a potential drug to alleviate the hypercoagulable state and thus permit the treatment of myriad effects resulting from sepsis, however, the clinical use is limited due to the pleiotropic effects of plasma serine protease (Yan et al. 2001). Proteases as digestive aids have been applied in patients with cystic fibrosis originated from a deficiency in pancreatic enzymes. Pancreatic enzyme replacement involves a defined mixture of proteases, lipases and amylases which can be used as a therapy. The commercial drug, Zenpep® (Eurand), is an approved pancreatic enzyme for cystic fibrosis (Wooldridge et al. 2009). Proteases can also be used to improve the digestion through the combination of proteases and other digestive enzymes for the treatment of pancreatic insufficiency (Craik, Page and Madison 2011).

Lipases

Lipases are triacylglycerol acyl hydrolases (EC3.1.1.3) catalysing the hydrolysis of fats and oils to yield glycerol and free fatty acids (Singh and Mukhopadhyay 2012). This type of enzymes is involved in catalytic reactions, such as aminolysis, alcoholysis, esterification, interesterification, transesterification, and acidolysis (Singh and Mukhopadhyay 2012). The hydrolysis essentially occurs at the aqueous/organic interface (Sharma and Kanwar 2014). Lipases can be found in nature and have been isolated from various sources. Lipases can feasibly be produced by filamentous fungi and yeasts. Fungal lipases are extracellular in nature, and they can be recovered without difficulty, which significantly reduces its production costs (Gopinath et al. 2013). Extracellular lipases have been produced by a high variety of fungi, such as *Lipomyces starkeyi*, *Rhizopus* sp., *Geotrichum candidum*, *Pencillium* sp., *Acremonium strictum*, *Candida rugosa*, *Humicola lanuginosa*, *Cunninghamella verticillata*, and *Aspergillus* sp. (Sztajer, Maliszewska and Wieczorek 1988; Gopinath et al. 2003; Helena Sztajer and Maliszewska 1989; Okeke and Okolo 1990; Wu, Guo and Sih 1990; Iizumi, Nakamura and Fukase 1990; Gopinath et al. 2002; Gopinath, Hilda and Anbu 2000; Thota et al. 2012). For instance, lipase from *C. rugosa* has been used for the synthesis of drugs, such as lovastatin (reduction of cholesterol), via a regioselective acylation of a diol-lactone precursor with 2-methylbutyric acid (Yang et al. 1997; Gurung et al. 2013). In fat, fungal lipases have gained a great attention as a therapeutic agent and have high potential in medicine due to their substrate specificity and unique properties (Lott and Lu 1991; Gurung et al. 2013). Moreover, lipases are used in cancer treatment since some types of cancer as colorectal and pancreatic, may be influenced by the levels of triglycerides, and consequently, the role of lipases, that catalyse the hydrolysis of plasma triglycerides is also realized (Takasu et al. 2012). Lipases are also used for the treatment of pancreatic insufficiency, a condition affecting patients with cystic fibrosis and for the treatment of fat malabsorption in patients with human immunodeficiency virus (HIV) (Schibli, Durie and Tullis 2002; Carroccio et al. 2001). This enzyme is commercialised (TheraCLEC Total™) as a mixture of pancreatic enzymes (lipase, amylase and protease mix). In addition to this, lipases are used in the treatment of malignant tumours. Furthermore, lipases can also be applied in diagnosis, since its presence or high level can be the sign of a specific infection or disease such as pancreatic injury and acute pancreatitis (Lott and Lu 1991; Gurung et al. 2013).

Glucose Oxidase

Glucose oxidase (GOx) (EC 1.1.3.4) is an endogenous oxidase-reductase broadly distributed in living organisms, including fungus such as *Penicillium notatum*, whose

non-toxicity, biocompatibility and particular catalysis against β-D-glucose, enables its use in cancer diagnosis and therapeutics methods (Fu et al. 2018). Particularly, GOx catalyzes the oxidation of glucose into gluconic acid and H_2O_2, which drives reactive oxygen species (ROS) stimulation promoting cancer cell death (Huggett and Nixon 1957; Imlay, Chin and Linn 1988; Fu et al. 2018). Cancer cells demand glucose due to their high energy need for growth, as they experience low adenosine triphosphate-productive anaerobic glycolysis in the absence of oxidative phosphorylation (Warburg 1956; Fu et al. 2018). Therefore, tumor growth and proliferation are inferred from cancer cells glucose levels (Fu et al. 2018). Due to cancer cells high energy need for growth, uncontrolled proliferation and altered metabolic pathways, more glucose is required, whose proliferation can be monitored via its glucose use (Fu et al. 2018). In the presence of oxygen, GOx catalyzes the oxidation of glucose and production of gluconic acid and H_2O_2, multiple types of therapy such as cancer starvation therapy, hypoxia-activated therapy, pH-responsive drug release, and oxidation therapy have been developed (Fu, Qi, Lin and Huang 2018). Tumor microenvironment (TME) acidity enhances too, which helps in the activation of a pH-responsive drug delivery system (pH-responsive drug release) (Fu et al. 2018; Sato, Yoshida, Takahashi and Anzai 2011). However, tumor heterogeneity, diversity and complexity require the development of multimodal synergistic therapies, in which several types of therapies are combined, as is shown below (Fanet al. 2017; Fu et al. 2018). Zhao et al. successfully developed a glucose-responsive nanomedicine of GOx-polymer nanogels, which modulates H_2O_2 production for melanoma starving and oxidation therapy via constraining GOx in the tumor. This new therapeutic strategy revealed an high anti-melanoma efficacy, while not revealing systemic toxicity (Zhao et al. 2017). In another work, it was proposed a starvation and hypoxia-activated therapy alliance via the co-administration of liposome-GOx and liposome-AQ4N, a hypoxia-activated prodrug, which achieved effective tumor growth inhibition, without important toxic side effects in the mouse tumor model (Zhang et al. 2018). Li et al. managed to amplify the synergistic effects of long-term cancer starvation therapy, along with photodynamic therapy (PDT), creating a cancer targeted cascade bioreactor, mCGP, by inserting GOx and catalase in the cancer cell membrane-camouflaged porphyrin metal−organic framework (MOF) of a porous coordination network (PCN-224) (Li et al. 2017). Zhou et al. established a tumor-targeted nanoplatform, which takes advantage of both, tumor starvation and low-temperature photothermal therapy (PTT) by packing porous hollow Prussian Blue nanoparticles with GOx, followed by redox-cleavable linkage of hyaluronic acid (HA) to their surface, allowing CD44-overexpressing tumor cells specific bind, enhancing antitumor efficacy (Zhou et al. 2018). Fan et al. developed an unparalleled coefficient cancer starving-like/gas therapy, through the use of hollow mesoporous organosilica nanoparticle (HMON), which co-delivers GOx and L-Arg, allowing L-Arg oxidation into nitric oxide (NO) by generated acidic H_2O_2, enhancing gas therapy with minimal adverse effects

(Fanet al. 2017). Li et al. orchestrated a tumor-based oxidation/chemotherapy treatment by specific activation at tumor sites, based on GOx-loaded polymersome nanoreactors (GOD@PCPT-NR), which are exclusively triggered by tumor acidity to in situ generate H_2O_2 and further cause the fast release of camptothecin (CPT), an anticancer drug (Li et al. 2017). Nevertheless, there are still unexploited potential of enzyme reactions, which can be applied in many medical research areas (Gurung et al. 2013).

Since GOx displays high selectivity and sensitivity towards glucose, this enzyme can also be used for electrochemical cancer and diabetes mellitus diagnosis and as a biosensor. These methodologies are viable since the catalysis of glucose by GOx, using an electrode, induces an electric current in ratio of the glucose concentration (Wang 2008; Fu et al. 2018). GOx-based biosensors show massive potential for diagnosis of cancer, because GOx catalysis reaction allows the amplification of cancer biomarkers signals via specific target ligands which recognize these biomarkers (Fu et al. 2018). GOx-based biosensors can be classified as oxygen-based, pH sensitive, H_2O_2 dependent such as: H_2O_2-based electrochemiluminescence (ECL) biosensors, H_2O_2-based photoelectrochemical (PEC) immunosensors, H_2O_2 regulates metal-based biosensors, and GOx-based electrochemical biosensors (Fu et al. 2018). Hereby, oxygen consumption analysis using a specific probe by oxygen-based biosensors is followed by glucose levels extrapolation of tumor cells; medium pH decrease due to glucose oxidation to gluconic acid can be detected by a pH-sensitive transducer, which converts pH changes into an electrical signal, allowing single cancer cell glucose concentration calculation (Fu et al. 2018). Furthermore, H_2O_2-based ECL biosensors allow DNA target detection through sensitive ECL signal-change of the $Ru(bpy)_3^{2+}$–tripropylamine (TPrA) system due to H_2O_2 concentration changes, taking advantage H_2O_2 is an ECL quencher for $Ru(bpy)_3^{2+}$. H_2O_2 designed as an ultrasensitive PEC immunosensor for cancer biomarkers detection, since H_2O_2 is able of photocurrent amplifying. H_2O_2 -induced growth of small-sized metal nanoparticles are applied in biosensors development, when in reaction to a biorecognition event, there is a shift in their size, aggregation, and localized surface plasmon resonance (LSPR) (Fu et al. 2018). Additionally, GOx specificity and unique reactivity of manganese dioxide (MnO_2) nanosheets allow glucose detection through trimodal self-indication method, namely fluorescence, ultraviolet-absorbance and magnetic resonance signals (Chen et al. 2017; Fu et al. 2018).

Laccases

Laccases (EC 1.10.3.2) (*p*-diphenol: dioxygen oxidoreductases; benzenediol dioxygen oxidoreductases) are multicopper oxidases catalyzing both phenolic and non-phenolic compounds (Giardina et al. 2010). This type of enzyme only uses molecular oxygen as the electron acceptor and the substrate to initiate catalysis, i.e., electrons are

removed from the reducing substrate molecules and transferred to oxygen to produce water (Giardina et al. 2010). Laccase is an extracellular enzyme secreted by various fungi during their secondary metabolism. Among fungi, ascomycetes, basidiomycetes, and deuteromycetes can produce laccase, and white-rot basidiomycetes are the best laccase producers (Arora and Sharma 2010). Laccase production can be achieved by submerged or solid-state fermentation processes. Laccase has received great attention from both academia and industry due to these simple requirements and ability to degrade a diversity of substrates (Chaurasia, Bharati and Sarma 2017). Recently, laccase has a high potential application in the therapeutic field, principally against cancer (Guest and Rashid 2016). Laccases have shown anti-proliferative activities, primarily against breast cancer and liver carcinoma cell lines (Guest and Rashid 2016). Laccase from *Pleurotus cornucopiae* was evaluated for the deactivation of HIV-1 reverse transcriptase and the enzyme showed HIV-1 inhibitory activity (Wong et al. 2010). In another study, laccase from *P. ostreatus* was able to inhibit hepatitis C virus entry into peripheral blood cells and hepatoma cells (El-Fakharany et al. 2010).

PURIFICATION PROCESSES OF THERAPEUTIC FUNGAL ENZYMES

As part of the production of therapeutic fungal enzymes, there are three core technologies areas, namely production, purification and the biological activity of the purified enzymes (Figure 1), being the purification the critical process to apply these enzymes in the pharmaceutical industry. In fact, the high-cost production of biopharmaceuticals is usually associated with the purification steps (downstream process). Thus, it has become crucial to investigate how to replace traditional methods with efficient and cost-effective alternative techniques for recovery and purification of fungal enzymes from the fermentation medium. One of the major challenges of the production of therapeutic fungal enzymes is closely related to the reduction of the purification steps in a way to obtain one single-step process. In fact, different purification techniques having different conditions become suitable for one but not for other enzymes, i.e., a slight change in pH above or below the optimum value may change the activity of the enzyme, which can be a reason for a variation in the percentage yield of the same enzymes using different purification strategies (Polizeli, Jorge and Terenzi 1991). Thus, after the purification process of the enzyme is a pre-requisite to study their structure-function relationships and biochemical properties (Gupta et al. 2003). Moreover, after the purification process, the purity of the enzyme and its molecular weight is usually checked using SDS-PAGE (molecular weight is determined by running the marker and purified enzyme) (Patil 2010).

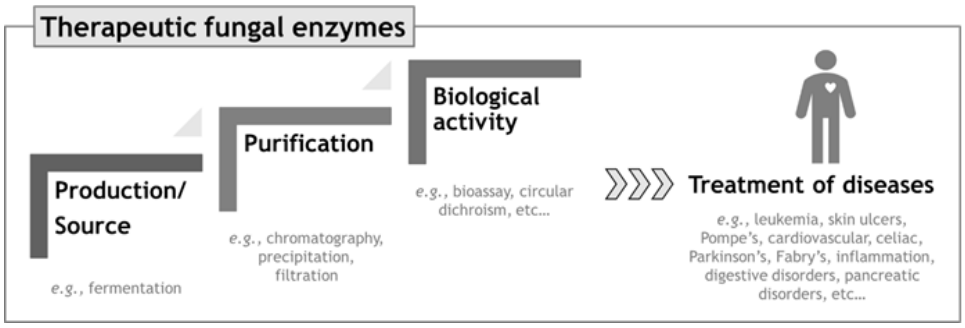

Figure 1. Core technology areas for the production of therapeutic fungal enzymes.

Traditional Processes to Purify Fungal Enzymes

Different processes are used to purify fungal enzymes, since in the pharmaceutical industry a high purity level is required. The purification of fungal enzymes usually includes a first step regarding the concentration of proteins from the crude enzyme extract, by precipitation with organic solvents (ethanol, acetone, among others) or ammonium sulphate (Table 2). Subsequently, a sequence of various steps has been applied, including dialysis and chromatography, e.g., ion exchange, hydrophobic, and gel filtration chromatography (Table 3).

Table 2. Principal techniques used in the purification of enzymes (adapted from Kiiskinen et al. 2004)

Property	Method	Scale
Size or mass	Centrifugation	Large or small
	Dialysis, Ultrafiltration	Mainly small
	Gel filtration chromatography (GFC)	Mainly small
Polarity		
(a) Charge	Ion-exchange chromatography (IEC)	Large or small
	Electrophoresis	Mainly small
(b) Hydrophobic character	Hydrophobic chromatography (HIC)	Mainly small
Solubility / Precipitation	Change in pH	Mainly large
	Change in ionic strength	Large or small

Usually, ammonium sulphate is used to precipitate the enzymes. This phenomenon is related to the 'salting out' effect, i.e., the addition of salt in excess in the aqueous extract leads to the "competition" of hydrophilic solutes (salt and enzyme) for the water molecules, with the formation of hydration complexes between the salt and the water. Consequently, the enzymes stay without water and occurs their precipitation. The quantity of salt required for the precipitation of specific enzyme is directly dependent on its molecular weight. Most of the authors have tried 30–80% (w/v) ammonium sulphate

concentration for fungal enzyme extraction (Table 3). Although salt precipitation process brings about conformational changes in the protein, it does not denature them. In fact, these protocols are usually performed at lower temperatures up to 4°C.

Table 3. Summary of enzyme purification from fungi using sequential multi-step purification processes

Enzyme		Fungus	Purification method	Specific activity (U/mg)	Purification fold	Yield of protein (%)	Ref.
Chitinases	---	*Trichoderma viride*	IEC + GFC	1.7	14.3	11.7	(Omumasaba, Yoshida and Ogawa 2001)
	Type I	*Stachybotrys elegans*	Precipitation + IEC + HIC	5.6	13.5	8	(Duo-Chuan, Chen and Jing 2005)
	Type II			4.3	9	6.1	
	Type I	*Penicillium aculeatum NRRL 2129*	IEC + GFC	8	94.1	29.1	(Binod et al. 2005)
	Type II			0.7	7.6	9.6	
	Type III			5.3	62.8	31.4	
	Type IV			3.7	43.7	29.9	
	---	*T. lanuginosus*	Precipitation + IEC + GFC	35.5	10.6	1.4	(Guo et al. 2008)
	---	*G. catenulatum HL-1-1*	Precipitation + chromatography + electrophoresis	12.2	10.1	3.2	(Gui-Zhen Ma 2012)
	---	*Rhizopus oryzae*	IEC + GFC	165.2	4.3	19.7	(Nagpure and Gupta 2013)
	---	*Aspergillus terreus*	Precipitation + GFC + IEC	182.1	5.2	12	(Farag et al. 2016)
L-asparaginase		*F. velutipes*	Ultrafiltration + GFC	n.d.	n.d.	n.d.	(Eisele et al. 2011)
		Aspergillus aculeatus	Precipitation + dialysis + GFC	207	267.8	0.5	(Dange and Peshwe 2011)
		Aspergillus aculeatus	Precipitation + GFC + IEC + filtration	29.6	38.2	7.9	(Dange and Peshwe 2011)
		Cladosporium sp.	Precipitation + IEC + GFC	83.3	867.7	n.d.	(Kumar and Manonmani 2013)
		R. miehei	Nickel-iminodiacetic acid column	1984.8	2.6	48.8	(Huang et al. 2014)
		Aspergillus flavus	Precipitation + GFC + IEC	176.5	7.8	25	(Patro et al. 2014)
		Aspergillus fumigatus WL002	Ultrafiltration + precipitation + GFC	355	232	n.d.	(Ghosh and Pramanik 2015)
		Aspergillus sp. *ALAA-2000*	Precipitation + GFC	n.d.	8.3	43.6	(Ahmed 2015)

Table 3. (Continued)

Enzyme		Fungus	Purification method	Specific activity (U/mg)	Purification fold	Yield of protein (%)	Ref.
		Fusarium culmorum ASP-87	Precipitation + IEC + GFC	16.7	14	2.6	(Janakiraman 2015)
		Penicillium cyclopium	Precipitation + GFC	39480	52.3	4.5	(Shafei et al. 2015)
		Streptomyces brollosae NEAE-115	Precipitation + IEC	76.7	7.8	7.3	(El-Naggar et al. 2018)
Cytosine Deaminases		*Saccharomyces cerevisiae*	Precipitation + chromatography + GFC	56.3	21.7	60	(Hayden et al. 1998)
Proteases		*Aspergillus parasiticus*	Precipitation + GFC + IEC	3530	200	17	(Tunga, Shrivastava and Banerjee 2003)
		Engyodontium album BTMFS10	Precipitation + IEC	3148	16	0.6	(Chellappan et al. 2011)
		Aspergillus clavatus ES1	Precipitation + precipitation + GFC + IEC	37600	7.5	29	(Hajji et al. 2007)
		H. rhossiliensis	Precipitation + GFC + IEC	123.1	16	7.1	(Wang, Wu and Liu 2007)
	---	*G. putredinis*	Precipitation + GFC	14.9	8.6	36.5	(Savitha et al. 2011)
	---	*T. harzianum*	Precipitation + GFC	14.5	11.5	29.4	(Savitha et al. 2011)
	---	*Beauveria* sp.	Precipitation + IEC	60.4	10	38.6	(Savitha et al. 2011)
	---	*Botrytis cinerea*	Dialysis + IEC + GFC	58216	19	5.6	(Abidi et al. 2011)
	---	*Aspergillus parasiticus*	Precipitation + dialysis + IEC	106232	2.2	2.5	(Anitha and Palanivelu 2013)
Laccase		*Aspergillus nidulans*	IEC + GFC + IEC	892.7	557.3	9	(Scherer and Fischer 1998)
		M. albomyces	Ultrafiltration + IEC + HIC + GFC	1136	292	17	(Kiiskinen et al. 2004)
		M. grisea	Precipitation + IEC + GFC	225.9	282	11.9	(Iyer and Chattoo 2003)
		Mauginiella sp.	Precipitation + IEC + HIC	1449	100	40	(Palonen et al. 2003)
		M. albomyces	HIC + IEC + GFC	560	11	40	(Kiiskinen et al. 2004)
		Trametes sanguinea MU-2	Dialysis + IEC + GFC	689	n.d.	73	(Han et al. 2005)

Table 3. (Continued)

Enzyme		Fungus	Purification method	Specific activity (U/mg)	Purification fold	Yield of protein (%)	Ref.
		Trametes versicolor CCT 452	Precipitation + IEC + GFC	101	34.8	38.4	(Minussi et al. 2002)
		P. sajor-caju MTCC 141	Precipitation + ultrafiltration + GFC	n.d.	10.7	3.5	(Sahay, Yadav and Yadav 2008)
		Ganoderma sp. *MK05*	Precipitation + IEC	2.3	3.1	13.6	(Khammuang and Sarnthima 2009)
		Pleurotus sp.	Precipitation + IEC + GFC	2600	72.2	22.4	(More et al. 2011)
		Marasmius sp. *BBKAV79*	Dialysis + GFC + IEC	n.d.	376.7	13.5	(Vantamuri and Kaliwal 2016)
		Pestalotiopsis sp. *CDBT-F-G1*	Precipitation + Precipitation	31700	14	84.0*	(Yadav et al. 2019)

*partial purification.
n.d.- not determined.

In the chromatographic methods, the selection of the appropriate method among the variety of chromatographic methods is dependent upon the type of enzyme, impurities, charge, size of the molecules and purity of the extract. Hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), size exclusion chromatography (SEC) and gel filtration chromatography (GFC) are the chromatographic methods more used for the purification of fungal enzymes from various sources as mentioned in Table 3. The combination of more than one chromatographic operation is usually employed to improve the purification fold. Kiiskinen et al. (2004) used HIC, IEC, as well as GFC for purification of laccase from *Trichoderma reesei* increasing the purification fold from 2 to 11. However, laccase from *Pleurotus* sp. was purified up to 72.2 fold using IEC and GFC, but only after the precipitation with ammonium sulphate (More et al. 2011). Thus, pre-treatment of the crude extract is essential to achieve an efficient purification with chromatography, with many authors applying extraction methods such as, ammonium sulphate precipitation, before adopting chromatography (Table 3), as mentioned before. The application of chromatography seems to be very efficient to obtain a high enzyme purity. More recently, a different chromatography was reported, i.e., affinity chromatography using a nickel-iminodiacetic acid column (More et al. 2011).

Among the selected studies summarized in Table 3, the purification of fungal enzyme employs at least three 3 steps, (1) precipitation, (2) GFC and/or (3) IEC, to obtain a high purity. However, these protocols involve several chromatographic steps, make the

process costly and time-consuming (Martínez-Aragón et al. 2009). A solution to suppress these and other shortcomings related with the chromatographic methods, can be the synergism between different unit operations involving easier and cheaper techniques that can be scaled in an industrial context (Dux et al. 2006). More specifically, other low-resolution separation methods have been studied, precipitation and aqueous two-phase systems (ATPS).

ALTERNATIVE PROCESSES TO PURIFY FUNGAL ENZYMES

Precipitation

Besides the addition of the salts, the organic solvents such as acetone and ethanol are used to precipitate the proteins, as mentioned before. The solvent percentage change can also be used for the separation of different type of proteins. Kumarevel et al. (2005) reported a stepwise purification strategy for fungal lipases from *C. verticillata*, using precipitation with 50% acetone with a gradual increment of 5% acetone as the important step to minimize the impurities as much as possible, avoiding many chromatographic purification steps. Moreover, Yadav et al. (2019) could also partially purify laccase from *Pestalotiopsis* using precipitation method, through two steps: first with a mixture of ammonium sulfate (13-fold purification) and then with acetone (14-fold purification). However, in both studies presented here, the enzyme obtained was only partially purified, demonstrating therefore, the need to associate other techniques to obtain a pure enzyme.

Phase Separation

Aqueous Two-Phase Separation

Liquid-liquid extraction (LLE) seems to be more viable than traditional methods since several features of the early processing steps can be combined into a single operation. LLE consists in the transference of certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. Aiming to avoid the use of organic solvents in LLE, in 1958, Albertson introduced the ATPS concept for the separation of (bio)molecules by their partitioning between two liquid aqueous phases (Albertsson 1958). An ATPS consists of two immiscible aqueous-rich phases based on polymer/polymer, polymer/salt or salt/salt combinations.

The practical strategies for the design of an appropriate recovery process using ATPS can be divided into four stages, namely the initial physicochemical characterization of the

feedstock, selection of the type of ATPS, selection of the system parameters, and evaluation of the influence of the process parameters upon the product recovery/purity (Benavides and Rito-palomares 2008) (Figure 2). More specifically, different physicochemical properties affect the partition of the biomolecules in the two-phase systems, like surface hydrophobicity, molar mass, isoelectric point and components of the system and some other factors that influence partitioning are concentration of polymer or surfactant, salt addition and pH.

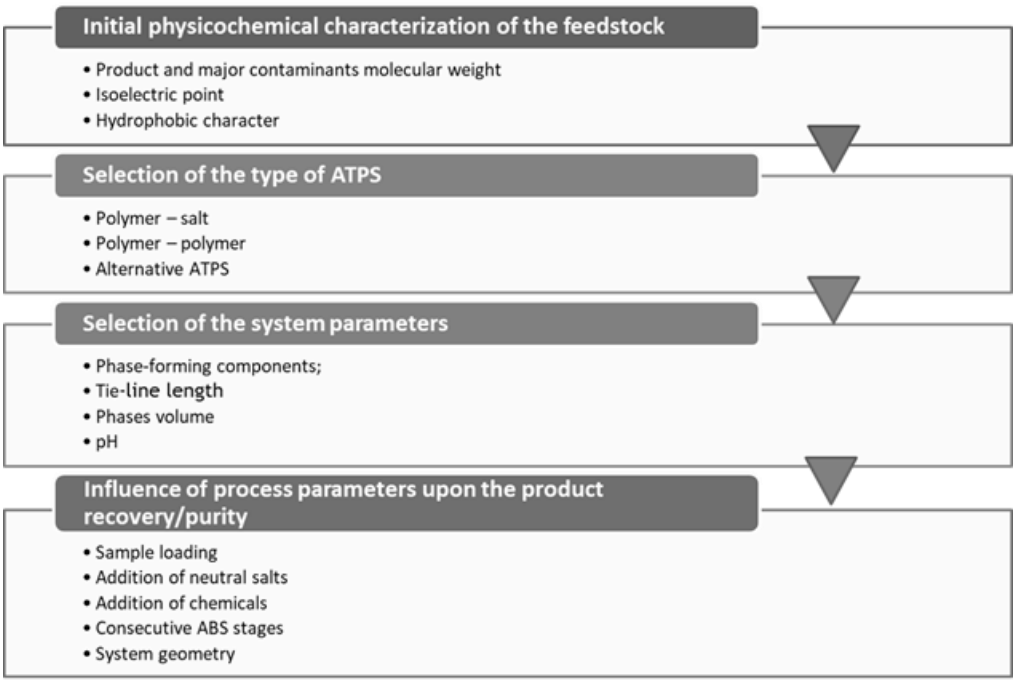

Figure 2. Simplified representation of the strategies for the design of the recovery of biological products using aqueous biphasic systems (ABS) (adapted from Benavides and Rito-palomares 2008).

ATPS are favourable for the extraction of enzymes due to the high amount of water present in the phases (Freire et al. 2012). Moreover, these systems are of low-cost when compared with chromatographic strategies, more environmentally benign since the use of volatile organic compounds is avoided, allow the scale-up and lead to high extraction performance and purity levels. For instance, a comparison between a purification process using IEC, with a previous acetone fractionation, and an ATPS extraction, demonstrated superior overall yield of the enzyme α-galactosidase in ATPS (11.5 vs 87.6%, respectively) (Naganagouda and Mulimani 2008). Other widely used technique for the purification of enzymes, as mentioned before, consists on the precipitation of the target molecule with ammonium sulphate. A comparison between the two methods was already performed and ATPS exceeded the precipitation method, achieving a greater recovery yield (184 % vs 53%) and purification factor (7.2 vs 4.8) of laccase (Schwienheer et al.

2015). Thus, it is clear that ATPS constitutes an interesting alternative method over other conventional separation processes (Figure 3), and in particular for enzymes, and so, these systems have been subject of increased attention and research.

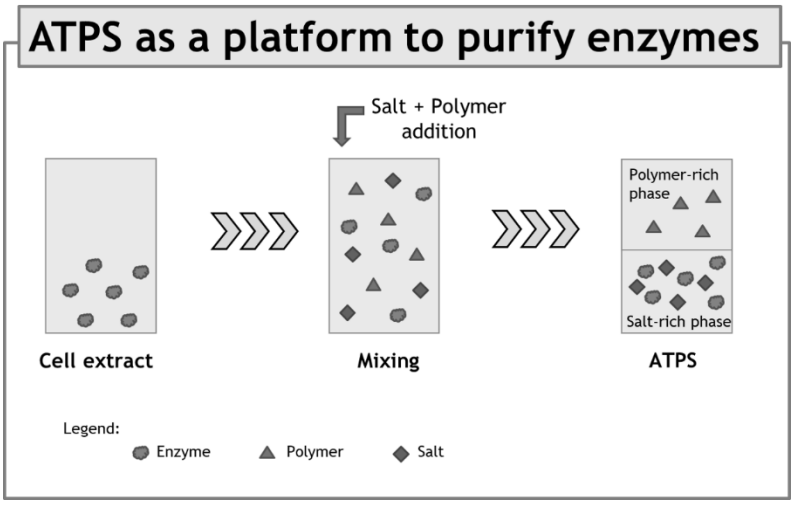

Figure 3. A proposed strategy for the purification of enzymes from fermentation broth.

For the fungal enzyme extraction by ATPS, most of the authors have used polymer-salt based ATPS as mentioned in Table 4. These systems are mainly composed of biodegradable organic salts, such as sodium citrate (Table 4). The maximum of protein yield (130%) using the conventional ATPS was observed using PEG 10000 and buffer citrate salt concentration of 15-20% and 8-15%, respectively (Porto et al. 2008). Moreover, Alhelli et al. (2016) have used ATS composed of PEG, a sodium citrate salt and added a third component, sodium chloride to successfully purify protease from *Penicillium candidum* in the salt-rich phase, increasing the purification factor. The authors observed that the sodium chloride concentrations can be a factor that display a significant influence on the purification factor (Alhelli et al. 2016).

Polymer-polymer ABS have been also investigated for the purification of fungal enzymes, for instance chitinases (Chen and Lee 1995). However, these systems display high viscosities at the coexisting phases (Martínez-Aragón, Goetheer and de Haan 2009). Furthermore, dextran is too expensive as a phase-forming component to scale-up the extraction process (Liu et al. 2012). To overcome these drawbacks, most works in literature describe the use of polymer–salt systems (Table 4) thereby decreasing the viscosity of the coexisting phases, providing a higher density difference, and thus faster separation rates, as well as by providing lower cost systems and their scale-up (Martínez-Aragón, Goetheer and de Haan 2009). These systems are mainly composed of inorganic salts, especially phosphate-based, and some biodegradable organic salts, such as sodium citrate (Table 4).

Table 4. Summary of enzyme purification from fungi using ATPS as alternative purification processes

Enzyme	Microorganism	Purification		Purification factor	Yield of protein (%)	Ref
		Type of ATPS	Additive			
Chitinases	*Neurospora crassa*	PEG 6000 22.0% + K_2HPO 10.0%	---	38.0	88.0	(Teotia, Lata and Gupta 2004)
Proteases	*Penicillium roqueforti*	PEG 4000 15.5% + Sodium Phosphate 20.0% - pH 7.5	---	3.5	n.d.	(Pericin, Madjarev-Popovic and Vastag 2008)
	R. mucilaginosa L7	PEG 6000 15.5% + Sodium Tartrate 11.5 %	---	2.5	81.1	(Lario et al. 2016)
	Penicillium candidum	PEG 8000 9.0% + Sodium Citrate 15.9%	Sodium chloride	6.8	93.0	(Alhelli et al. 2016)
	Mucor subtilissimus UCP1262	PEG 6000 30.0% + Sodium Citrate 13.2 wt%	---	10.0	100.0	(Nascimento et al. 2016)
Laccase	*A. bisporus*	PEG 1000 18.2% + Buffer Phosphate 15.0% - pH 7	---	2.5	95.0	(Mayolo-Deloisa, Trejo-Hernández and Rito-Palomares 2009)
	L. polychrous	PEG 4000 12.0% + Phosphate salt 16.0%	---	3.0	99.1	(Ratanapongleka and Phetsom 2011)
	P. sapidus	PEG 3000 13.3% + Phosphate salt 6.3%	---	1.7	92.0	(Prinz et al. 2014)
	Trametes versicolor	PEG 3000 13.3% + Phosphate salt 6.3%	---	1.9	90.0	(Prinz et al. 2014)

n.d.- not determined.

One of the most used polymers in ATPS is PEG (Table 4). Polymers offer some degree of design, for instance, by varying the length of the polymeric chains, *i.e.*, by changing their average molecular weight, or by changing the structure of the monomer unit. PEG also displays some attractive properties, such as biodegradability, low toxicity, low volatility, low melting points, high water solubility and low cost (Ferreira et al. 2016). However, the hydrophilic nature of PEG limits the polarity range between the coexisting phases in the ATPS. To overcome this limitation, recent works have introduced ionic liquids to tune the properties of PEG through the modification of its chemical structure and thus increasing the extraction yield. The use of ILs in ATPS leads to the possibility of controlling the phases' polarities by an adequate choice of the constituting ions, and so, this high tunability makes them a desirable class of extraction solvents in liquid-liquid extraction processes. In addition, it was already shown that ionic liquids could be used as adjuvants to tailor the selectivity and extraction aptitude for target biomolecules. In summary, it is clear that low amounts of ionic liquids in the

formulation of ATPS are enough to trigger complete extractions of target compounds in a single step. ATPS composed of PEG, salts and ILs (as adjuvants) are a promising alternative and more efficient method for the purification of biopharmaceuticals. Additionally, we believe that there is a requirement to study further ATPS made up of ionic liquids for the purification of fungal enzymes which appears to be a predominantly promising substitute. However, the commercial purification of fungal enzymes using ATPS still requires more exploration for its implementation. Santos et al. (2018) demonstrated that high purification performance, usually required in pharmaceutical industry, was achieved through the design of an integrated process comprising the steps production, cell disruption, and purification with an ammonium sulphate precipitation followed by the application of ATPS with ionic liquid as adjuvant, and culminating in the L-asparaginase isolation and reuse of the various phases. Additionally, the study of enzyme recuperation from phase, as well as the recycling nature of the ATPS used needs to be more explored in the future. Additional investigations regarding the effects of the phase-forming components through the protein stability and activity are also required.

Three-Phase Partitioning

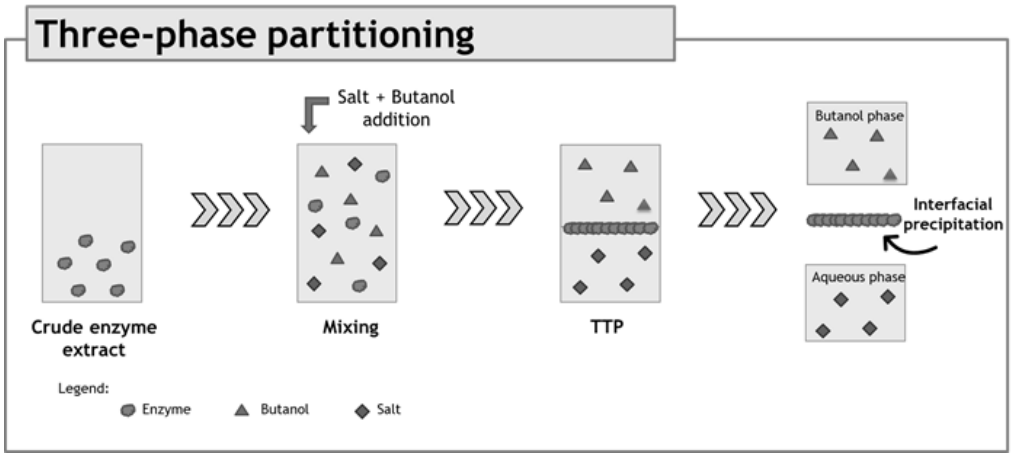

Figure 4. Scheme of three phase partitioning recovery experiment from crude enzyme extract to three distinct separated phases.

Three-phase partitioning (TPP) is an upcoming bio-separation technique developed for the extraction of proteins, especially enzymes from multi-component systems, due to their ability to concentrate proteins from crude broths with higher purification than conventional concentration methods (Gagaoua and Hafid 2016). The principle of this emerging tool consists in mixing the crude protein extract with solid salt (mostly ammonium sulphate) and an organic solvent, usually butanol in order to obtain three phases, i.e., involves the accumulation of the target enzyme at the liquid–liquid interface while the contaminants mostly partition to t-butanol (top phase) and to the aqueous phase

(bottom phase) (Figure 4) (Ketnawa, Rungraeng and Rawdkuen 2017). Kumar et al. (2011) revealed that butanol provided the purity (7.2-fold) and recovery (184%) of laccase from *Pleurotus ostreatus*. However, the main drawback of TPP is that the use of a volatile organic solvent such as t-butanol may limit the large-scale use of this technique (Alvarez-Guerra et al. 2014); without forgetting that, some enzymes may lose their activity in the presence of high amount of t-butanol (Ketnawa, Rungraeng and Rawdkuen 2017).

CHARACTERIZATION OF THERAPEUTIC FUNGAL ENZYMES

The activity of each enzyme depends on several parameters, such as pH, temperature, substrate, among others (Figure 5). The enzymes structure influences the parameters in which its activity is optimum, and therefore a deep knowledge on the characterization of enzymes is required. Specifically, for therapeutic fungal enzymes, its characterization is even more important, since the efficiency of a therapy depends on the knowledge of the target and the therapeutic.

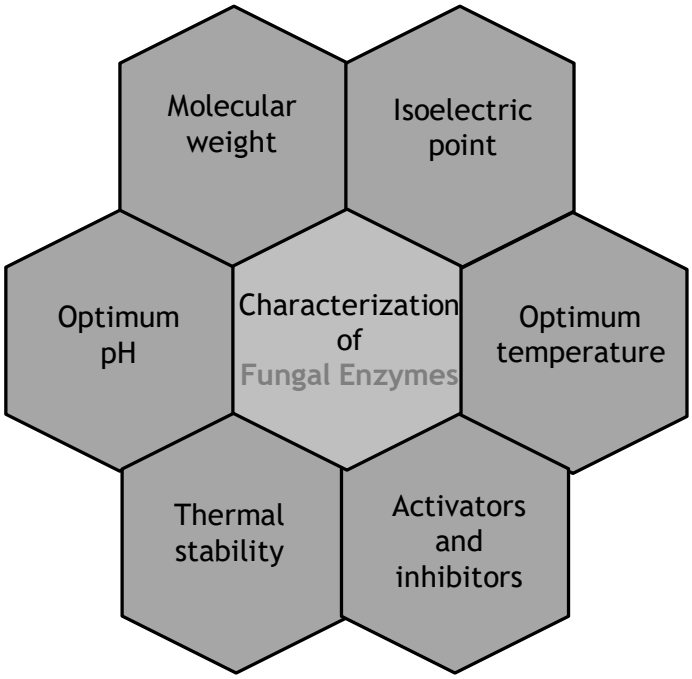

Figure 5. Parameters influencing enzyme activity.

α-Amylases

The properties of α-amylases are highly dependent on the microorganism where the enzyme is expressed. Regarding to the molecular weight, α-amylase from *A. oryzae* was estimated with 51kDa by the combined use of high-pressure silica gel chromatography and the low angle laser light scattering technique (Patel et al. 2005). The α-amylase from halophilic *Engyodontium album* was found to have a single band with relative molecular mass of 50 kDa (Ali et al. 2014). On the other hand, α-amylase from *T. lanuginosus* reveal by electrophoretic experiments a higher molecular weight of 61 kDa (Nguyen et al. 2002). Other fungal α-amylases described, for instance from *Cryptococcus flavus* presented an apparent molecular mass of 75 and 32.5 kDa (Wanderley et al. 2004; Balkan and Ertan 2010). Electro focusing of α-amylase of *A. niveus* revealed an isoelectric point of 6.6 (Silva et al. 2013), in contrast, the α-amylase from *A. flavus* presented an isoelectric point of 3.5 (Khoo et al. 1994).

Substrate Specificity and Effect of Substrate on α-Amylases Activity

As holds true for the other enzymes, the substrate specificity of α-amylase varies from microorganism to microorganism. In general, α-amylases display highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen and maltotriose (Saranraj and Stella 2013). The α-amylase activity from *A. niveus* against various substrates, such as, soluble starch, amylose, amylopectin, and glycogen was investigated by Silva et al. (Silva et al. 2013). The enzyme preferentially hydrolyzed maltopentose, maltotriose, maltotetraose, and malto-oligosaccharide (G10), but sucrose, trehalose, α-cyclodextrin, β-cyclodextrin, and p-nitrophenyl α-D-glucopyranoside were not hydrolyzed (Silva et al. 2013). α-Amylase produced by *A. oryzae* reveal a maximum activity of 36.13 U/mg with 1% starch as the substrate concentration (Patel et al. 2005).

Effect of pH on α-Amylases Activity

Optimum pH is required for maximum enzyme activity (Patel et al. 2005). The pH optima of α-amylases vary from 2 to 12. α-amylases from most bacteria and fungi have pH optima from the acidic to neutral range (Saranraj and Stella 2013), since in their catalytic mechanism, an oxidation-reduction reaction is involved and for this particular reaction, the H^+ concentration should be optimum for the proper catalysis (Patel et al. 2005). The optimum pH of an extracellular amylase secreted by *A. niveus* was 6.0 (Silva et al. 2013), while α -amylase produced by *A. oryzae* showed that the maximum specific activity was obtained at pH 5 (Patel et al. 2005). Optimum α-amylase from *T. lanuginosus* activity was found in the pH range between 4.6 and 6.6 with changes less than 10% (Nguyen et al. 2002). Enzyme activity decreased drastically at pH below 4.0 or above 7.0 (Nguyen et al. 2002).

In contrast, α-amylase obtained from halophilic *E. album* showed that this enzyme was able to work better in neutral and alkaline pH ranges (Ali et al. 2014). A steady increase in enzyme activity was observed from pH 5 to 9, with the highest enzyme activity observed at pH 9.0 (Ali et al. 2014).

Effect of Temperature on α-Amylases Activity

The optimum temperature and the activity of α-amylase is related to the growth of the microorganism (Saranraj and Stella 2013). The lowest optimum temperature for α-amylases is reported to be 25 to 30°C for *Fusarium oxysporum* amylase (Saranraj and Stella 2013). The α-amylase produced by *A. oryzae* showed a maximum activity at 50°C (Patel et al. 2005) while, the optimum temperature for α-amylase from *T. lanuginosus* is exhibit at 70ºC (Nguyen et al. 2002). The α-amylase from halophilic *E. album* has been found to have optimum activity at 60ºC and retain more than 85% of its activity at high temperatures of 70-80ºC, which are considered as thermophilic range for enzymes (Ali et al. 2014).

Activators and Inhibitors on α-Amylases Activity

α-amylase is a metalloenzyme, which contains at least one Ca^{2+} ion. Many fungal amylases described in the literature are activated by metal ions (Saranraj and Stella 2013; Silva et al. 2013). It has been reported that partially purified α-amylase, particularly those of fungal origin, lose activity above 50°C but the activity could be retained in the presence of Ca^{2+} (Patel et al. 2005). In fact, α-amylase from *A. oryzae* has a specific activity of 22.03 U/mg at 50ºC and 20.93, 12.10 and 11.78 at 60, 65 and 70ºC, respectively. However, when the reaction was carried out at 65ºC in the presence of $CaCl_2$ 10 mM, the enzyme activity was even better than at 50ºC (Patel et al. 2005).

$BaCl_2$, $CaCl_2$, $HgCl_2$ and $MgCl_2$ increased the amylase activity from halophilic *E. album*, but not greater than 110% (Ali et al. 2014). In contrast, β-mercaptoethanol, EDTA, $FeCl_2$ and $ZnCl_2$ decreased the enzyme activity. The greatest inhibition occurred in the presence of $ZnCl_2$. The decrease in enzyme activity was never less than 60% by the addition of any inhibitor (Ali et al. 2014).

The activities of α-amylase from *T. lanuginosus* decreased significantly by adding 10mM of Zn^{2+} ion to reaction mixture (Nguyen et al. 2002). Moreover, Co^{2+} showed inhibitor and Ca^{2+} and Ba^{2+} activator effects (Nguyen et al. 2002).

The α-amylase from *A. niveus* showed a slight increase in its activity in the presence of many salts (Silva et al. 2013). This enzyme was activated 17, 14, 80, 28, 39, and 61 % in presence of 1 mmol/L of NH_4F, NaBr, $MnCl_2.4H_2O$, $NaH_2PO_4H_2O$, $ZnCl_2$ and β-mercaptoethanol, respectively. At 10 mmol/L, the α-amylase activity was increased in 23, 20, 16, 12, and 16%, in the presence of NH_4F, KH_2PO_4, NH_4Cl, NaCl, and $CoCl_2.6.H_2O$, respectively. $HgCl_2$, $AgNO_3$, and $Fe_2(SO_4)_3$ drastically inhibited the enzyme activity (Silva et al. 2013).

Chitinases

Chitinases, glycosyl hydrolases, have sizes ranging from 20 kDa to about 90 kDa (Javed et al. 2013). Different molecular masses ranging from 38 to 45 kDa have been reported for fungal chitinases derived from *P. cinnabarinus* (Ohtakara 1988), *T. harzianum* (Ulhoa and Peberdy 1992), *A. obclavatum* (Gunaratna and Balasubramanian 1994) and *P. communis* (Masaru Sakurada et al. 1996). De La Cruz et al. (1992) isolated three chitinases from *T. harzianum* with molecular masses ranging from 33 to 42 kDa with isoelectric points, determined by chromatofocusing and isoelectrofocusing, between 5.0 and 7.8, depending on the enzyme (Cruz et al. 1992). Cytosolic chitinase from *P. communis* were purified and a molecular mass of 42 kDa and an isoelectric point of 4.9 was estimated (Sakurada et al. 1996).

Substrate Specificity on Chitinases Activity

Activity of chitinase from *Fusarium chlamydosporum* on both colloidal and pure chitins was high (Mathivanan, Kabilan and Murugesan 1998). This is possibly due to the availability of a larger number of active sites or termini for the enzyme in the purified and colloidal chitins than in crude chitin and cell wall fragments. Chitinases from *T. harzianum* were able to hydrolyze colloidal and glycol-chitin, a β-(1-4)-N-acetylglucosamine polymer (Cruz et al. 1992). Chitinase with 33 kDa was only active on colloidal and glycol-chitin, and almost inactive, on β-(1-4)-N-acetylglucosamine. Chitinases with 37 and 42 kDa were active in colloidal, glycol-chitin and β-(1-4)-N-acetylglucosamine, and less glycol-chitosan, perhaps because chitosan is only partially deacetylated (Cruz et al. 1992).

Effect of pH and Temperature in the Chitinases Activity

Chitinase of *F. chlamydosporum* showed an optimum activity at pH 5 and was stable from pH 4 to 6 with more than 80% activity (Mathivanan, Kabilan and Murugesan 1998). The optimum temperature for this chitinase activity was at 40°C and the activity was stable up to 40°C, above which the activity sharply declined. However, chitinase from *P. communis* showed maximum activity at 60°C and stability from 40 to 60°C (Sakurada et al. 1996). Chitinases from *T. harzianum* also revealed optimal temperature and heat-inactivation temperature quite similar at 50-60ºC (Cruz et al. 1992). Cytosolic chitinase from *P. communis* revealed a higher activity at pH 6.2 at 39ºC, with 50% of the chitinase activity maintained between pH 5 and 8 (Sakurada et al. 1996). However, at pH 6.2 the chitinase activity was greatest at 60°C and 50% chitinase activity remained from 40°C to 60°C. At 65ºC, the chitinase activity decreased to 12% of the activity at 60°C (Sakurada et al. 1996).

Effect of Activators and Inhibitors on Chitinases Activity

Chitinase activity from *F. chlamydosporum* was inhibited by metals and other inhibitors to varying degrees, ranging from 5 to 100%, with $HgCl_2$ totally inhibiting the enzyme activity. A similar effect of $HgCl_2$ on chitinases of *A. obclavatum* and *P. communis* was also reported (Gunaratna and Balasubramanian 1994; Sakurada et al. 1996). Cytosolic chitinase from *P. communis* decreased its activity, with 1 mM of Ag^+ or Hg^{2+}, more than 60 % (Sakurada et al. 1996). Its activity was also inhibited by allosamidin, an analogue of N-acetylglucosamine, which has been reported to be a chitinase inhibitor (Sakuda et al. 1987). Sodium dodecyl sulfate at low concentration (1 mM) had no effect on chitinase activity, however at 10 mM inhibited chitinase activity completely. N-Ethylmaleimide, iodoacetic acid, iodoacetamide and p-chloromercuribenzoic acid at 10 mM also inhibited chitinase activity by approximately 30% (Sakuda et al. 1987).

Fungal Chitinases Activity

The purified chitinase of *F. chlamydosporum* exhibited strong antifungal activity by inhibiting the uredospore germination of *Puccinia arachidis*, with this effect being dependent on the concentration of the enzyme (Mathivanan, Kabilan and Murugesan 1998). The chitinase of *F. chlamydosporum* completely inhibited the germination of uredospores at a concentration of 30 µg/mL. At 10 and 20 µg/mL, the enzyme caused inhibition of 78 and 92%, respectively (Mathivanan, Kabilan and Murugesan 1998). Gunaratna and Balasubramanian also reported the inhibition of uredospore germination of *P. arachidis* by the chitinase of *A. obclavatum* (Gunaratna and Balasubramanian 1994). The inhibition of uredospore germination might be due to the action of chitinase on the newly formed chitin in germ tube walls (Gunaratna and Balasubramanian 1994).

The antifungal activity of *T. harzianum* chitinases was tested using an assay based upon inhibition of hyphal extension of the phytopathogenic fungi *Rhizoctonia solani*, *F. oxysporum* and *Verticillium nigerensis*, all of which have chitin in their cell walls (Cruz et al. 1992). However, none of the three chitinases caused inhibition of hyphal extension.

L-Asparaginases

LA occurs abundantly in nature from prokaryotic microorganisms to vertebrate (Eisele et al. 2011). In fact, LA can be obtained from a variety of sources, including, many mitosporic fungi genera such as *Aspergillus*, *Fusarium* and *Penicillium* (Luhana, Dave and Patel 2013). The variability in LA molecular weight from different organisms may be inferable to its genetic diversities. LA from *Fusarium culmorum* showed homogeneity and the molecular mass was estimated as 90 kDa, by SDS-PAGE analysis (Janakiraman 2015). The molecular weight of LA from *F. culmorum* (Janakiraman 2015)

is similar to LA from *Penicillium brevicompactum* (94 kDa) (Elshafei 2012) and *Trichoderma viride* (99 kDa) (Thakur et al. 2011). On the other hand, LA from *Cladosporium* sp. (Sarquis et al. 2004) and *Aspergillus niger* (Akilandeswari, Kavitha and Vijayalakshmi 2012) has a molecular weight of 117 kDa and 48 kDa, respectively.

Effect of pH on L-Asparaginases Activity

A critical factor for stability and activity of purified enzyme is the pH, as it impacts on the ionic form of the enzyme active site residues. The effect of pH on the activity of purified LA from *F. culmorum* was done over a wide range of pH from 3.0 to 11.0 at 30°C (Janakiraman 2015). The results revealed that LA was active over a broad range of pH, optimum being pH 8.0, and 100% of activity at pH 8.0 up to 24 h of incubation. Similar results were reported by LA from *P. brevicompactum* (Elshafei 2012) and *Streptomyces* sp. (Sabha, Nadia and Tarek 2013). Thakuret al. reported the opposite, with pH 7.0 as the optimum pH for the activity of LA from *T. viride* with 82% of its activity maintained after 24 h of incubation (Thakur et al. 2011). More et al. also demonstrate pH 7.0 as the optimum pH for the activity of LA from *Mucorhiemalis*, however, its stability is only retained during 4 h (More et al. 2013). Eisele et al. reported similar results to More et al., 2013, with the optimum pH for LA from *F. velutipes* being pH 7, a high stability over the broad range of pH 3–9 where was retained at least 85% of its maximum activity after 16 h (Eisele et al. 2011). LA from *A. niger* showed maximum activity at pH 6 and lowest activity at pH 3 (Luhana, Dave and Patel 2013).

Effect of Temperature on L-Asparaginases Activity

Temperature is an important physical parameter which influences the enzyme activity. The optimum temperature for LA purified from *F. culmorum* was 40 °C with a high stability during 120 min at 30-40°C and 50% of its activity retained at 60 °C for 1h (Janakiraman 2015). However, increasing the temperature, a sharp decline in the reaction rate is observed. Similar results were reported for LA purified from *Aspergillus nidulans* (Archana rani and Raja rao 2014). Native LA from *F. velutipes* showed an optimum temperature at 40ºC, being the hydrolysis of L-glutamine and L-asparagine optima at 30 ºC and 40 ºC, respectively (Eisele et al. 2011). After 1 h at 60ºC, native and recombinant LA from *F. velutipes* displayed 39% and 45% of residual activity, compared to their respective values at 37ºC (Eisele et al. 2011). On the other hand, 37ºC was reported as the optimum temperature for the activity of LA in *T. viride* (Thakur et al. 2011), *M. hiemalis* (More et al. 2013) and *P. brevicompactum* (Elshafei 2012), with this last one, being stable up to 1h at 37°C. LA from *A. niger* also reveal a high activity at 37ºC, but at 4ºC and 50ºC lost its activity (Luhana, Dave and Patel 2013).

Effect of Activators and Inhibitors on L-Asparaginases Activity

Different metal ions have been investigated as enhancers/inhibitors of LA activity. In fact, Mn^{2+} increases the activity of LA from *F. culmorum* by 18%, while Cu^{2+} and Hg^{2+} inhibited its activity by 84% and 80%, respectively (Janakiraman 2015). Metal ions like Ca^{2+} and Mg^{2+} did not have any effect on the LA from *A. nidulans* (Archana and Raja rao 2014), EDTA inhibited the activity of LA from *T. viride* by 88% while β-mercaptoethanol did not have any effect on the enzyme activity (Thakur et al. 2011). Non-ionic surfactant, such as tween 80 was found to enhance the activity of LA from *F. culmorum* by 16%, whereas, the anionic surfactant, sodium dodecyl sulphate, completely inhibited the enzyme activity (Janakiraman 2015). Kumar and Monica also reported similar results with tween 80 at 2mM inducing the production of LA in *Cladosporium* sp. and *M. hiemalis* (Kumar and Manonmani 2013; Thakuret al. 2013).

Cytosine Deaminases

Cytosine deaminase from *Aspergillus fumigatus* was the first cytosine deaminase to be found in a mould (Yu et al. 1991). The enzyme was a monomer of 32 KDa with an optimum activity at pH 7 and 35˚C. Beside cytosine, the enzyme also hydrolyses 5-methylcytosine and 5-fluorocytosine. The activity of the enzyme in the presence of heavy metal ions, such as, Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}, is inhibited.

Cytosine deaminase from *A. parasiticus* has an increased activity at pH 7.2 (Zanna et al. 2012). Although at pH 4 and 7 the enzyme activity was appreciable. Highest cytosine deaminase activity was verified between 40ºC and 45ºC, with an enzyme activity decrease at 50ºC but stable up to 80ºC. Cytosine deaminase from *A. parasiticus* is strongly inhibited by some metal ions, losing 47% of its activity in the presence of Ca^{2+}, 58% in the presence of Hg^{2+} and 40% in the presence of Co^{2+} and Zn^{2+}. Cu^{2+} and Fe^{2+} at 50mM completely inhibited the enzyme activity (Zanna et al. 2012). The study on ionizable groups in the active site of *A. parasiticus* cytosine deaminase revealed the presence of groups with enthalpy of ionization of 43.01 KJ/mole, suggesting histidine in or around the active site of the enzyme (Zanna et al. 2012).

Proteases

As already described in this book chapter, a great number of fungal strains have been used to produce proteases belonging to the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Humicola*, *Thermoascus*, *Thermomyces*, among others (Souza et al. 2015). Moreover, for each genera, different types of proteases have been reported, namely, acid,

alkaline, neutral, serine, aspartate, among others. Therefore, the proteases produced describe different properties (Souza et al. 2015).

Effect of pH on Proteases Activity

Acid proteases have an optimum activity in a pH range between 3.0 and 5.0 (Souza et al. 2015). Aleksieva and Peeva report an acid protease from *Humicola lutea* with an optimum activity at pH 3.0-3.5 (Aleksievaa and Peeva 2000), while Negi and Banerjee describe an acid protease from *Aspergillus awamori* with an optimum pH at 5.0 (Negi and Banerjee 2009). Aspartate protease also present an optimum and stable activity at pH ranges between 3.0 and 5.5 (Souza et al. 2015).

The majority of alkaline proteases have been reported to have optimum/stable activity in the pH range between 7.0 and 9.0 (Souza et al. 2015). However, Chellapan et al. characterized a protease from marine *E. album* with a higher optimum pH between 10.0 and 11.0 (Chellappan et al. 2011). Neutral proteases have an optimum activity at pH 7.0 (Souza et al. 2015). Serine proteases, as alkaline proteases reveal an optimum/stable activity at alkaline pH values (7.0-8.0). Particularly, serine protease from *T. lanuginosus* present an increased activity at pH 5.0 (Li, Yang and Shen 1997).

Effect of Temperature on Protease Activity

Fungal proteases are usually thermolabile and show reduced activities at high temperatures (Souza et al. 2015). Acid proteases reveal a temperature optimal in a wide range between 25 and 70ºC. For instance, Larsen et al. report a protease from *Penicillium roqueforti* with an optimum temperature at 25ºC (Larsen, Kristiansen and Hansen 1998), while Negi and Banerjee describe a protease from *A. awamori* with an optimum temperature at 55ºC (Negi and Banerjee 2009), and Merheb-Dini et al. report a protease from *T. indicae-seudaticae* with an activity increased at 70ºC (Merheb-Dini et al. 2010). Serine proteases like acid proteases have very different optimum temperatures from 28 to 70ºC, with a higher number of proteases more active between 40 and 50ºC. Alkaline proteases are more active at lower temperatures and the major of reported proteases have an optimum temperature between 30-36ºC (Souza et al. 2015). Aspartate proteases have an optimum temperature at 50-55ºC (Souza et al. 2015).

Proteases Inhibitors

Several compounds have been reported in the inhibition of proteases activity. Protein proteases inhibitors are divided in 71 families. Among the 71 families, 27 include members of microbial and fungal origin, with 7 families including members exclusively of bacterial origin, and 5 families being exclusively of fungal origin. In addition to protein protease inhibitors, other small-molecule inhibitors synthesized in the laboratory have been described (Sabotič and Kos 2012). Protease from the nematode-trapping fungus *A. oligospora* was completely inhibited by the serine protease inhibitor

phenylmethylsulfonyl fluoride (Wang et al. 2006). The amino acid aldehydes chymostatin and antipain with a Phe and Arg residue, respectively, were also inhibitory. Proteases from *Sporotrichum pulverulentum* were almost completely inhibited by Ag^{+} and Hg^{2+} at 1 mM concentrations while Cu^{2+} at the same concentration was less inhibitory (Eriksson and Pettersson 2005). The inhibition by p-chloromercuribenzoate was almost completely restored for proteases by the addition of stoichiometric amounts of reduced glutathione or dithiothreitol. Partial inhibition was also observed with EDTA and α,α'-dipyridyl (Eriksson and Pettersson 2005). Trypsin-like protease (serine protease) from *T. harzianum* was strongly inhibited by 1 mM phenylmethylsulfonyl fluoride (78% inhibition) (Suarez et al. 2004). Aspartic-peptidase, cysteine-peptidase and metallo-peptidase inhibitors (0.1 mM pepstatin,1 mM iodoacetamide, and 1 mM EDTA, respectively) had a weak effect on this protease, with less than 11% of inhibition (Suarez et al. 2004).

Lipases

The number of available lipases has increased since the 1980s and their use as an industrial biocatalyst has also increased, due to their properties like biodegradability, high specificity, high catalytic efficiency, temperature, pH dependency, activity in organic solvents and nontoxic nature (Mehtaand Gupta 2017).

Effect of pH and Temperature on Lipases Activity

Lipases are active in a large range of pH and temperatures (Barriuso et al. 2016). They possess stability from pH 4.0 to 11.0 and temperature optima between 10 to 96°C. The extracellular lipase produced by *A. niger* is particularly active at low pH (Barriuso et al. 2016). Falony et al. reported the influence of various pH on the activity of *A. niger* lipase (Falony et al. 2006). A higher lipase activity was achieved at pH 6.0, and this enzyme was 100% stable within a pH range from 4.0 to 7.0 during 24 h. Ülkeret al. describe that pH 8.5 was found to be excellent for maximum activity of lipase from *T. harzianum* (Ülker et al. 2011). Lipase activity was declined by changing the pH above or below the pH optima. Lipases from *A. niger* (Fukumotoand Tsujisaka 1963) and *Rhizopus japonicas* (Aisaka and Terada 1981) are stable at 50°C, and lipase of thermotolerant *H. lanuginosa* is stable at 60°C (Mehta and Gupta 2017).

Activators and Inhibitors on Lipases Activity

Aspergillus japonicus lipase activity is inhibited by 1 mM of Mn^{2+} and Hg^{2+} while Ca^{2+} was found to be the best for maximum activity after pre-incubation for 1h (Jayaprakash and Ebenezer 2012). *T. harzianum* lipase is stable after pre-incubation for 1h in several metal ions solutions (1 mM) (Ülker et al. 2011). In particular, Ca^{2+} and

Mn^{2+} increased the activity of lipase up to 25% and 15%, respectively, while K^{+} and Cr^{3+} inhibited the lipase activity by 22% and 21%. Ca^{2+} also increases the activity of *Rhizopus chinensis* (Yu, Wang and Xu 2009) and *A. oryzae* (Ohnishi et al. 1994) lipases. This might be because the enzyme requires Ca^{2+} as a cofactor for its biological activity.

The activity of *A. oryzae* lipase is inhibited by Cu^{2+}, Fe^{3+}, Hg^{2+}, Zn^{2+} and Ag^{+} (Toida et al. 1995). Extracellular lipase activity from *C. kikuchii* has increased in the presence of ions like Al^{3+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Hg^{2+}. Residual lipase activity was increased to 129.3% in presence of Al^{3+} ion as compared to control (Costa-Silva et al. 2014).

Glucose Oxidases

The most studied and commercialized glucose oxidase (GOx) is obtained from the fungus *A. niger*. The GOx extracted has a high substrate specificity and is stable over a wide range of pH and temperature (Yuivar et al. 2017). The molecular weight of native glucose oxidase from *A. niger* is approximately 160 kDa with two equal subunits (Singh and Verma 2013). The molecular mass of GOx from *P. ostreatus* was found to be 290 kDa consisting in four subunits with a molecular mass of 70 kDa (Shinet al. 1993).

Effect of Temperature on Glucose Oxidases Activity

GOx from *A. niger* has optimal activity at 25°C and exhibited more than 90% of the maximum activity between 20-35°C (Singh and Verma 2013). However, above 45°C its activity decreased rapidly. GOx maintained 90% of its optimum activity at 37°C, when compared to optimal activity of this enzyme between 25 and 30°C. On the contrary, GOx from *A. tubingensis* and a recombinant GOx from *Penicillium amagasakiense* presented highest activity at 60°C (Courjean and Mano 2011). The residual activity of purified GOx from *A. niger* remained relatively unchanged over 10 h at 25°C, whereas exhibiting a half-life of approximately 30 min at 50°C (Singh and Verma 2013). The enzyme is stable up to 40ºC but its stability decreased at higher temperatures. On the other hand, GOx from *P. ostreatus* has stability at 70ºC during 120 min (Shinet al. 1993).

Activators and Inhibitors on Glucose Oxidases Activity

The activity of GOx from *A. ninger* is highly specific for D-glucose, however, other sugars, such as maltose, fructose, are oxidized at lower rate (Singh and Verma 2013). Similar results have been reported for glucose oxidase from *P. ostreatus* (Shinet al. 1993).

GOx from *A. niger* was inhibited 56.5 and 48% by Cu^{2+} and Ag^{2+}, respectively (Singh and Verma 2013). Similar results were reported for the enzyme from

Phanerochaete chrysosporium, with the enzyme being inhibited by Ag^{2+} (10 mM) and o-phthalate (100 mM), but not by Cu^{2+}, NaF, or KCN (10 mM) (Kelley and Reddy 1986). The inhibition of glucose oxidase by Ag^{2+} ions is due to reaction of Ag^{2+} with thiol group of the enzyme, essential for the enzymatic activity which is close to the FAD binding region of protein (Singh and Verma 2013).

Laccases

Laccase is currently seen as highly interesting industrial enzymes because of their broad substrate specificity. The molecular weight of most fungal laccases is between 43 and 110 kDa (Thurston 1994). The molecular mass of laccase from basidiomycete *Trametes* sp. strain AH28-2A was estimated to be 62 kDa with an isoelectric point of 4.2 (Xiao et al. 2003). A similar molecular weight was determined for laccase produced by *Mycena purpureofusca* (Shujing et al. 2013).

Effect of pH and Temperature on Laccases Activity

Purified laccase from *Pleurotus* sp. is a monomer with a molecular mass of 40 kDa, active in a pH range between 3 and 5 with optimum activity at pH 4.5 (More et al. 2011). Similar results were obtained for laccase from basidiomycete *Trametes* sp. strain AH28-2A, stable in a pH range between 4.2 to 8.0, and an optimum pH at 4.5 in citrate-Na_2HPO_4 (Xiao et al. 2003). Laccase from the ascomycete *Thielavia* sp. is highly stable at acidic pH range, with an optimum activity at pH 5.0 and 6.0 (Mtibaà et al. 2018).

Laccase from *Pleurotus* sp. is stable in a temperature range between 35 and 70°C and an optimum temperature at 65°C (More et al. 2011), like laccases from *Sclerotium rolfsii* (Ryan et al. 2003). Temperature kinetics of this enzyme suggests that the enzyme activity increases sharply from 60 to 65°C followed by a decline after 70°C. The laccase was stable at 60°C during 8 h, while at 75°C was stable up to 30 min, and after 90 min it retained 38% of the activity. *Pleurotus* sp. was stable for 20 days at room temperature and stable for 60 days when stored at −4°C (More et al. 2011). Laccase from basidiomycete *Trametes* sp. strain AH28-2A has an optimum activity at 50ºC and the enzyme is stable at 70ºC for more than 1 h. The activity of laccase is 2.5 times higher at 50ºC than at 20ºC (Xiao et al. 2003).

Activators and Inhibitors on Laccases Activity

Laccase from *Pleurotus* sp. is more inhibited by sodium EDTA (More et al. 2011), similar to laccases from *Chaetomium thermophilum* (Chefetz, Chen and Hadar 1998). The activity of laccase from basidiomycete *Trametes* sp. strain AH28-2A is totally inhibited by 0.1 mM of sodium azide or cyanide, 59.6% inhibited by 25 mM of SDS, and almost unaffected by 25 mM of EDTA (Xiao et al. 2003). Fe^{3+}, Mn^{2+}, Cu^{2+}, Ag^{+}, Ca^{2+},

Ba^{2+} and Zn^{2+} at 0.05M have a slightly stimulating effect on laccase from *M. purpureofusca* (Shujing et al. 2013). The enzyme activity can be enhanced by 18.7% and 130.5% when Ag^{+} was added to the medium at 0.05 and 0.5 M, respectively. On the contrary, Fe^{2+} strongly inhibited enzyme activity up to 98% at 0.05 and 0.5 mM. Laccase from the ascomycete *Thielavia* sp. is inhibited by Hg^{2+} and Fe^{2+}, while the presence of Mn^{2+} at concentrations of 5 and 10 mM promoted the enzymatic activity (Mtibaà et al. 2018).

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