J Ind Microbiol Biotechnol (2014) 41:1467–1478
DOI 10.1007/s10295-014-1496-2

REVIEW

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Endophytic fungi: expanding the arsenal of industrial enzyme producers

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Received: 22 April 2014 / Accepted: 27 July 2014 / Published online: 13 August 2014

Abstract Endophytic fungi, mostly belonging to the Ascomycota, are found in the intercellular spaces of the aerial plant parts, particularly in leaf sheaths, sometimes even within the bark and root system without inducing any visual symptoms of their presence. These fungi appear to have a capacity to produce a wide range of enzymes and secondary metabolites exhibiting a variety of biological activities. However, they have been only barely exploited as sources of enzymes of industrial interest. This review emphasizes the suitability and possible advantages of including the endophytic fungi in the screening of new enzyme producing organisms as well as in studies aiming to optimize the production of enzymes through well-known culture processes. Apparently endophytic fungi possess the two types of extracellular enzymatic systems necessary to degrade the vegetal biomass: (1) the hydrolytic system responsible for polysaccharide degradation consisting mainly in xylanases and cellulases; and (2) the unique oxidative ligninolytic system, which degrades lignin and opens phenyl rings, comprises mainly laccases, ligninases and peroxidases. The obvious ability of endophytic fungi to degrade the complex structure of lignocellulose makes them useful in the exploration of the lignocellulosic biomass for the production of fuel ethanol and other value-added commodity chemicals. In addition to this, endophytic fungi may become new sources of industrially useful enzymes such as lipases, amylases and proteases.

Keywords Biomass · Endophytes · Environmental biotechnology · Fungal bioactives · Fungal diversity · Ligninolytic enzymes · Lignocellulosic waste · Hydrolytic enzymes

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Introduction

Filamentous fungi are the preferred source of industrial enzymes because of their excellent capacity for extracellular protein production. Glucoamylase, cellulase, lipase, glucose-oxidase, pectinase, laccase, catalase, phytase and proteases are only a few examples of a wide range of enzymes produced by filamentous fungi that are commercially available. Complete lists can be seen in recently published reviews [43, 62, 74, 93, 111, 115]. In spite of the great heterogeneity of filamentous fungi in nature, not more than a few dozen of species have been commercially exploited as enzyme producers. *Aspergillus* species, and particularly *A. niger* and *A. oryzae*, play a dominant role in the production of many enzymes [11, 20, 23].

Genetic manipulation of filamentous fungi

Industrial processes require generally robust enzymes, able to act in a wide range of conditions (extreme pH,

temperature, osmolarity, pressure, etc.). It should be noted that enzymes must be produced with high yield by means of simple and low-cost fermentative processes. Programs to select new microorganisms for enzyme production are increasing around the world. In the last years, several prospecting studies evaluating the diversity of fungi isolated or associated with species from desertic, Alpine, marine, Arctic and tropical environments, among others, have been done [2, 20, 27, 30, 77]. All these searches are, at least in principle, useful for discovering new enzyme producers with specific properties, and the natural habitats of microorganisms often provide clues about the properties of the enzymes [38, 96]. For example, a heat-tolerant xylanase was discovered from a bacterium originating in hot springs [53], and a cold-tolerant lipase was produced by an Antarctic fungus [24].

Fungi are ubiquitous in plants and can be classified as epiphytes, the fungi found on the plant surfaces, and endophytes, those fungi that necessarily inhabit the healthy tissues of aboveground plant parts without causing any visible sign of infection during all or part of the plant life cycle [8, 10, 12, 99, 101, 102, 112]. Some authors also designate the interactions of mycorrhizal fungi with the roots of their hosts as being endophytic [114]. The position adopted in the present revision is in agreement with Brundrett [25], who distinguishes mycorrhizal from endophytic interactions, the former having synchronized plant–fungus development and nutrient transfer at specialized interfaces. Both epiphytes and endophytes have important implications for the fungal biodiversity and plant health, but the two communities have rarely been compared. Although some species are more frequently found as epiphytes and others more frequently as endophytes, a few studies have reported species that are common to both leaf tissues and surfaces [66, 91, 109]. This includes species of the genera *Aspergillus* and *Trichoderma* [66, 104]. Frequently applied in enzyme production due to the GRAS status, high productivity and facility of cultivation [83, 129], *A. niger* has been recently found as endophyte in several plants [59, 104, 133].

Several endophytic species have demonstrated to be mutualistic by enhancing resistance of the host plant against herbivores and pathogens [48, 108, 126]. They utilize several hydrolytic enzymes to degrade the cell wall [54]. The endophytic fungal hyphae reside within the intra- and intercellular spaces of the aerial plant parts, particularly in leaf sheaths, sometimes even within the bark and root system. Knowledge about the factors that affect the colonization and the exchange of nutrients between the plants and the fungi is still limited [100]. The endophytic fungi which are associated with plants reduce the damage from the pathogens probably due to the accumulation of secondary metabolites [26]. Furthermore, the endophytes are able to inhibit pathogen infection and proliferation within the host directly or indirectly by inducing resistance responses intrinsic to the host defense [45]. The symbiotic association leads to increases in the biomass as well as to the development of aerial parts in the form of flowerings [37].

The best known endophyte fungi are members of the Clavicipitaceae (Ascomycota), which are endophytes of some temperate grasses [63]. However, endophytes have been found in every plant species studied so far, and it has been suggested that 1 million or more species exist in nature [88, 108, 118, 125, 131]. As a consequence, studies on endophytic fungi of plants are necessary to provide fundamental information for the assessment of the global fungal diversity and distribution, as well as for the description of new species [116, 117]. An example of the interaction between an endophytic fungus and its host plant, as well as the morphologic aspects of endophytic isolates, is presented in Fig. 1.

The terrestrial environment has been largely scrutinized for drugs for several decades, and the possibilities in this direction seem to have been almost exhausted. Recently, scientists have identified oceans as a novel and rich source of natural products with potential as drugs [27]. Numerous promising and structurally unprecedented bioactive compounds have been isolated from the oceans and some have been identified as preclinical anticancer compounds [49, 84]. Marine

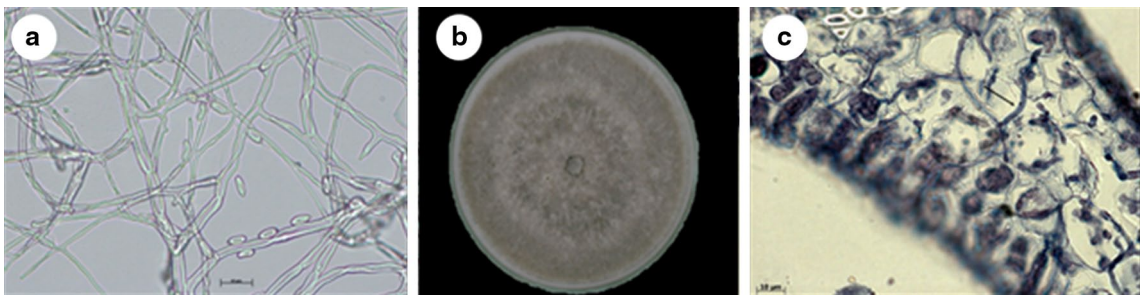

Fig. 1 Morphological characteristics of endophytic fungi. **a** Microscopic aspect of hyphae and spores of endophyte isolated from *Trichilia elegans*. **b** Macroscopic visualization of endophyte fungus isolated from *Trichilia elegans*. **c** Histologic visualization of leaf showing endophyte hyphae

microalgae, cyanobacteria, and heterotrophic bacteria living in association with invertebrates (e.g., sponges, tunicates, and soft corals) have been pointed out as sources of bioactives and compounds of general interest [132]. The exploitation of endophytes and associated marine-derived fungi as promising sources of novel bioactive natural products constitutes a new broad possibility for science [39].

Techniques for the isolation and identification of endophytic fungi

The surface disinfection is the most important step when working with endophytic microorganisms [6]. Thus, the choice of the disinfecting agent (alcohol, sodium hypochlorite, hydrogen peroxide, UV light) is crucial to the success of the endophyte isolation. The most frequently used procedure for disinfecting the surface tissues consists basically in: (a) flushing the tissue with sterile distilled water to remove dust and other debris; (b) treating the plant material with 70 % ethanol during 30–60 s; (c) treating the sample with 3–4 % sodium hypochlorite for 3–4 min. After treatment, washes with sterile distilled water are performed and an aliquot of this water is used as a negative control [9].

For the isolation of endophytic fungi, it is essential to maintain the viability of the internal microbiota while eliminating the epiphytic microbiota. This process varies depending on the part of the plant used, its texture, age, among others. The biggest challenge is to achieve the ideal conditions to isolate the greatest possible diversity of endophytic fungi present in the material.

Small pieces of the materials are then transferred to a nutrient solid medium such as potato dextrose agar, malt extract agar, yeast malt agar or Sabouraud agar, supplemented with antibacterial agents (chloramphenicol, penicillin, ampicillin, tetracycline and streptomycin, among others) to prevent bacterial growth. Sporulating fungi can be identified via standard morphological and molecular biological techniques. Around 50 % of endophytic fungi do not produce conidia or spores when cultured on common mycological media. In these cases, endophytic fungi can be identified based on the sequence of the internal transcribed spacer (ITS) region of the large subunit of the rRNA gene. After sequencing the ITS1–5.8S–ITS2 region, the sequence of the endophytic fungus is compared with the sequences deposited in public databases, the GenBank database being the major source of nucleotide sequences [14, 52, 101, 102].

Evaluation of the potential of endophytic fungi as enzyme producers

Over more than 20 years the endophytic fungi have been explored as biofactories of novel bioactive substances. The substances produced by endophytic fungi, which originate from different biosynthetic pathways, belong to various structural groups, such as terpenoids, steroids, xanthones, quinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasins and enniatins [112, 113]. In fact, endophytic fungi represent a reservoir for discovering new compounds, such as antibiotics, antioxidants, immunomodulators, and anticancer and antiparasitic compounds, for use in the pharmaceutical and agrochemical industries [12, 85, 88, 105].

Currently, the main focus in endophytic fungi research is associated with the ability of these microorganisms to produce and accumulate secondary metabolites. Several of these compounds present biological activities of interest for application in environment, agriculture, medicine and food industry [51, 69–71, 97] Table 1 lists current representative studies in which several compounds produced by endophytic fungi were evaluated for their biological activities.

Although the ability of fungi to produce unique bioactive metabolites is well known, endophytes have not yet been extensively exploited, perhaps because we are only beginning to understand their distribution and biology. Recent reviews have emphasized the need of routinely include endophytic fungi in the screening of organisms for bioactive metabolites and novel drugs [1, 4, 21, 22, 120, 121, 123, 128]. Most studies so far have, thus, focused on the production or at least of the perspectives of production of such compounds by endophytic fungi. However, the possible use of endophytic fungi as sources of industrial enzymes has received much less attention, in spite of the fact that they have since long been recognized as enzyme producers for their natural needs, more specifically as producers of a series of enzymes necessary for penetrating and colonizing their plant hosts, including hydrolytic and oxidative enzymes [8, 29, 120, 121].

Endophytic fungi produce enzymes such as amylases, lipases and proteases, as part of their mechanism to overcome the defense of the host against microbial invasion and to obtain nutrients for their development [119, 122, 124]. Over the past few years (2005–2014) various researchers have made systematic attempts to catalogue endophytes as enzyme producers with potential for practical application (Table 2).

Many of these enzymes are involved in the degradation of components of lignocellulosic materials. Apparently endophytic fungi possess the two types of extracellular enzymatic systems necessary to degrade the lignocellulosic fibers: (1) the hydrolytic system responsible for polysaccharide degradation consisting mainly in xylanases and cellulases; and (2) the unique oxidative ligninolytic system, which degrades lignin and opens the phenyl rings and that comprises mainly laccases, ligninases and peroxidases. The obvious ability of endophytic fungi to degrade the complex

Table 1 Recent studies reporting biological properties of secondary metabolites produced by endophytic fungi

Biological activity	Endophytic fungi	Source plant	Metabolites	References
Help in biomass recovery and osmotic stress mitigation	*Penicillium resedanum*	*Capsicum annuum* L.(pepper)	ND	[65]
Potential anticancer activity	*Colletotrichum gloeosporioides*	*Barringtonia acutangula*	ND	[71]
Biological control	*Nodulisporium* sp.	*Thelypteris angustifolia*	A group of volatile organic compounds (VOCs)	[103]
Soil bioremediation	*Gaeumannomyces cylindrosporus*	*Astragalus adsurgens* Pall.	Melanin	[10]
Biological control (insecticide)	*Hypoxylon pulicicidum* sp.	ND	Nodulisporic acids	[18]
Improves plant growth/plant defense	*Paecilomyces formosus*	*Cucumis sativus* (cucumber)	Gibberellins and indole acetic acid	[64]
Improves plant growth/plant defense	*Phoma glomerata*	*Cucumis sativus* (cucumber)	Gibberellins and indole acetic acid	[128]
Improves plant growth/plant defense	*Penicillium* sp.	*Cucumis sativus* (cucumber)	Gibberellins and indole acetic acid	[128]
Antioxidant and antidiabetic activities	ND	*Taxus sumatrana*	ND	[7]
Plant defense	*Fusarium* sp.	*Cajanus cajan* (L.) Mill sp.	Cajaninstilbene acid	[50]
Plant defense	*Neonectria* sp.	*Cajanus cajan* (L.) Mill sp.	Cajaninstilbene acid	[50]
Potential anti-dementia drug (β-secretase inhibitor)	*Cytospora rhizophorae*	Malasian medicinal plant	ND	[55]
Potential anti-dementia drug (acetylcholinesterase inhibitor)	*Shiraia* sp.	*Huperzia serrata*	Huperzine A	[134]
Potential anticancer activity	*Halorosellinia* sp.	Mangrove plant	Anthracenedione derivatives	[132]
Potential anticancer activity	*Guignardia* sp.	Mangrove plant	Anthracenedione derivatives	[132]

ND not determined

structure of lignocellulose makes them potentially useful in the exploration of the lignocellulosic biomass for the production of fuel ethanol and other value-added commodity chemicals. In addition to this, endophytic fungi may become new sources of industrially useful enzymes such as lipases, phytases, amylases and proteases.

Studies aiming to find new enzyme producers start by screening microorganisms for the desired activity using appropriate selection procedures. After isolation and identification, fragments of the pure endophyte are transferred to Petri dishes containing solid medium with specific substrates for each enzyme, for example, citric pectin for pectinases [126], carboxymethylcellulose for cellulases [86, 90], xylan for xylanases [61] and casein or gelatin for proteases [110, 131]. The cultures are then evaluated for the zone of enzyme activity. However, to produce elevated amounts of an enzyme or group of enzymes, cultivation techniques of filamentous fungi such as submerged cultivation and solid-state cultivation have been widely used, first in laboratory scale, later in industrial scale [74].

The production of industrial enzymes in large scale has generally been carried out using well-established submerged fermentation systems, where the fungi are grown in a fully liquid system which has the advantage of allowing control over process parameters such as pH, temperature and aeration [34, 62]. However, solid-state fermentation systems appear as a promising technology. Solid-state fermentation (SSF) is defined as the cultivation process in which microorganisms grow on solid materials without the presence of free liquid [17, 135]. Solid-state cultivation using different agro-industrial residues appear to be the most adequate technique for culturing endophytic fungi. Selection of agro-industrial residues for utilization in SSF depends on physical parameters such as particle size, moisture level, intra-particle spacing and nutrient composition within the substrate. In recent years, several important agro-industrial residues such as cassava bagasse, sugarcane bagasse, sugar beet pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, coir pith, etc., have been used as substrates for solid-state cultivation [17].

After successful fermentation, the desired enzymes must be separated and purified. This final step is commonly known as downstream processing or bioseparation, which can account for up to 60 % of the total production costs, excluding the cost of the purchased raw materials [33]. The downstream processing includes methods such as extraction, concentration, purification and stabilization. Although endophytes have been used in a series of screening studies to examine their catalytic repertoire [120, 121], similar

Table 2 Endophytic fungi with potential for producing industrial enzymes

Endophytic fungi	Source plant	Enzymes	References
Preussia minima	*Eremophilia longifolia*	amylase	[132]
Alternaria sp.	*Eremophilia longifolia*	amylase	[132]
Cladosporium cladosporioides	*Costus igneus, Lawsonia inerims*	amylase, cellulase	[4]
Curvularia brachyspora	*Adathoda vasica*	amylase, laccase, lipase	[4]
Curvularia vermiformis	*Coleus aromaticus*	cellulase, lipase, protease	[4]
Drechslera hawaiiensis	*Adathoda vasica*	amylase, lipase, protease	[4]
Nigrospora sphaerica	*Costus igneus, Lawsonia inerims*	amylase	[4]
Colletotrichum carssipes	*Lawsonia inerims*	amylase, protease	[4]
Colletotrichum falctum	*Lawsonia inerims*	lipase, protease	[4]
Colletotrichum gleosporioides	*Costus igneus, Lawsonia inerims*	amylase, protease	[4]
Phyllosticta sp.	*Adathoda vasica, Lawsonia inerims*	amylase, lipase	[4]
Xylaria sp.	*Coleus aromaticus*	amylase, laccase, protease	[4]
Acrimonium terrícola	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Aspergillus japonicus	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, pectinase, protease, xylanase	[15]
Cladosporium cladosporioides	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Cladosporiums phaerospermum	*Opuntia ficus-indica* Mill. (Cactaceae)	protease, xylanase	[15]
Fusarium lateritium	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Monodictyscastaneae	*Opuntia ficus-indica* Mill. (Cactaceae)	xylanase	[15]
Nigrosporasphaerica	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Penicillium aurantiogriseum	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Pestalotiopsis guepinii	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Phoma tropica	*Opuntia ficus-indica* Mill. (Cactaceae)	protease, xylanase	[15]
Phomopsisarcheri	*Opuntia ficus-indica* Mill. (Cactaceae)	protease, xylanase	[15]
Tetraploa aristata	*Opuntia ficus-indica* Mill. (Cactaceae)	protease, xylanase	[15]
Xylaria sp. 1	*Opuntia ficus-indica* Mill. (Cactaceae)	cellulase, protease, xylanase	[15]
Xylaria sp. 2	*Opuntia ficus-indica* Mill. (Cactaceae)	protease, xylanase	[15]
Talaromyces flavus	*Potentilla fulgens*	lipase, protease, xylanase	[16]
Mortierella hyalina	*Osbeckia stellata*	cellulase, lipase, protease, xylanase	[16]
Paecilomyces variabilis	*Osbeckia chinensis*	amylase, lipase, protease, xylanase	[16]
Penicillium sp.	*Camellia caduca*	cellulase, lipase, protease, xylanase	[16]
Penicillium sp.	*Schima khasiana*	cellulase, lipase, protease, xylanase	[16]
Penicillium sp.	*Centella asiatica*	cellulase	[40]
Cylindrocephalum sp.	*Alpina calcarata*(Haw) Roscoe	amylase	[119]
Discosia sp.	*Calophyllum inophyllum*	amylase	[57]
Fusariumoxysporum	*Musa* sp.	protease	[87]
Rhizoctonia sp.	*Glycine max*(L.) Merril	phytase	[79]
Fusarium verticillioides	*Glycine max*(L.) Merril	phytase	[79]
Acremonio zeae	*Zea mays*	xylanase	[19]
Chaetominum globosum	*Glinus lotoides*	laccase	[46]
Bjerkandera sp.	*Drimys winteri*	cellulase, phenoloxidase	[90]
Acremonium sp.	*Acrostichum aureum*	amylase, cellulase, lipase	[78]
Alternaria chlamydosporus	*Acanthus ilicifolius*	cellulase, lipase, protease	[78]
Alternaria sp.	*Acrostichum aureum*	amylase, cellulase, lipase, protease	[78]
Aspergillus sp.	*Acrostichum aureum*	cellulase, lipase, protease	[78]
Aspergillus sp.	*Acanthus ilicifolius*	cellulase, lipase	[78]
Fusarium sp.	*Acrostichum aureum*	amylase, cellulase, lipase	[78]
Pestalotiopsis sp.	*Acanthus ilicifolius*	amylase, cellulase, lipase, protease	[78]

efforts have not been invested in upstream and downstream processing of their enzymes. The literature is scarce in this area and we have selected the most representative studies where the authors went beyond the application of qualitative tests for enzyme production.

Amylases

Amylases (α-amylases, β-amylases and glucoamylases) represent one of the most important enzyme groups within the field of biotechnology. A variety of industries (e.g., food, chemical, detergent, textile) employ microbial amylolytic enzymes to convert starch into different sugar solutions. Several types of enzymes are involved in the degradation of starch, mainly α-amylase (1,4 α-glucan glucanohydrolase, EC 3.2.1.1), β-amylase (1,4 α-glucan maltohydrolase, EC 3.2.1.2), and glucoamylase (1,4 α-glucan glucohydrolase, EC 3.2.1.3) [93]. Among the amylases produced by diverse microorganisms, the α-amylases from *Bacillus* sp. have been the most extensively studied considering especially their extreme thermostability [106]. Glucoamylases are common in fungi, and *Aspergillus* sp. and *Rhizopus* sp. are often used as sources of industrial amylases [93].

Among several endophytic fungi, *Fusicoccum* sp. BCC4124, showed strong amylolytic activity when cultivated on multi-enzyme induction-enriched media and agro-industrial substrates [29]. Both α-amylase and α-glucosidase activities were highly induced in the presence of maltose and starch. An α-amylase was purified to apparent electrophoretic homogeneity and showed strong hydrolytic activity on soluble starch. The characteristics of this enzyme, such as high activity at 70 °C and the resistance to inhibition by glucose up to 1 mol/L, make it useful for biotechnological applications.

The endophyte *Cylindrocephalum* sp. isolated from the medicinal plant *Alpinia calcarata* (Haw.) Roscoe was cultivated in liquid conditions and the production of amylase was evaluated under several conditions. The maximal amylase production was found to occur at 30 °C and at pH 7.0. Among the various carbon and nitrogen sources tested, maltose at 1.5 % and sodium nitrate at 0.3 %was the best combination for the production of amylase [119].

Four newly isolated strains of endophytic fungi, namely *Gibberella pulicaris*, *Acremonium* sp., *Synnematous* sp. and *Nodilusporium* sp., were compared for their production of amylase in submerged conditions [81]. Analyses showed that the raw starch-degrading enzyme from *Acremonium* sp. had a broad activity towards both small and large granule sized raw starches, whereas the enzyme from the other strains showed high activity toward starches of smaller granule size. Analysis of the end products by thin layer chromatography showed that enzymes from *G. pulicaris*, *Acremonium* and *Nodilusporium* sp. hydrolysed raw sago starch to produce solely glucose while the amylase of *Synnematous* sp. produced glucose and maltose. The same group of researchers tested different nitrogen and carbon sources to improve the growth of *Acremonium* sp. and the production of the raw sago starch degrading enzyme (RSSDE) [80, 81]. They found that growth and enzyme activity levels were highest with peptone and sodium nitrate as the nitrogen sources and raw sago starch as the carbon source for which the optimum concentrations were 0.5, 3, and 20 g/L, respectively. Cell growth and RSSDE production reached their optimum at pH 5.0 and incubation temperature of 30 °C. Under these conditions, the enzyme production was significantly increased by 19- to 22-fold compared to the activity obtained in the original basal medium. The endophytic fungus, *G. pulicaris* was also described as a producer of an amylase able to degrade raw starches from cereals and other crops including raw potato, sago, tapioca, corn, wheat and rice starch, glucose being the main final product [82]. The highest amylase production (260 units/mg protein) was achieved when the concentration of raw potato starch was increased to 60 g/L.

Lipases

Lipases EC (3.1.1.3) are hydrolytic enzymes that in vivo break the ester bond of triacylglycerol, releasing free fatty acids and glycerol, being then classified as a special class of esterases [60, 89]. Besides the hydrolysis reaction, these enzymes are also able to catalyze interesterification, alcoholysis, acidolysis, esterification and aminolysis reactions when under proper conditions [5, 35, 36, 42]. The microbial lipases, which are usually obtained from certain species of bacteria and filamentous fungi, present an outstanding potential for being used in bioprocesses due to a series of highly desirable characteristics. For example, they are highly stable in organic solvents, do not require cofactors and can act on an ample variety of substrates [56, 60].

Endophytes able to produce lipases have been the target of research in the last years. A screening of endophytic fungi isolated from Mediterranean plants rendered a mycelium-bound lipase from a strain of *Rhizopus oryzae* that catalyzes the esterification of fatty acids in isooctane [124]. The influence of various factors (water content, temperature, and pH) on the ester synthesis was investigated. The catalytic activity inversely correlates with the water content. The enzyme shows optimal activity at pH 4–7 and 60 °C.

In another recent work, effort was done to improve the production and stabilization of a lipase from the endophyte *Cercospora kikuchii* [122]. Maximum enzyme production (9,384 U/mL) was obtained after 6 days in a medium supplemented with 2 % soybean oil. The lipases were spray

dried with different adjuvants, and their stability was studied. The residual enzyme activity after drying with 10 % (w/v) of lactose, β-cyclodextrin, maltodextrin, mannitol, gum arabic, and trehalose ranged from 63 to 100 %. The enzyme activity was lost in the absence of adjuvants. Most of the adjuvants kept up at least 50 % of the enzymatic activity at 5 °C and 40 % at 25 °C after 8 months. The lipase dried with 10 % of β-cyclodextrin retained 72 % of its activity at 5 °C. The lipases were separated in a butyl-Sepharose column into four pools, and pool 4 was partially purified (33.1 %; 269.5 U/mg protein). This pool was also spray dried in maltodextrin DE10, and it maintained 100 % of activity.

The endophytic yeast *Candida guillermondi* was isolated from castor leaves (*Ricinus communis* L.) and used for the production of a lipase under submerged fermentation in a medium containing soybean oil as the main nutrient [89]. The obtained enzyme was partially purified and freeze-dried before immobilization on agarose and silica gel supports. The immobilized lipase showed potential for being used in food and biofuel industries as well as in laboratorial applications.

A screening of endophytic fungi isolated from Mediterranean plants rendered a mycelium-bound lipase from a strain of *R. oryzae* that catalyzed the esterification of fatty acids in isooctane [124]. The influence of various factors (water content, temperature, and pH) on the ester synthesis was investigated. The catalytic activity correlates inversely with the water content. This enzyme is active over a wide pH range, pH 3–8, and its activity is maximal at pH 4 and 7. The enzyme is thermostable, with maximal activity at 60 °C.

In a study in which 65 endophytic fungal isolates were identified [94], ten were found to produce extracellular lipase activity, and *Fusarium oxysporum* PTM7 isolated from the leaves of *Croton oblongifolius* Roxb. (Plao yai), was selected for further studies. The lipase activity in the basal culture medium of *F. oxysporum* was highest with 1 % (v/v) olive oil, 1 % (w/v) peptone and 0.5 % (w/v) sodium nitrate as the carbon, organic and inorganic nitrogen sources, respectively. The 37.4 kDa enzyme was purified using chromatographic methods and showed optimal activity at pH 8 and 30 °C, with reasonable stability at 40 °C and over a wide range of pH (from pH of 8.0 to 12). The enzyme is stimulated by Ca^{2+}, Mg^{2+} and Mn^{2+} and inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+}. When using *p*-nitrophenyl palmitate as the substrate, the enzyme revealed K_m and V_{max} values of 2.78 mM and 9.09 μmol min^{-1} mg $protein^{-1}$, respectively. However, the enzyme presents low transesterification activity.

Proteases

The term protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. The proteases form a large group of enzymes belonging to the class of hydrolases and are ubiquitous in nature. In addition to their physiological importance, they are also widely employed for commercial, industrial and health purposes [73, 76]. Proteases are largely used in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes [13]. During the last few years, studies about the fibrinolytic enzymes from microorganisms have attracted significant attention because of their potential use in thrombosis therapy. Fibrinolytic enzymes were discovered in various microorganisms, the most important among them being the genus *Bacillus* from traditional fermented foods [95]. A novel fibrinolytic enzyme with high activity, however, was obtained from the endophyte *Fusarium* sp. CPCC 480097, which was isolated from chrysanthemum stems [130]. The purified enzyme has a molecular weight of 28 kDa, isoelectric point of 8.1 and maximal activity at 45 °C and pH 8.5. This protease may have potential applications in thrombolytic therapy and in thrombosis prevention.

In an interesting work of bioremediation, several endophytic fungi were evaluated for their ability to degrade efficiently the polyester polyurethane in both solid and liquid suspensions [107]. Polyester polyurethane (PUR) is a plastic widely used in industry and manufacture that has been shown to be susceptible to biodegradation. Among several endophytes, two *Pestalotiopsis microspora* isolates were selected due to the high capability to grow on PUR as the sole carbon source under both aerobic and anaerobic conditions. According the authors, the main enzyme responsible for the capability of these endophytes to degrade PUR is a serine protease.

Biomass converting enzymes

The increasing demand for energy and the depletion of the fossil fuel reserve surge us to find large quantities of alternative precursors for the petrol-based chemical industry and transportation sectors. Cellulosic biomass, derived from non-food sources, such as trees and grasses, is being explored as a feedstock for cellulosic ethanol production. In nature, cellulose is found in combination with lignin and hemicellulose resulting in a complex and recalcitrant solid called lignocellulose. Fungi are the major decomposers of lignocellulosic material in several ecosystems and play an essential role in the cycling of carbon and other nutrients. The wood-decaying fungi comprise mainly basidiomycetes and ascomycetes and usually are saprotrophic, utilizing dead wood, or parasitic, attacking living trees. The key for exploring the chemical value of lignocellulosics is to depolymerise the lignocellulosic matrix in order to obtain smaller molecules that can be used, or further converted

to platform chemicals and biofuels, especially bioethanol. The biological degradation of the carbohydrates within the biomass is achieved using multiple enzymes in defined ratios to convert the carbohydrates into their monomer sugars [44, 98]. The main hydrolytic enzymes involved in the lignocellulose degradation are exo- and endo-glucanases, β-glucosidases, exo- and endo-xylanases and β-xylosidases. For the complete degradation of lignocellulosic materials, a series of oxidative enzymes are also necessary, namely laccase, manganese peroxidase and lignin peroxidase [75]. Additional hemicellulases (e.g., acetylesterase, β-glucuronidase, endo-1,4-β-mannanase, α-galactosidase) and oxidoreductases (e.g., aryl alcohol oxidase, glucose-1-oxidase, glyoxal oxidase, pyranose-2-oxidase) may also participate in the degradation process. Endophytic fungi also produce ligninocellulolytic enzymes and can, thus, be regarded as potential alternative sources [120, 121]. The application of qualitative tests in the evaluation of enzyme activity has revealed that both cellulolytic and xylanolytic activities are common among endophytes (Table 1). In a very recent study, 110 endophytic fungi were explored concerning their abilities to produce hemicellulases and related enzymes, suitable for lignocellulosic biomass deconstruction [104]. The best six strains were identified as *Aspergillus niger* DR02, *Trichoderma atroviride* DR17 and DR19, *Alternaria* sp. DR45, *Annulohypoxylon stigyum* DR47 and *Talaromyces wortmannii* DR49. Other recent work described the production of cellulase from a new strain of *Acremonium strictum* isolated from the Brazilian Biome using different substrates [54]. Two endophytes, *Colletotrichum* sp. and *Alternaria* sp. were described as cellulase producers with the additional capability of producing substantial amounts of lipids when cultured on rice straw and wheat bran in solid-state fermentation [40, 41]. In the last study, the authors suggest the potential utilities of endophytic fungi as biodiesel feedstock.

The use of lignocellulosic residues to produce bioethanol is hindered by the presence of lignin. Lignin has a highly recalcitrant structure with several carbon–carbon and other bonds that make the enzymatic and chemical degradation highly problematic. For this reason, the previous degradation of lignin is a pre-requisite for the utilization of carbohydrates in biomasses. Pretreatments are required to remove or to modify lignin into a lignocellulosic fiber structure in order to facilitate the access of the hydrolytic enzymes to the polysaccharides. Ideally, these pretreatments should modify predominantly lignin without causing major breaks in the structural carbohydrates, making the latter available for fermentation processes. Mild pretreatments, avoiding the generation of waste and pollutants are desirable. Several chemical and physicochemical pretreatment processes, such as acid pretreatment, alkaline pretreatment, steam explosion and ammonia fiber explosion, have been used to enhance the enzymatic hydrolysis of lignocellulose. However, these processes usually require high temperatures and pressures, resulting in high costs and undesirable products [3]. In this respect biological pretreatment could be an interesting alternative. The biological pretreatment of lignocellulosic residues, where saprophytic microorganisms degrade plant cell walls under natural conditions, is an interesting alternative. In this context, the white rot basidiomycete should be remarked. These fungi degrade lignin efficiently because they are able to produce highly efficient ligninolytic enzymes. The most studied producers of ligninolytic enzymes belong to the genus *Phanerochaete, Trametes, Pleurotus*, *Ganoderma*, and *Bjerkandera* [75]. Studies have shown that some endophytic fungi can also produce laccase or to use plant materials such as lignin or cellulose [28, 67, 68, 72, 92]. Two endophytic fungi that are laccase producers were recently more properly characterized. In the first study, the endophytic fungus *Monostospora* sp. isolated from *Cynodon dactylon* was cultivated in submerged cultures to produce laccase [127]. In this study, maltose (2 g/L) and ammonium tartrate (10 g/L) were the most suitable carbon and nitrogen sources, respectively, for enzyme production. Under optimal culture conditions, the maximal laccase activity was determined to be 13.55 U/mL. In a second study, a laccase from *Pestalotiopsis* sp. J63, isolated from sea mud samples collected in the East China Sea, was investigated under submerged and solid-state fermentation using various lignocellulosic by-products as substrates [31]. The most effective solid-state culture was that one with rice straw powder, which accumulated a laccase activity of 10,700 IU/g substrate. In submerged fermentation the maximal amount of laccase activity (2,000 IU/mL) was found when untreated sugarcane bagasse was used. The *Pestalotiopsis* laccase shows a moderate halotolerance, a useful property for biotechnological applications.

The endophytic fungus *Phomopsis liquidambari*, which grows on phenolic 4-hydroxybenzoic acid as the sole carbon and energy source, is able to produce the ligninolytic enzymes laccase and lignin peroxidase when cultured in submerged fermentation [32]. This endophyte can be regarded as a species with bioremediation potential, considering that it was able to degrade indole, which has an N-heterocyclic structure. Indole is widely present in the natural environment due to the fact the compound as well as its derivatives are important plant hormone precursors and microbial signaling molecules [22, 47]. However, due to its use in several industrial products, including pharmaceuticals, fuels, cosmetics, pesticides, disinfectants and dyestuffs, indole and its derivatives are now considered pollutants. They are released into the environment through cigarette smoke, coal tar, sewage, cooking and dye-stuff wastewater [58].

Concluding remarks

Endophytic fungi have recently received more attention, but they remain yet an unexplored group of filamentous fungi. Up to now, their potential as enzyme producers has not yet been accordingly explored. Not less important appears to be the potential of using the endophytic fungi in bioremediation processes. The question if these microorganisms will be used in the future as efficient industrial enzyme sources cannot be answered at the present stage of knowledge. New and more extensive studies using the well-known methods of fungal cultivation to optimize the production of enzymes are necessary. This must be followed by the characterization of the physicochemical properties of these enzymes to evaluate the real potential of endophytic fungi as sources of industrial biocatalysts.

Acknowledgments This work was supported by the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq, Proc. 563260/2010-6 and Proc. 477825/2012-5) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). R.M.P., M.L.T.M.P. and A.B. are research fellows of CNPq. R.C.G.C. and T.R.M. are research fellows of CAPES.

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