Homologous and Heterologous Expression of Basidiomycete Genes Related to Plant Biomass Degradation

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1 Introduction

The necessity to move towards a bio-based economy creates many challenges for industry and academia. Industries dedicated to supplying the population with food, feed, detergents, textile and other products collaborate with researchers to develop efficient biotechnological tools. This includes the development of optimal enzyme sets to convert plant biomass into valuable products. Recently there has been a strong increase in the demand of these enzymes due to their growing biotechnological significance (Harris et al. 2014).

Fungi play an important role in plant biomass degradation; therefore improving the production of fungal enzymes is of great interest for biotechnology. Fungal enzymes have been mainly studied in saprobic ascomycetes, and much less extensively in basidiomycetes. This is largely caused by the better manageability of ascomycetes in industrial fermentations and the availability of transformation systems for many ascomycetes, while these aspects are still a major challenge in basidiomycetes. Despite this, basidiomycetes form a promising source of novel enzymes with different biochemical properties than ascomycete enzymes.

Plant biomass consists mainly of polysaccharides, proteins and lignin (Pauly and Keegstra 2010). Ascomycete enzyme systems focus largely on the polysaccharide

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fraction but they rarely modify the aromatic lignin polymer, due to the absence of lignin-modifying peroxidases in their genomes (Floudas et al. 2012). They do produce laccases, which have been implicated to potentially modify lignin (Xie et al. 2014). In contrast, basidiomycetes include the most efficient lignin decomposers, but they also degrade polysaccharides. The lignin-modifying enzymes of basidiomycetes are of fundamental importance for efficient bioconversion of plant residues that are rich in lignin, such as wheat straw. They are therefore highly relevant for various biotechnological applications, for example pulp and paper, and textile industries, bioremediation, and the production of biofuels and biochemicals (Jolivalt et al. 2005; Raghukumar et al. 2008). With respect to plant biomass degradation, basidiomycetes are classified either as white-rot, brown-rot or litter-decomposing fungi. White- and brown-rot refer to the appearance of wood after fungal decomposition. Brown-rot fungi efficiently degrade wood polysaccharides but are only slightly capable of modifying lignin, resulting in a brownish residue of partly decayed wood. In contrast, white-rot fungi are able to decompose all wood polymers, including lignin, resulting in a white residue, consisting mainly of cellulose (Lee et al. 2014; Ruiz-Dueñas et al. 2013; Rytioja et al. 2014).

1.1 Plant Polysaccharide Degrading Enzymes

Cellulose is the most abundant polysaccharide in plant biomass. This polymer gives rigidity to the plant cell wall and is formed by chains of β-1,4-linked D-glucose molecules that create linear crystalline structures (microfibrils) and less crystalline, amorphous regions (Kolpak and Blackwell 1976).

Several types of hemicelluloses are present in plant biomass: xyloglucan, xylan, mannan, glucomannan, and β-1,3/1,4-glucans. They are named after the monosaccharides that form their backbone (de Vries and Visser 2001). In addition, they contain various branches consisting of monomers and short oligomers.

Pectin is a heteropolysaccharide mainly composed of three principal structures (Caffal and Mohnen 2009). The simplest one is homogalacturonan, a galacturonic acid based linear polysaccharide. Xylogalacturonan is modified form of homogalacturonan in that it contains xylose branches. Rhamnogalacturonan I has a backbone of alternating galacturonic acid and rhamnose residues, with arabinan and (arabino-)galactan side chains attached to the rhamnose residues.

Each plant polysaccharide degrading fungus has a specific set of carbohydrate-active enzymes involved in the degradation of the backbone and branching structures (Culleton et al. 2013; van den Brink and de Vries 2011). The different enzymes involved in this process with their CAZy family (Lombard et al. 2014), EC number and substrate are listed in Table 1. While some enzymes act only on a single polysaccharide (e.g. cellobiohydrolase on cellulose), others are involved in the degradation of several polysaccharides (e.g. β-galactosidase for xylan, pectin, xyloglucan and galactomannan). An overview of the available knowledge on plant polysaccharide degrading enzymes from basidiomycetes was recently presented (Rytioja et al. 2014).

Table 1 Fungal CAZymes involved in plant biomass degradation. Based on (Coutinho et al. 2009; Lombard et al. 2014)

CAZy families	Enzyme code	Enzyme	EC number	Substrate
Glycoside hydrolases				
GH1,3	BGL	β-1,4-D-Glucosidase	3.2.1.21	Cellulose, xyloglucan, galactoglucomannan
GH2,35	LAC	β-1,4-D-Galactosidase	3.2.1.23	Xylan, xyloglucan, galactomannan, pectin
GH2	MND	β-1,4-D-Mannosidase	3.2.1.25	Galactomannan
GH3,39,43	BXL	β-1,4-D-Xylosidase	3.2.1.37	Xylan, pectin
GH5,7,12,61,131	EGL	β-1,4-D-Endoglucanase	3.2.1.4	Cellulose
GH5,26	MAN	β-1,4-D-Endomannanase	3.2.1.78	Galactomannan
GH5	GLN	β-1,6-Endogalactanase	3.2.1.164	Pectin
GH6,7	CBH	Cellobiohydrolase	3.2.1.91	Cellulose
GH10,11	XLN	β-1,4-D-Endoxylanase	3.2.1.8	Xylan
GH12,74	XEG	Xyloglucan-active β-1,4-D-endoglucanase	3.2.1.151	Xyloglucan
GH13	AMY	α-Amylase	3.2.1.1	Starch
GH15	GLA	Glucoamylase	3.2.1.3	Starch
GH27,36	AGL	α-1,4-D-Galactosidase	3.2.1.22	Xylan, xyloglucan, galactomannan
GH28	PGA	Endopolygalacturonase	3.2.1.15	Pectin
GH28	PGX	Exopolygalacturonase	3.2.1.67	Pectin
GH28	RGX	Rhamnogalacturonan galaturonohydrolase/ exorhamnogalacturonase	3.2.1.40	Pectin
GH28	RHG	Rhamnogalacturonan hydrolase/endorhamnogalacturonase	3.2.1.–	Pectin
GH29,95	AFC	α-L-Fucosidase	3.2.1.51	Xyloglucan
GH30	XBH	Xylobiohydrolase	3.2.1.–	Xylan

(continued)

Table 1 (continued)

CAZy families	Enzyme code	Enzyme	EC number	Substrate
GH31	AGD	α-1,4-D-Glucosidase	3.2.1.20	Starch
GH31	AXL	α-D-Xylosidase	3.2.1.–	Xyloglucan
GH32	INU	Endo-inulinase	3.2.1.7	Inulin
GH32	INX	Exo-inulinase	3.2.1.80	Inulin
GH32	SUC	Invertase/fructofuranosidase	3.2.1.26	Inulin
GH43,51,54	ABF	α-L-Arabinofuranosidase	3.2.1.55	Xylan, xyloglucan, pectin
GH43	ABN	Endoarabinanase	3.2.1.99	Pectin
GH43	XTG	β-1,3-Exogalactanase	3.2.1.145	Pectin
GH53	GAL	β-1,4-Endogalactanase	3.2.1.89	Pectin
GH62	AXH	Arabinoxylan arabinofuranohydrolase	3.2.1.55	Xylan
GH67,115	AGU	α-Glucuronidase	3.2.1.139	Xylan
GH78	RHA	α-Rhamnosidase/rhamnogalacturonan rhamnohydrolase	3.2.1.40	Pectin
GH88	UGH	D-4,5-Unsaturated glucuronyl hydrolase	3.2.1.–	Pectin
GH93	ABX	Exoarabinanase	3.2.1.–	Pectin
GH105	URH	Unsaturated rhamnogalacturonan hydrolase	3.2.1.–	Pectin
GH131	AMG	Amylo-α-1,6-glucosidase	3.2.1.33	Starch
–	XFG	β-1,4-Exogalactanase	–	Pectin
–	XSG	β-1,6-Exogalactanase	–	Pectin
Carbohydrate esterases				
CE1,2,3,4,5,6	AXE	Acetyl xylan esterase	3.1.1.72	Xylan
CE1	FAE	Feruloyl esterase	3.1.1.73	Xylan, pectin
CE8	PME	Pectin methyl esterase	3.1.1.11	Pectin
CE12	RGAE	Rhamnogalacturonan acetyl esterase	3.1.1.–	Pectin
CE12	PAE	Pectin acetyl esterase	3.1.1.–	Pectin
CE15	GE	Glucuronoyl esterase	3.1.1.–	Xylan

–	GMAE	Galactomannan acetyl esterase	–	Galactomannan
–	XGAE	Xyloglucan acetylesterase	–	Xyloglucan
Polysaccharide lyases				
PL1	PEL	Pectin lyase	4.2.2.10	Pectin
PL1,3,9	PLY	Pectate lyase	4.2.2.2	Pectin
PL4,11	RGL	Rhamnogalacturonan lyase	4.2.2.–	Pectin
Auxiliary activities				
AA1_1	LCC	Laccase	1.10.3.2	Lignin
AA2	MnP	Manganese peroxidase	1.11.1.13	Lignin
AA2	VP	Versatile peroxidase	1.11.1.16	Lignin
AA2	LiP	Lignin peroxidase	1.11.1.14	Lignin
AA3,8	CDH	Cellobiose dehydrogenase	1.1.99.18	Cellobiose
AA9,10,13	LPMO	Lytic polysaccharide monooxygenase		Cellulose , xylan, xyloglucan, starch

1.2 Lignin-Modifying Enzymes

Lignin is a complex heterogenous aromatic polymer that is among the most abundant natural materials on earth (Martinez et al. 2004). Lignin is present in all layers of woody cell walls and is composed of three monomers: coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol. Plant laccases and peroxidases polymerize these monomers to guaiacyl-, syringyl- and hydroxyphenyl-type of lignin subunits (Higuchi 2006). The most common linkage between the subunits is a β-aryl-ether linkage (Adler 1977), but other linkages also occur (Brunow et al. 1998). The structure and composition of lignin varies significantly depending on the plant species (Martínez et al. 2008; Ralph et al. 2004). Lignin is an undesirable component e.g. in the conversion of wood into pulp and paper and remova1 of lignin is a major step in the paper making process (Campbell and Sederoff 1996). White-rot basidiomycetes are studied due to their efficient and complete degradation of woody plant cell walls (Ruiz-Dueñas et al. 2008). In contrast to other fungi and bacteria they are capable of mineralising lignin to carbon dioxide and water by producing extracellular, oxidative lignin-modifying enzymes. These include class II heme-containing peroxidases (AA2 in CAZy database), i.e. manganese peroxidases (MnPs), lignin peroxidases (LiPs) and versatile peroxidases (VPs), which catalyse H_2O_2-dependent unspecific reactions (Table 1) (Mäkelä et al. 2014). Various phenolic compounds are oxidised to organic radicals via the action of MnPs, while LiPs are able to oxidise non-phenolic lignin substructures. VP is a hybrid type of peroxidase combining the activities of both MnP and LiP (Kamitsuji et al. 2005; Ruiz-Dueñas et al. 2001).

Fungal laccases (AA1_1 in CAZy database) belong to the widely distributed family of multicopper oxidases and participate in many processes from fungal morphogenesis to melanin synthesis (Giardina et al. 2010; Kües and Rühl 2011). Laccases catalyse the oxidation of phenolic compounds and aromatic amines coupled to the reduction of molecular oxygen to water (Giardina et al. 2010). In the presence of small molecular weight mediator molecules laccases can also oxidise non-phenolic compounds. Although the role of laccases in lignin degradation is still debatable, they are usually regarded as lignin-modifying enzymes (Lundell et al. 2010). Laccases can be used in various biotechnological processes. For example the white-rot fungus *Trametes pubescens* is considered an excellent producer of industrial laccases (Galhaup et al. 2002). Applications of laccases include bleaching of textiles (Vinod 2001) and wood pulp (Widsten and Kandelbauer 2008), clearing of fruit juice, beer and wine (Minussi et al. 2002), biosensors (Kulys and Vidziunaite 2003), hair dyes, degradation of plastics and decontamination of soils (Kunamneni et al. 2008).

Despite the huge biotechnological potential of fungal lignin-modifying enzymes, the production of both laccases and peroxidases to levels required by industry using homologous and heterologous expression remains a challenge and has been the topic of numerous research projects.

2 Homologous and Heterologous Expression of Basidiomycete Genes in Basidiomycetes

Overexpression of genes in basidiomycetes is less commonly performed than in ascomycetes. This is largely due to the availability of transformation systems for many ascomycetes, while only a relatively small number of basidiomycetes can be transformed. A well-developed transformation system based on auxotrophic mutants was generated for the white-rot fungus *Phanerochaete chrysosporium* that is the best studied basidiomycete with respect to plant biomass degradation (Mayfield et al. 1994). Transformation systems are also being developed for other basidiomycete species and significant progress has been made (e.g. Burns et al. 2005, 2006; Eastwood et al. 2008; Salame et al. 2012; Sharma and Kuhad 2010; Shi et al. 2012; Wang et al. 2008), but their efficiency is still significantly less than those of ascomycetes.

2.1 Homologous Expression

Homologous transformation of plant cell wall degradation related genes is limited to a small number of basidiomycete species and this involves mainly genes related to lignin degradation. The advantage of homologous overexpression is that the genes do not need to be modified to accommodate optimal codon usage or gene structure as is sometimes the case for heterologous overexpression (Kajita et al. 2004). Homologous expression in basidiomycetes has been reported for several genes (Irie et al. 2001; Li et al. 2000; Ma et al. 2003; Mayfield et al. 1994; Sollewijn Gelpke et al. 1999), and some examples are discussed in more detail below and listed in Table 2.

Laccases have been overproduced in some basidiomycete species. The genome of the white-rot fungus *Trametes* (*Coriolus*) *versicolor* contains several laccases but the highest secretion level was observed for laccase III (CVL3, Kajita et al. 2004). Improved production of this enzyme was achieved by transformation using a chimeric gene for laccase III under glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter of *T. versicolor* and hygromycin B phosphotransferase (*hph*) gene that encodes resistance to phleomycin and hygromycin B as a selection marker. In addition, a further improvement was made by the addition of copper (II) sulphate to the growth medium (Kajita et al. 2004).

Overexpression of laccase genes in the white-rot species *Polyporus brumalis* resulted in a three to fourfold increase in laccase activity (Ryu et al. 2008b). In this case, transformants were generated using the restriction enzyme mediated integration (REMI) method (Leem et al. 1999) and using hygromycin resistance selection. The *lcc1* gene of the coprophilic fungus *Coprinopsis cinerea* has been homologously expressed using different homologous and heterologous promoters. The highest activity of one of the *C. cinerea* transformants was reached under control of *gpd* II promoter from the litter-decomposing fungus *Agaricus bisporus* and with addition of copper to the medium (Kilaru et al. 2006).

Table 2 Homologous overproduction of basidiomycete enzymes involved in plant biomass degradation

	Enzyme	Enzyme	Protein/gene accession number	Species	Production level	References
Lignin modifying enzymes	Laccase (AA1_1)	Cvl3	BAA22153	*Coriolus* (*Trametes*) *versicolor*	0.95 μmol/min/mL oxidized guiacol	Kajita et al. (2004)
		Pblac1	EF362634	*Polyporus brumalis*	nr	Ryu et al. (2008b)
		Pblac2	EF362635		nr	
		Lcc1	AAR01242	*Coprinopsis cinerea*	3 μmol/min/mL ABTS[a] radical	Kilaru et al. (2006)
		Lac1	AAT28344	*Pycnoporus cinnabarinus*	4660 nkat/mL, 1.2 g/L	Alves et al. (2004)
	Lignin peroxidase (AA2)	LiPH8	ACY82388	*Phanerochaete chrysosporium*	2 mg/L	Sollewijn Gelpke et al. (1999)
		YK-LiP1	AB455006	*Phanerochaete sordida*	0.09 nkat/mL	Sugiura et al. (2009)
		YK-LiP2	AB455007	*P. sordida*	nr	Sugiura et al. (2009)
	Versatile peroxidase (AA2)	MnP4	AAK69187	*Pleurotus ostreatus*	0.12 μmol/min/mL oxidized phenol red	Salame et al. (2012)
	Manganese peroxidase (AA2)	MnP3	AAB39652	*P. ostreatus*	1.560 U/mL[b]	Irie et al. (2001)
		MnP4	AB585997	*P. sordida*	nr	Sugiura et al. (2012)

nr production level (mg/L) not reported

[a]2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

[b]One unit was defined as the amount of enzyme that results in an increase of one absorbance unit per minute at 465 nm

The production of a laccase of the white-rot fungus *Pycnoporus cinnabarinus* was significantly increased by overexpression under control of its own promoter, and *Schizophyllum commune GPD* and hydrophobin (*SC3*) promoters (Alves et al. 2004). The maximum levels (4660 nkat mL^{-1}) are the highest reported so far, highlighting the potential of this species for enzyme production.

As mentioned above, *P. chrysosporium* has been the most extensively studied white-rot basidiomycete and a transformation system for this fungus was already developed many years ago (Mayfield et al. 1994). The *P. chrysosporium* genome does not have laccase encoding genes, but this species produces a number of lignin-modifying heme peroxidases (Martinez et al. 2004).

Homologous production of lignin peroxidase (LiPH8) of *P. chrysosporium* has been successful (Sollewijn Gelpke et al. 1999). LiPs are major extracellular components of the lignin-degrading system of *P. chrysosporium*, but there are still open questions about their mechanisms of action, like the presumptive role of veratryl alcohol as a mediator in LiP-catalysed reactions.

Another species of the genus *Phanerochaete*, *Phanerochaete sordida*, exhibits greater ligninolytic selectivity during growth on beech wood than either *P. chrysosporium* or *T. versicolor*, and produces two LiPs (YK-LiP1 and YK-LiP2) under manganese-deficient conditions. Cloning and homologous expression of the YK-LiP1 and YK-LiP2 encoding genes in *P. sordida* YK-624 were carried out to obtain higher levels of these enzymes allowing characterization of the recombinant protein (Sugiura et al. 2009).

A manganese peroxidase (MnP3) from the white-rot fungus *Pleutorus ostreatus* was also homologously overproduced using a drug-resistant selection marker. This marker was based on a nucleotide substitution in *P. ostreatus sdi1*, encoding an iron sulfur protein that confers dominant resistance to the fungicide carboxin (Honda et al. 2000). The recombinant *mnp3* was expressed under control of the *P. ostreatus sdi1* promoter and terminator (Irie et al. 2001).

Due to the lower transformation efficiency of (filamentous) basidiomycetes compared to ascomycetes, relatively few studies have been reported on gene disruption of plant biomass degradation related genes in basidiomycetes. However, a homologous gene-replacement system was developed for *P. ostreatus* (Salame et al. 2012). This highlighted the limitations of homologous recombination in basidiomycetes due to the low frequency (2.3 %) of site-specific recombination of DNA integration and the low transformation efficiency using protoplast-mediated transformation.

2.2 Heterologous Expression in Other Basidiomycetes

Because of the limited number of easily transformable basidiomycete species only few basidiomycete genes encoding plant biomass degrading enzymes have been heterologously expressed in other basidiomycetes (Table 3). One example is the expression of a MnP encoding gene from the white-rot fungus *Dichomitus squalens* in *P. chrysosporium*. Most (82 %) of the transformants exhibited MnP activity and further analysis demonstrated that the recombinant protein was fully functional (Li et al. 2001)

Table 3 Heterologous production of basidiomycete enzymes involved in plant biomass degradation in filamentous fungi

	Enzyme	Enzyme	Protein accession number	Host	Source of the native protein	Highest production level	References
Lignin modifying enzymes	Laccase (AA1_1)	Lac1	AF170093	*Aspergillus niger*	*Pycnoporus cinnabarinus*	0.7 1 µmol/min/mL ABTS radical, 85 mg/L	Record et al. (2002)
						7.1 µmol/min/mL ABTS radical, 51 mg/L	Sigoillot et al. (2004)
		Pel3	AY686700		*Pleurotus eryngii*	2.4 U/mg[a] protein	Rodríguez et al. (2008)
		Lcc2	Q12718		*Trametes versicolor*	2.7 µmol/min/mL ABTS radical	Bohlin et al. (2006)
		Lcc4	U44431			0.6 µmol/min/mL ABTS radical	Téllez-Jurado et al. (2006)
		POXA1b	CAA06291		*Pleurotus ostreatus*	583.3 nkat/mL, 13 mg/L	Macellaro et al. (2014)
		1H6C	–			1000 nkat/mL, 20 mg/L	
		Lcc1	AAD30964	*Aspergillus oryzae*	*Coprinopsis cinerea*	3 µmol/min/mL ABTS radical, 135 mg/L	Yaver and Golightly (1996)
		Lcc1, Lcc4	P56193, Q02081		*Rhizoctonia solani*	nr	Wahleithner et al. (1996)
		LacA	AB015758		*Schizophyllum commune*	774 U/mL[b]	Hatamoto et al. (1999)
		Lac1	AF170093		*P. cinnabarinus*	7.8 µmol/min/mL ABTS radical, 51 mg/L	Sigoillot et al. (2004)
		Lcc1	AF170093		*Pycnoporus coccineus*	2.8 U/mL	

		LacA	AY839936.1	*Trichoderma reesei*	*Trametes* sp.	3.62 µmol/min/mL ABTS radical	Zhang et al. (2012)
		Lcc	AAR21094		*P. ostreatus*	237.134 U/mL[a]	Dong et al. (2012)
		Lac1	CAE00180		*Melanocarpus albomyces*	193 nkat/mL, 230 mg/L	Kiiskinen et al. (2004)
		Lcc	CAA36379		*Phlebia radiata*	7.7 nkat/mL, 19.5 mg/L	Saloheimo and Niku-Paavola (1991)
		Lac	ACC43989	*Penicillium canescens*	*Trametes hirsuta*	3 U/mL[c]	Abianova et al. (2010)
	Versatile peroxidase (AA2)	VPl	AF007222	*Aspergillus nidulans*	*P. eryngii*	466 U/L[d]	Eibes et al. (2009)
	Manganese peroxidase (AA2)	MnP1	Q02567	*A. niger*	*Phanerochaete chrysosporium*	3 U/L[a]	Cortes-Espinosa et al. (2011)
				A. oryzae	*P. chrysosporium*	0.33 µmol/min/mL	Stewart et al. (1996)
				Amylomyces rouxii	*P. chrysosporium*	130 U/L[b]	Montiel-González et al. (2009)
		MnP2	AAF31330	*P. chrysosporium*	*Dichomitus squalens*	0.65 µmol/min/mL	Li et al. (2001)
	Lignin peroxidase (AA2)	LiP	M27884	*A. rouxii*	*P. chrysosporium*	72.14 U/L	Montiel-González et al. (2009)
			CAA32616	*Phlebia radiata*	*T. reesei*	nd	Saloheimo et al. (1989)
	Aryl-alcohol oxidase (AA3_2)	AAO	AAC72747	*A. nidulans*	*P. eryngii*	400–500 mU/mL[a]	Varela et al. (2001)

(continued)

Table 3 (continued)

	Enzyme	Enzyme	Protein accession number	Host	Source of the native protein	Highest production level	References
(Hemi-) cellulose active enzymes	Cellobiohydrolase I (GH7)	CBH1	AB103461	*A. oryzae*	*Athelia* (*Corticium*) *rolfsii*	0.122 U/mL[a]	Yasokawa et al. (2003)
	β-Glucosidase (GH3)	Uebgl3A	AB618734	*A. oryzae*	*Ustilago esculenta*	Nr	Nakajima et al. (2012)
	Cellobiose dehydrogenase (AA3)	CDH	XP_001835032	*A. niger*	*Coprinopsis cinerea*	127 nkat/mL	Turbe-Doan et al. (2013)
	Pyranose dehydrogenase	PDH1	AY753307	*A. niger*	*Agaricus meleagris*	nr	Pisanelli et al. (2010)
				A. nidulans		5.5 U/ mL[a]	
	Glucuronoyl esterase (carbohydrate esterase)	GE1	130517 (JGI[f])	*Aspergillus vadensis*	*P. chrysosporium*	0.036 U/mL[e]	Duranová et al. (2009)
				P. cinnabarinus		0.417 U/mL[e]	
				S. commune		0.453 U/mL[e]	
		GE2	6482 (JGI[f])	*S. commune*	*P. chrysosporium*	0.530 U/mL[e]	

nr production level not reported, *nd* not detected

[a]Unit definition unknown

[b]One unit of enzyme activity is defined as a change in A_{690} of 0.001 per minute

[c]One unit of enzyme activity equals with an increase in the optical density of the reaction mixture over 1 min calculated per mg of protein

[d]One unit of activity is defined as the amount of enzyme oxidizing 1 µmol of Mn^{2+} per minute

[e]One unit of activity is defined as the amount of enzyme de-esterifying 1 µmol of 4-nitrophenyl-2-*O*-(methyl-4-*O*-methyl-α-D-glucopyanosyluronate)-β-D-xylopyranoside per minute

[f]Available through the fungal genome portal of the Joint Genome Institute of the Department of Energy of the USA (http://genome.jgi.doe.gov/programs/fungi/index.jsf)

Glucuronoyl esterases are suggested to break the linkage between lignin and xylan through glucuronic acid. Two genes (*ge1* and *ge2*) from *P. chrysosporium* encoding these enzymes were successfully expressed in *P. cinnabarinus* under control of the *S. commune GPD* promoter and in *S. commune* under control of the *S. commune SC3* promoter to produce the recombinant protein (Duranová et al. 2009). The highest glucoronoyl esterase activity was found in the transformants of *ge2* in *S. commune*.

3 Heterologous Expression of Basidiomycete Genes in Ascomycetes

3.1 Expression in Yeasts

Pichia pastoris and *Saccharomyces cerevisiae* are the most commonly used yeast hosts for heterologous expression of plant biomass modifying enzyme encoding genes from basidiomycetes. This is because of the various production strains and vectors that are available for these species. In few cases, *Kluyveromyces lactis*, *Pichia methanolica* and *Yarrowia lipolytica* have been employed as hosts for the production of basidiomycete plant biomass modifying enzymes. *K. lactis*, *S. cerevisiae* and *Y. lipolytica* are generally regarded as safe (GRAS) organisms, which is beneficial when producing food or medical related proteins. The methylotrophic yeasts *P. pastoris* and *P. methanolica* both have two alcohol oxidase (AOX, AUG) encoding genes and can utilize methanol as a sole carbon source (Cregg et al. 1989; Raymond et al. 1998). The strong and regulated *AOX* (or *AUG*) promoter regions of these yeasts are widely employed for the heterologous expression of recombinant proteins.

In contrast to the other eukaryotic heterologous protein production systems, the yeast systems are fast and inexpensive ways to achieve recombinant proteins. One of the main advantages is also the availability of a wide variety of well-developed tools for genetic manipulation of yeasts. In addition, unicellular yeasts are easy to cultivate in bioreactors, unlike filamentous fungi. Yeasts can be cultivated on a comparatively simple and inexpensive medium at a relatively fast growth rate (Daly and Hearn 2005). As eukaryotic organisms, yeasts are more suitable for the production of eukaryotic proteins than bacteria, since yeasts are able to perform several post-translational modifications, which are often crucial for the biochemical properties of the target proteins. These include e.g. phosphorylation, glycosylation, formation of disulfide bonds and proteolytic processing of the signal sequence (Cregg et al. 2000; Gasser et al. 2008; Huang et al. 2012).

Although yeasts are able to perform these modifications, the patterns of O- and N-linked sugar units added to the synthesized proteins by yeasts differ from those of other eukaryotes (Berends et al. 2009; Cereghino and Cregg 2000; Gasser et al. 2008; Gellissen et al. 2005; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). This can be a drawback when using yeasts as production hosts since the different glycosylation patterns may alter the folding of the recombinant protein and

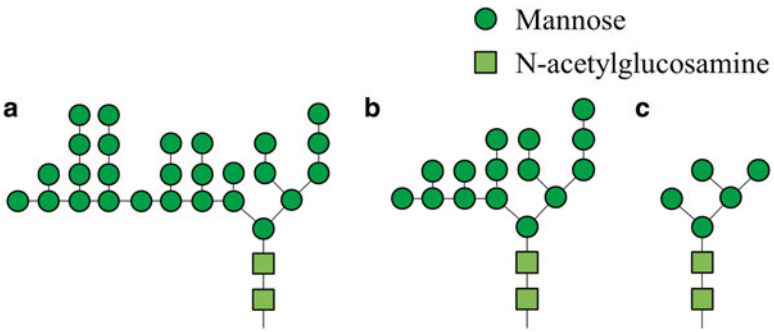

Fig. 1 Simplified structure of N-linked glycosylation pattern of (**a**) *Saccharomyces cerevisiae*, (**b**) *Pichia pastoris*, and (**c**) basidiomycete fungi (Berends et al. 2009; Hamilton and Gerngross 2007; Herscovics and Orlean 1993)

by that change its biochemical and functional properties (Berends et al. 2009; Bohlin et al. 2006; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). The O-linked glycosylation of the proteins produced by yeasts consist only of mannose subunits. In contrast, higher eukaryotes, including basidiomycete fungi, add also other O-linked sugar units, such as galactose, N-acetylgalactosamine and N-acetylglucosamine, to the proteins (Lengeler et al. 2008). Yeasts may also attach excess N-linked mannose subunits to the recombinant proteins (Fig. 1) (Berends et al. 2009; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). Especially, *S. cerevisiae* is known to hyperglycosylate secreted proteins (Mattanovich et al. 2009).

Yeast can produce intracellular recombinant proteins or secrete proteins out of the cells (Macauley-Patrick et al. 2005). However, secretion of the recombinant proteins is usually preferred, since targeting of the recombinant protein into the yeast eukaryotic secretory pathway is necessary especially when the recombinant protein requires post-translational modifications. Yeasts also secrete relatively low levels of native proteins, thus simplifying the recovery and purification of the target protein from the extracellular culture medium (Cregg et al. 2000; Huang et al. 2011a).

3.1.1 Yeast Expression Systems Used for the Heterologous Production of Plant Biomass Modifying Enzymes from Basidiomycetes

Recombinant Protein Production in Saccharomyces Cerevisiae

Multiple *S. cerevisiae* strains (such as CEN.PK2-1C, CEN.PK113-17A, W303-1A, INVSc1, Y294, BJ5465 and BJ3505) have been used for the heterologous expression of basidiomycete plant cell wall modifying enzymes (Table 4). From these strains, BJ5465 and BJ3505 are protease deficient, thus making the recombinant proteins less susceptible to degradation by extracellular proteases. All the previously mentioned *S. cerevisiae* strains are auxotrophic mutants which are unable to synthetize uracil or uridine, indispensable compounds for ribonucleic acids, or one of the essential amino acids tryptophan or leucine. These features have been exploited as selection markers in the *S. cerevisiae* expression vectors (Table 5). Most of the

Table 4 Yeast strains used for the heterologous expression of plant biomass modifying enzymes from basidiomycetes

Host	Strain	Genotype	Phenotype	References
Saccharomyces cerevisiae	CEN.PK2-1C	MATa; ura3-52; trp1-289; leu2-3112; his3Δ 1; MAL2-8^{c}; SUC2	Unable to synthetize uracil, tryptophan, leucine and histidine	Bleve et al. (2008, 2013)
	CEN. PK113-17A	MATa; ura3; leu2	Unable to synthetize uracil and leucine	Cassland and Jönsson (1999)
	CEN. PK113-13D	MATa; ura3-52; MAL2-8c; SUC2	Unable to synthetize uracil	Wilde et al. (2012)
	W303-1A	MATa; leu2-3112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15	Unable to synthetize leucine, tryptophan, uracil, adenine and histidine; sensitive to canavanine	Klonowska et al. (2005), Necochea (2005), Pezzella et al. (2009)
	INVSc1	MATa; his3D1; leu2; trp1-289; ura3-52	Unable to synthetize histidine, leucine, tryptophan and uracil	Cassland and Jönsson (1999), Tang et al. (2001)
	BJ5465	MATa; ura3-52; trp1; leu2-delta1; his3-delta200; pep4::HIS3; prb1-delta1.6R; can1; GAL	Unable to synthetize uracil, tryptophan, leucine and histidine; sensitive to canavanine; protease-deficient	Ryu et al. (2008a)
	BJ3505	pep4::HIS3; prb-δ1.6R HIS3; lys2-208: trp1-δ101; ura3-52; gal2; can1	Unable to synthetize tryptophan and uracil; sensitive to canavanine; protease-deficient; able to utilize alpha-aminoadipate as a nitrogen source	Toda et al. (2005)
Pichia pastoris	X-33	Wild type		Bey et al. (2011), Ding et al. (2002), Garg et al. (2012), Herter et al. (2011), Huy et al. (2013c), Joo et al. (2008), Nishibori et al. (2013), Salinas et al. (2011), Toda et al. (2008), Wang and Wen (2009)

(continued)

Table 4 (continued)

Host	Strain	Genotype	Phenotype	References
	GS115	his4	Unable to synthetise histidine	Brown et al. (2002), Colao et al. (2006, 2009), Ding et al. (2002), Hildén et al. (2013), Hong et al. (2006), Huang et al. (2011b), Huy et al. (2011, 2013a, b, c), Jiang et al. (2008), Jönsson et al. (1997), Kim et al. (2012), Li et al. (2007), Lin et al. (2013), O'Callaghan et al. (2002), Saito et al. (2013), Sun et al. (2012), You et al. (2013)
	KM71H	aox1::ARG; arg4	Reduced ability to metabolise methanol	Bao et al. (2013), Igarashi et al. (2008)
	KM71	his4; aox1::ARG4; arg4	Unable to synthetise histidine; reduced ability to metabolise methanol	Kawai et al. (2003), Kotake et al. (2009), Tang et al. (2001), Yoshida et al. (2001)
	SMD1168H	pep4	Protease-deficient	Gu et al. (2003)
	SMD1168	his4; pep4	Unable to synthetise histidine; protease-deficient	Bohlin et al. (2006), Gelo-Pujic et al. (1999), Jönsson et al. (1997)
Pichia methanolica	PMAD11	ade2-11	Unable to synthetize adenine	Guo et al. (2006, 2008)
	PMAD16	ade2-11; pep4; prb1	Unable to synthetize adenine; protease-deficient	Guo et al. (2005, 2006)
Kluyveromyces lactis	CMK5	MATa; thr; lys; pgi1; adh3; adh1::URA3; adh2::URA3	Unable to synthetize threonine and lysine	Camattari et al. (2007), Faraco et al. (2008), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005), Ranieri et al. (2009)
Yarrowia lipolytica	Polg	MATa; leu2-270; ura3-302c; xpr2-322; axp-2	Unable to synthetise leucine and uracil; protease-deficient	Jolivalt et al. (2005), Madzak et al. (2000, 2005, 2006)

Table 5 Yeast expression vectors used for the heterologous expression of basidiomycete plant biomass modifying enzymes

Host	Vector	Promoter		Selection marker	Signal peptide	References
S. cerevisiae	pYES2	GAL1	Inducible	Uracil synthesis	Native	Bleve et al. (2008, 2013), Tang et al. (2001), Wilde et al. (2012)
	pSAL4	CUP1	Inducible	Uracil synthesis	Native	Autore et al. (2009), Festa et al. (2008), Pezzella et al. (2009)
	pFL60	PGK	Constitutive	Uracil synthesis	Native	Cassland and Jönsson (1999)
	pAJ401	PGK	Constitutive	Uracil synthesis	Native	Cassland and Jönsson (1999)
	pDP51	GAL10	Inducible	Uracil synthesis	Native	Cusano et al. (2009), Klonowska et al. (2005)
	pAC1	ADH1	Constitutive	uracil synthesis	Native	Klonowska et al. (2005)
	pYEX-S1	PGK	Constitutive	Uracil synthesis	Native	Ryu et al. (2008a)
	YEp-FLAG-1	ADH2	Inducible	Tryptophan synthesis	*S. cerevisiae* mating type α-leader sequence	Toda et al. (2005)
	YEp352	PGK	Constitutive	Uracil synthesis	Native	Hill et al. (1986)
	Yep351	GAL10	Inducible	Leucine synthesis	Native	Hill et al. (1986)
	pJRoC30-α	GAL1	Inducible	Uracil synthesis	*S. cerevisiae* mating type α-leader sequence	Garcia-Ruiz et al. (2012)
	pBM258	GAL1	Inducible	Uracil synthesis	Native	Cusano et al. (2009)
P. pastoris	pPIC9	AOX1	Inducible	Histidine synthesis	*S. cerevisiae* mating type α-leader sequence	Bao et al. (2013), Brown et al. (2002), Colao et al. (2006, 2009), Hong et al. (2006), Huang et al. (2011b), Jönsson et al. (1997), Joo et al. (2008), Kawai et al. (2003), Lin et al. (2013), Sun et al. (2012), Yoshida et al. (2001), You et al. (2013)
	pPIC3.5	AOX1	Inducible	Histidine synthesis	Native	Huang et al. (2011b), Saito et al. (2013)
	pPICZ	AOX1	Inducible	Zeocin resistance	Native	Ding et al. (2002), Hildén et al. (2013), Huy et al. (2013b), Lu et al. (2009), Nishibori et al. (2013)

(continued)

Table 5 (continued)

Host	Vector	Promoter		Selection marker	Signal peptide	References
	pPICZα	AOX1	Inducible	Zeocin resistance	*S. cerevisiae* mating type α-leader sequence	Bey et al. (2011), Ding et al. (2002), Garg et al. (2012), Hildén et al. (2013), Huy et al. (2013b), Igarashi et al. (2008), Kim et al. (2012), Kotake et al. (2009), Salinas et al. (2011), Tang et al. (2001), Toda et al. (2008), Wang and Wen (2009)
	pHIL-D2	AOX1	Inducible	Histidine synthesis	Native	Brown et al. (2002), Colao et al. (2006, 2009), Gelo-Pujic et al. (1999), Jönsson et al. (1997), O'Callaghan et al. (2002)
	pGAPZ	GAP	Constitutive	Zeocin resistance	Native	Bohlin et al. (2006), Gu et al. (2003)
	pGAPZα	GAP	Constitutive	Zeocin resistance	*S. cerevisiae* mating type α-leader sequence	Gu et al. (2003), Herter et al. (2011), Jiang et al. (2008)
P. methanolica	pMETαA	AUG1	Inducible	Adenine synthesis	*S. cerevisiae* mating type α-leader sequence	Guo et al. (2005, 2006, 2008), Raymond et al. (1998)
K. lactis	pYG132	KlADH4	Inducible	Geneticin resistance	Native	Faraco et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005), Saliola et al. (1999)
	pLC12	KlADH4	Inducible	Uracil synthesis	Native	Camattari et al. (2007)
Y. lipolytica	pINA1267	pLEU2	Inducible	Leucine synthesis	XPR2 extracellular protease prepro sequence	Madzak et al. (2000, 2005)
	pINA1296	pLEU2	Inducible	Leucine synthesis	XPR2 extracellular protease prepro sequence	Jolivalt et al. (2005), Madzak et al. (2000, 2005, 2006)

vectors applied for the expression of the basidiomycete lignocellulose modifying genes contain the *ura3* gene enabling the uracil auxotrophic strain to grow on a medium that lacks uracil or uridine.

The *S. cerevisiae* expression vectors employ both constitutive and inducible promoter regions of the yeast. The constitutive promoters applied are alcohol dehydrogenase I (*ADH1*) and phosphoglycerate kinase (*PGK*) (Derynck et al. 1983; Hitzeman et al. 1981; Romanos et al. 1992). These promotes are active in the presence of glucose and the genes cloned downstream of them are then expressed. The inducible promoters include galactokinase (*GAL1*), UDP galactose-4-epimerase (*GAL10*), alcohol dehydrogenase II (*ADH2*) and copper-metallothionein (*CUP1*) (Butt et al. 1984; Johnston and Davis 1984; Mascorro-Gallardo et al. 1996; Romanos et al. 1992; West et al. 1984). The *GAL1* and *GAL10* promoters are activated by galactose and repressed by glucose. The expression of *ADH2* promoter is activated by a non-fermentable carbon source such as ethanol or glycerol, whereas the addition of Cu^{2+} ions activates the expression of *CUP1* promoter region.

Recombinant Protein Production in Pichia Pastoris

All *P. pastoris* strains are derivatives of the wild type strain Y-11430 from Northern Regional Research Laboratories (NRRL, Peoria, IL, USA). Both wild type and auxotrophic strains of *P. pastoris* have been applied for the production of lignocellulose converting basidiomycete enzymes (Table 4). X-33 is a wild type strain, whereas GS115, KM71 and SMD1168 have a mutated histidinol dehydrogenase (*his4*) gene and are unable to grow without histidine supplementation. In addition, SMD1168 and SMAD1168H are protease deficient strains that lack protease A activity, thus often improving the yield of recombinant proteins. The strains KM71 and KM71H contain a mutated *AOX1* promoter. The remaining *AOX2* promoter is weaker and therefore the cells exhibit a slower growth rate with decreased consumption of toxic methanol (Chiruvolu et al. 1997; Cregg et al. 1989). The *his4* gene of *P. pastoris* and a gene encoding resistance against the antibiotic zeocin have been employed as selection markers in the expression vectors (Table 5). In these vectors, alcohol oxidase (*AOX*) and glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter regions of *P. pastoris* are used. The *AOX1* promoter is induced when methanol is used as the sole carbon source (Cregg et al. 1989) while the constitutive *GAP* promoter is active in the presence of glucose (Waterham et al. 1997). *P. pastoris* vectors pPIC3.5, pHIL-D2, pPICZ and pGAPZ use the native signal sequence of the heterologously produced protein for the secretion, whereas in the vectors pPIC9, pPICZα and pGAPZα the signal sequence of *S. cerevisiae* mating type α-factor is included (Cregg and Higgins 1995).

Compared to other yeasts, *P. pastoris* is capable of growing at higher cell density (OD_{600} 500) therefore often resulting in elevated yield of recombinant protein (Cereghino and Cregg 2000; Jahic et al. 2002). In fact, the amount of the target protein can be up to 30 % of all the extracellular proteins secreted by *P. pastoris* (Cregg and Higgins 1995; Gellissen 2000).

Other Recombinant Protein Production Systems

K. lactis, *P. methanolica* and *Y. lipolytica* are yeast species more rarely applied for the production of plant biomass modifying proteins from basidiomycetes. *K. lactis* CMK5 strain is a uracil auxotroph and possess only one functional alcohol dehydrogenase (KlADH) encoding gene (Table 4) (Saliola et al. 1999). *K. lactis* expression vectors include the inducible *KlADH4* promoter region (Table 5), which is activated in the presence of ethanol (Saliola et al. 1991). In *K. lactis* expression vectors, *ura3* and a gene encoding antibiotic resistance against geneticin are used as selection markers (Van Ooyen et al. 2006).

The *P. methanolica* PMAD11 and PMAD16 strains are adenine auxotrophic mutants. In addition, PMAD16 strain is protease deficient. In the *P. methanolica* expression vector, the *ade2* gene from *S. cerevisiae* has been used to complement adenine auxotrophy in the host strain (Table 5) (Raymond et al. 1998). Similarly with *P. pastoris*, the methanol inducible promoter region of alcohol oxidase (*AUG*) has been applied for the production of recombinant proteins in *P. methanolica* (Raymond et al. 1998). The pMETα vector utilizes the signal sequence of *S. cerevisiae* mating type α-factor for the secretion of the recombinant protein.

The protease deficient and leucine auxotrophic strain of *Y. lipolytica*, Polg, has been used for the production of recombinant plant cell wall degrading enzymes from basidiomycetes (Table 4) (Madzak et al. 2004). The expression vectors pINA1267 and pINA1296 both contain the inducible leucine promoter region (*pLEU2*) of *Y. lipolytica* which is activated by the presence of leucine precursors (Table 5) (Madzak et al. 2000, 2004). These vectors complement *leu2* deficiency providing a wild type phenotype. Recombinant proteins are secreted by employing the signal sequence of extracellular protease (XPR2) of *Y. lipolytica* (Madzak et al. 2000).

3.1.2 Examples of Recombinant Proteins Involved in Plant Biomass Degradation Produced in Yeasts

Biochemically active plant biomass modifying enzymes including hydrolases, oxidases and lyases from various basidiomycete species has been successfully produced in *S. cerevisiae* and *P. pastoris* (Table 6). Most of these enzymes originate from wood-decaying white-rot fungal species. Recently, the *P. pastoris* expression system has gained an increasing interest when compared to *S. cerevisiae* and other yeast systems.

Laccases (AA1_1) are the most commonly produced basidiomycete lignin modifying enzymes in yeasts (Table 6). From lignin modifying oxidoreductases, the highest activity levels, up to 7200 U/L, have been achieved with laccase from the saprobic fungus *Cyathus bulleri* produced in *P. pastoris* (Garg et al. 2012). The highest protein yield, up to 340 mg/L, has been reported when laccase of the white-rot fungus *Trametes trogii* has been produced in *P. pastoris* (Colao et al. 2009). Laccase production in *P. pastoris* has been significantly optimized. Supplementation

Table 6 Basidiomycete enzymes involved in plant biomass degradation produced in yeasts as recombinant enzymes

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
Lignin modifying enzymes	Laccase (AA1_1)	M60560	*S. cerevisiae*	*Colorius (Trametes) hirsutus*	nr	Kojima et al. (1990)
		AM773999 AM774000		*Pleurotus eryngii*	30–88 U/L[a]	Bleve et al. (2008, 2013)
		AJ005018 Z34848 Z34847 AJ344434		*Pleurotus ostreatus*	75 U/L[a]	Autore et al. (2009), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005)
		AB072703		*Pycnoporus coccineus* (syn. *Pycnoporus sanguineus*, *Trametes sanguinea)*	nr	Hoshida et al. (2001, 2005)
		Y18012 X84683 AAC49828 AY210894		*Trametes versicolor*	35 mU/L[a]	Cassland and Jönsson (1999), Necochea (2005), Uldschmid et al. (2003)
		AKY160 EMY162 EMY164		*Trametes* sp. C30	1120 U/L[a], 2 mg/L	Cusano et al. (2009), Klonowska et al. (2005)
		XM_001829459	*P. pastoris*	*Coprinopsis cinerea*	nr	Lin et al. (2013)
		EF439894 EF439893 HG764548 HG764549		*Coprinus comatus*	1465 U/L[a]	Bao et al. (2013), Gu et al. (2014)
		EU195884		*Cyathus bulleri*	600–7200 U/L[a]	Garg et al. (2012)
		AB253791		*Flammulina velutipes*	nr	Saito et al. (2013)
		AY167042		*Fome lignosus*	nr	Hu et al. (2007), Liu et al. (2003)
		AF185275 FJ473385		*Ganoderma lucidum*	680 U/L[a]	Joo et al. (2008), Sun et al. (2012), You et al. (2013)

(continued)

Table 6 (continued)

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
		JQ027726 JQ027727		*Physisporinus rivulosus*	nr	Hildén et al. (2013)
		AF297228		*Pleurotus sajor-caju*	nr	Soden et al. (2002)
		FJ473384		*Polyporus grammocephalus*	320 U/L[a]	Huang et al. (2011b)
		AF170093		*Pycnoporus cinnabarinus*	nr	Herter et al. (2011), Otterbein et al. (2000)
		AB072703		*Pycnoporus sanguineus (Trametes sanguinea)*	1733–4706 U/L[a]	Lu et al. (2009), Hoshida et al. (2001)
		AJ294820 AM292415		*Trametes trogii*	17–340 mg/L	Colao et al. (2006, 2009)
		X84683 Y18012 U44431 U44851 AY693776 Y18012 AB539566 AY049725		*T. versicolor*	14–1500 U/L[a]	Bohlin et al. (2006), Brown et al. (2002), Gelo-Pujic et al. (1999), Herter et al. (2011), Jönsson et al. (1997), Nishibori et al. (2013), O'Callaghan et al. (2002)
		AF388910 AY846842		*Trametes* sp. AH28-2	5440 U/L[a]	Hong et al. (2006), Li et al. (2007)
		AY839942		*Trametes* sp. 420	nr	Hong et al. (2007), Xu et al. (2007), Zhou et al. (2007)
		AJ005018 Z34848 Z34847 AJ344434	*K. lactis*	*P. ostreatus*	12 U/L[a]	Faraco et al. (2008), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005)
		AJ294820		*T. trogii*	nr	Camattari et al. (2007), Ranieri et al. (2009)
		AF170093	*Y. lipolytica*	*P. cinnabarinus*	20 mg/L	Madzak et al. (2005)
		AF414109		*T. versicolor*	2.5 mg/L	Jolivalt et al. (2005), Madzak et al. (2006)
		na	*P. methanolica*	*T. versicolor*	nr	Guo et al. (2005, 2006, 2008)

	Lignin peroxidase (AA2)	na	*S. cerevisiae*	*Phanerochaete chrysosporium*	nr	Ryu et al. (2008a)
		X15599	*P. pastoris*	*P. chrysosporium*	nr	Wang and Wen (2009)
	Versatile peroxidase (AA2)	na	*S. cerevisiae*	*P. eryngii*	21 mg/L	Garcia-Ruiz et al. (2012)
	Manganese peroxidase (AA2)	J04980	*P. pastoris*	*P. chrysosporium*	nr	Gu et al. (2003), Jiang et al. (2008)
Oxalate degrading enzymes	Oxalate decarboxylase (EC 4.1.1.2)	na	*P. pastoris*	*Dichomitus squalens*	nr	Sietiö et al. (2015)
	Oxalate oxidase (EC 1.2.3.4)	AJ746412	*P. pastoris*	*Ceriporiopsis subvermispora*	4 mg/L	Moomaw et al. (2013), Moussatche et al. (2011)
Cellulose degrading enzymes	Cellobiohydrolase I (GH7)	na	*S. cerevisiae*	*P. chrysosporium*	nr	Van Rensburg et al. (1998)
	Cellobiohydrolase II (GH6)	AB370872	*P. pastoris*	*Irpex lacteus*	nr	Toda et al. (2008)
	Endoglucanase (GH5, GH45)	AB194135	*S. cerevisiae*	*I. lacteus*	2 mg/L	Toda et al. (2005)
		AB378504	*P. pastoris*	*P. chrysosporium*	nr	Igarashi et al. (2008)
		HM052796		*Gloeophyllum trabeum*	nr	Kim et al. (2012)
		na		*T. versicolor*	0.5 mg/L	Salinas et al. (2011)
		AF329732		*Volvariella volvacea*	65–100 mg/L	Ding et al. (2002)
	β-Glucosidase (GH1)	na	*S. cerevisiae*	*P. chrysosporium*	nr	Wilde et al. (2012)
		AB081121	*P. pastoris*	*P. chrysosporium*	nr	Kawai et al. (2003)
	Cellobiose dehydrogenase (AA3, AA8)	HQ825322	*P. pastoris*	*P. cinnabarinus*	52–350 mg/L	Bey et al. (2011)
		X88897		*P. chrysosporium*	79 mg/L	Yoshida et al. (2001)
		AY187939		*T. versicolor*	nr	Stapleton et al. (2004)

(continued)

Table 6 (continued)

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
Hemicellulose degrading enzymes	β-Mannanase (GH5)	AJ271862	*S. cerevisiae*	*Agaricus bisporus*	nr	Tang et al. (2001)
		AJ271862	*P. pastoris*	*A. bisporus*	nr	Tang et al. (2001)
		na		*Armillariella tabescens*	440 mg/L	Wang et al. (2009)
	Arabinanase (GH43)	JQ838072	*P. pastoris*	*P. chrysosporium*	8 mg/L	Huy et al. (2013b)
	Xylosidase/ arabinofuranosidase (GH43)	JX625152		*P. chrysosporium*	14 mg/L	Huy et al. (2013a)
	Acetyl xylan esterase (CE1)	JQ031636		*P. chrysosporium*	26 mg/L	Huy et al. (2013c)
	Endo-1,4-β-xylanase (GH10)	AQ993045		*P. chrysosporium*	nr	Huy et al. (2011)
	β-Galactanase (GH43)	AB461394		*I. lacteus*	nr	Kotake et al. (2009)

na gene accession number not available
nr production level (mg/L) not reported
[a]one unit (U) is defined as the amount of enzyme required to degrade 1 μmol of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in 1 min

of copper ions and lowered growth temperature (15–25 °C) has resulted in increased laccase activity (Cassland and Jönsson 1999; Hildén et al. 2013; Liu et al. 2003; O'Callaghan et al. 2002). While copper ions are indispensable for laccase activity by forming the enzyme catalytic site (Baldrian 2006), lower temperature may prevent denaturation and aggregation of the native proteins which decreases the need for protein folding mechanisms resulting in higher and more efficient secretion capacity (Dragosits et al. 2009). In addition, improved production of two laccase isoenzymes of *Coprinus comatus* has been obtained by using an N-terminal fusion of 10 amino acids from a *Volvariella volvacea* xylanase (Gu et al. 2014).

Different class II heme peroxidases (AA2), lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs) have been produced in *S. cerevisiae* and *P. pastoris* (Garcia-Ruiz et al. 2012; Gu et al. 2003; Jiang et al. 2008; Ryu et al. 2008a; Wang and Wen 2009). The highest production level, 21 mg/L, has been described for the white-rot fungus *Pleurotus eryngii* VP produced in *S. cerevisiae* (Garcia-Ruiz et al. 2012). Recombinant production of class II heme peroxidases has been challenging in yeasts (Conesa et al. 2002). However, they have been produced successfully in *Escherichia coli* and *Aspergillus* species (Conesa et al. 2002; Fernández-Fueyo et al. 2012, see 3.2.1). The main obstacle in producing recombinant class II heme peroxidases is the availability and incorporation of iron-containing heme as the prosthetic group (Franken et al. 2011; Gu et al. 2003). While the production of heme peroxidases increased by supplementation of hemoglobin or hemin to the medium (Conesa et al. 2000; Gu et al. 2003), the mechanism of heme uptake is poorly understood. Better knowledge of the heme biosynthetic pathway and its regulation is needed to overcome the limiting effect of heme.

The production of active oxalate metabolizing enzymes, oxalate decarboxylase (ODC) and oxalate oxidase (OXO), from the white-rot fungi *Dichomitus squalens* (Sietiö et al. 2015) and *Ceriporiopsis subvermispora* (Moomaw et al. 2013; Moussatche et al. 2011), respectively, has been successful in *P. pastoris* (Table 6). In wood-decaying fungi these enzymes have been associated with the regulation of intra- and extracellular concentrations of oxalic acid, which is one of the key components in biological decomposition of wood. The production level of *C. subvermispora* OXO was 4 mg/L (Moussatche et al. 2011).

In addition to the lignin modifying oxidoreductases, plant cell wall polysaccharide degrading enzymes of basidiomycetes have been produced in *S. cerevisiae* and *P. pastoris*. The production of cellulose hydrolysing enzymes such as cellobiohydrolase I (GH7), cellobiohydrolase II (GH6), endoglucanases (GH5, GH45) and β-glucosidase (GH1) have been described (Table 6). From those, the highest yield (100 mg/L) was obtained for endoglucanase from the straw-degrading basidiomycete *V. volvacea* produced in *P. pastoris* (Ding et al. 2002). Oxidative cellobiose dehydrogenases (CDH; AA3 and AA8) have been produced only in *P. pastoris* (Bey et al. 2011; Stapleton et al. 2004; Yoshida et al. 2001), and the highest yield (350 mg/L) was achieved for CDH from the white-rot fungus *P. cinnabarinus* (Bey et al. 2011).

Some hemicellulose degrading enzymes, such as β-mannanase (GH5), endo-β-1,4-xylanase (GH10), β-galactanase (GH43), arabinanase (GH43), an enzyme possessing

both xylosidase and arabinofuranosidase activities (GH43), and acetyl xylan esterase (CE1) have also been produced in yeasts (Table 6). The best production level, up to 440 mg/L, in *P. pastoris* has been reported for a β-mannanase from the plant pathogen *Armillariella tabescens* (Wang et al. 2009).

3.2 Expression in Filamentous Ascomycetes

The limitations of overexpression of genes in basidiomycetes has stimulated attempts to heterologously express basidiomycete genes in filamentous ascomycetes, in particular those species used in industry, due to their high secretion capacity and good fermentation properties. However, production levels of basidiomycete enzymes in these ascomycetes are often very low. In this section we will provide examples of successful expression and the production levels that were reached.

Many yeasts and filamentous fungi have been used as hosts to develop different heterologous protein production systems for basidiomycete enzymes (Hoshida et al. 2005). Particular emphasis has been on lignin-modifying enzymes, as they are largely absent in ascomycete fungi. However, most lignin-modifying enzymes are generally difficult to overproduce heterologously in an active form (Hoshida et al. 2005). Heterologous production of active lignin-modifying peroxidases has been reported in several species (Eibes et al. 2009; Montiel-González et al. 2009; Stewart et al. 1996), but in no cases the levels have been satisfactory enough taking into account the amount of enzyme required for its biotechnological application. One of the reasons of this setback is factors related to the hemoprotein nature of the peroxidases.

3.2.1 Heterologous Expression in *Aspergillus* and *Penicillium*

Aspergillus niger is a well-known fungal host that produces high levels of recombinant enzymes for many industrial applications. It is therefore not surprising that it has also been used for the expression of basidiomycete genes.

The enzyme that has received the most attention for heterologous production in ascomycetes is laccase, due to its potential in a variety of industrial applications (Kunamneni et al. 2008). While overall expression of basidiomycete laccases in *Aspergillus* and *Penicillium* is regularly successful, the production levels vary strongly (Table 3). Expression of a laccase from *P. cinnabarinus* in *P. pastoris* resulted in a protein that differed in some biochemical properties (e.g. molecular mass, isoelectric point, optimal temperature and pH) from the native enzyme (Otterbein et al. 2000). However, expression of this laccase in *A. niger* demonstrated highly similar properties to the native enzyme, suggesting that *A. niger* is a more suitable production host for this enzyme (Record et al. 2002). When comparing the production of the *P. cinnabarinus* laccase in *A. niger* and *Aspergillus oryzae*, higher protein yields were obtained for *A. oryzae* (Sigoillot et al. 2004). While the

biochemical properties were similar between the native and the recombinant enzymes, the recombinant enzymes had a higher K_m value than the native enzyme. Also, in the presence of a redox mediator, 1-hydroxybenzotriazole, both the native enzyme and the recombinant enzyme produced in *A. niger* were able to delignify Kraft pulp almost to 75 %, while no delignification was observed with the *A. oryzae* produced enzyme (Sigoillot et al. 2004). Other basidiomycete laccases were also expressed in *A. niger*, such as one from *P. eryngii* (*pel*3), although the activity level obtained was low (Rodríguez et al. 2008). The best result was obtained when the native signal peptide was replaced by the signal peptide from *A. niger* glucoamylase and a protease deficient *A. niger* strain was used, suggesting that the native signal sequence was not well recognized and/or processed by *A. niger*. Expression in a low-protease producing *A. niger* strain also resulted in high production levels for a laccase from *P. ostreatus* and a variant thereof that was obtained through directed evolution (Macellaro et al. 2014). Using a low protease strain was also beneficial for the production of a laccase from *S. commune*. Several ascomycete strains were tested in this study, but the best production was achieved with *Aspergillus sojae* 1860, which has low extracellular protease levels (Hatamoto et al. 1999). These results indicate that ascomycete proteases can significantly reduce the production of basidiomycete enzymes.

The fermentation approach also affects the production of laccases, as was demonstrated for the production of a *T. versicolor* laccase in *A. niger*. Solid state fermentation resulted in a much higher yield of laccase than submerged fermentation (Téllez-Jurado et al. 2006). In a different study, expression of two laccase genes from *T. versicolor* in *A. niger* under control of the *gpdA* promoter demonstrated much higher yield compared to expression of these genes in *P. pastoris* (Bohlin et al. 2006), confirming the high potential of *A. niger* for industrial enzyme production, while *P. pastoris* is more suitable for academic studies.

A *Trametes villosa* laccase (*lcc1*) was successfully expressed in another industrial *Aspergillus* species, *A. oryzae* and the properties of the recombinant enzyme were the same as those of the native enzyme (Yaver and Golightly 1996). This demonstrates that while there are differences in the glycosylation patterns of ascomycetes and basidiomycetes (Berends et al. 2009), these do not necessarily affect the biochemical properties of the heterologously produced enzymes. *A. oryzae* was also used for the heterologous production of a laccase from the white-rot fungus *Pycnoporus coccineus* using the maltose inducible promoter of α-amylase encoding *amyB* gene (Taka promoter) (Hoshida et al. 2005). The activity of this laccase was highly copper-dependent, even if the expression of the gene under control of the *amyB* promoter was not. When three laccase genes from the plant pathogenic basidiomycete *Rhizoctonia solani* were expressed in *A. oryzae* under control of the Taka amylase promoter, good production levels were only obtained for two of the enzymes (Wahleithner et al. 1996). For the third gene an initial activity level could be detected, but this disappeared in time, suggesting that this enzyme was not stable in *A. oryzae* cultures.

Penicillium is a sister genus of *Aspergillus* and species of this genus are more commonly used for the production of secondary metabolites than of enzymes.

However, heterologous production of a laccase from *Trametes hirsuta* in *Penicillium canescens* resulted in a high level of secreted protein (Abianova et al. 2010), indicating that species of this genus could be good alternatives for *Aspergillus* species with respect to enzyme production.

Heterologous expression of genes encoding lignin-modifying peroxidases from *P. chrysosporium* has been performed in several Aspergilli (Cortés-Espinosa et al. 2011). Stewart et al. (1996) were able to successfully produce active MnP and LiP from *P. chrysosporium* in *A. oryzae* under control of the Taka amylase promoter. To achieve this hemin was added to the culture medium. The same *mnp* containing expression vector was also introduced in *A. niger* where active protein was similarly detected when the culture medium contained hemoglobin (Cortés-Espinosa et al. 2011). However, the best *A. niger* transformant had less activity than the native *P. chrysosporium* strain.

The importance of temperature during bioreactor cultivation for the improvement of the recombinant proteins was demonstrated in a recent study using a versatile peroxidase from *Pleurotus eryngii*. The production of this enzyme in *A. nidulans* and *A. niger* was performed under different temperatures and the highest activity was obtained at 19 °C, which is significantly below the optimum growth temperature for *A. niger* (Eibes et al. 2009). Also in these cultures, hemin was added to the media.

Production levels of the flavoenzyme aryl-alcohol oxidase (AAO), also involved in lignin degradation, in *P. eryngii* are quite low and unsuitable for industrial applications. Heterologous production of the *P. eryngii* AAO encoding gene in *A. nidulans* was performed using the strong inducible alcohol dehydrogenase promoter (*alcA*). The recombinant protein had the same molecular mass and isoelectric point as the native protein, suggesting no significant modifications. In addition, the production of the recombinant enzyme was 10–50 times higher than in the original fungus, indicating that this strategy is highly promising for obtaining industrially relevant levels of lignin-related enzymes (Varela et al. 2001).

Cellobiose dehydrogenases (CDHs) are produced by many plant biomass degrading and phytopathogenic fungi, but the function of these enzymes are not fully deciphered. A putative *cdh* gene from *Coprinopsis cinerea* as well as one from the ascomycete *Podospora anserina* were successfully expressed in *A. niger* to enable comparison of their enzymatic properties (Turbe-Doan et al. 2013). Interestingly a much higher activity was observed for the transformant carrying the *C. cinerea cdh* and taking into account the specific activity of the enzymes, a fivefold higher enzyme production was observed for the basidiomycete protein (Turbe-Doan et al. 2013).

In many studies, the main purpose of heterologous expression of the gene is to allow the detailed characterization of the enzyme, since the production level in the original species is too low or the purification is too complicated to obtain sufficient amounts of pure enzyme. This was the case for the CDH described above, but for instance also for the heterologous expression of a cellobiohydrolase gene (*cbh1*) from the plant pathogen *Athelia rolfsii* (synonym *Corticium rolfsii*) in *A. oryzae*

under control of the phosphoglycerate kinase promoter *pgkA* (Yasokawa et al. 2003). The same motivation resulted in the expression of the UeBgl3A gene encoding a β-glucosidase from the plant pathogen *Ustilago esculenta*, in *A. oryzae* under control of the *amyB* promoter (Nakajima et al. 2012). The production levels are highly variable though (Table 3), and reflect the problems associated with the heterologous production of basidiomycete proteins in ascomycetes. While some are produced at high levels, others result in only minor amounts of protein that is barely sufficient for extensive characterization.

The major pyranose dehydrogenase (PDH) from the litter-decomposing fungus *Agaricus meleagris* was heterologously expressed in *A. nidulans* and *A. niger* under control of the glucoamylase promoter of the respective species (Pisanelli et al. 2010). Two versions were introduced in *A. nidulans*, one with its native signal sequence and one with the signal sequence of the *A. nidulans* glucoamylase, with the native signal sequence resulting in the highest extracellular activity. This is the opposite results as that obtained for the *P. eryngii* laccase (*pel*3, see above), where the glucoamylase signal peptide improved the production of the enzyme (Rodríguez et al. 2008). Introduction of this modified version of PDH in *A. niger* resulted in yields that were 10 times lower than those obtained for the best *A. nidulans* transformant (Pisanelli et al. 2010). As expression of *P. eryngii pel*3 was also performed in *A. niger*, this could imply that *A. nidulans* is more capable to processing basidiomycete signal peptides.

Expression of a *P. cinnabarinus* glucuronoyl esterase encoding gene was attempted in *A. niger* and *Aspergillus vadensis* under control of the *A. niger* glucoamylase promoter (Duranová et al. 2009). Activity was only detected in transformants of *A. vadensis*, although 10–20-fold lower than in transformants of *S. commune* and *P. cinnabarinus* (see 2.2). The better result for *A. vadensis* compared to *A. niger* could be due to the very low extracellular protease production in *A. vadensis* and the absence of acidification of the medium (de Vries et al. 2004), which may be the main reasons why no active protein was detected in *A. niger* transformants.

3.2.2 Heterologous Expression in *Trichoderma reesei*

Trichoderma reesei is with *Aspergillus* the most commonly used fungus for industrial production of heterologous and homologous proteins, in particular cellulases. Some successful examples of heterologous production of basidiomycete enzymes in *T. reesei* have been reported (Table 3). One example is the expression of a laccase gene (*lacA*) from *Trametes* sp. The gene was placed under control of the strong and constitutive *gpdA* promoter from *A. nidulans* and its native signal peptide was replaced by the signal peptide of *T. reesei* cellobiohydrolase I (CBHI). The production levels obtained for this construct were higher than those of most other studies (Zhang et al. 2012).

Dong et al. (2012) obtained a 28.6-fold higher production of a *P. ostreatus* laccase by producing it heterologously in *T. reesei*. The gene was codon optimized for *T. reesei* and expressed under control of the *cbh1* promoter. When this promoter and species was used for the heterologous production of a *Phlebia radiata* laccase, three additional isoforms were detected, suggesting variable phosphorylation or glycosylation of the recombinant enzyme (Saloheimo and Niku-Paavola 1991). Fusion of heterologous proteins of interest to homologous proteins of the expression host can improve the production of the target protein. To test this, a native form of a laccase from an ascomycete fungus *Melanocarpus albomyces* and one fused to a hydrophobin were expressed in *T. reesei* under control of the *cbh1* promoter (Macellaro et al. 2014). In this case, the fusion protein was accumulating intracellularly, likely due to the difficulties in secretion, and higher production of the native form was observed.

3.2.3 Promoters Used for Heterologous Expression of Basidiomycete Genes in Ascomycetes

The promoters used for expression of heterologous genes in filamentous ascomycetes can be divided in constitutive and inducible promoters, but all originate from genes that are highly expressed in the intended hosts. The constitutive promoters have the advantage that they result in high expression under most culture conditions, allowing more flexibility in the enzyme production. In contrast, inducible promoters require specific inducing conditions, but often result in higher enzyme production than the constitutive promoters. In addition, inducible promoters can be used for production of proteins that have a negative effect on the production hosts. In such a case, the production strain can initially be grown under non-inducing conditions and when sufficient growth has occurred the inducer can be added to result in a short period of high protein production.

Both types of promoters have been used for heterologous expression of basidiomycete genes encoding plant biomass degrading enzymes (Table 7). The most commonly used inducible promoters are the glucoamylase promoter from *A. niger* (Conesa et al. 2000) and the Taka amylase promoter from *A. oryzae* (Stewart et al. 1996), which both respond to the presence of maltose or starch. The most commonly used constitutive promoter is the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter from *A. nidulans* (Punt and van den Hondel 1992), although this promoter has also been used from other species. Recently, a new set of constitutive promoters from *A. niger* and *A. vadensis* were tested for enzyme production, resulting in several with a higher production levels than *gpdA* (Culleton et al. 2014), but these have not yet been tested for the expression of basidiomycete genes.

Table 7 Promoters used for heterologous expression of basidiomycetes genes in filamentous basidiomycetes and ascomycetes

Promoter	Corresponding gene	Species of origin	Reference	Host species	Expressed gene	Donor species	Reference
alcA	Alcohol dehydrogenase	*A. nidulans*	Fernández-Cañón and Peñalva (1995)	*A. nidulans*	Versatile peroxidase	*Pleurotus eryngii*	Eibes et al. (2009)
					Aryl-alcohol oxidase	*P. eryngii*	Varela et al. (2001)
				A. niger[a]	Versatile peroxidase	*P. eryngii*	Eibes et al. (2009)
amyB	α-Amylase	*A. oryzae*	Tada et al. (1991)	*A. oryzae*	β-Glucosidase	*Ustilago esculenta*	Nakajima et al. (2012)
					Laccase	*Pycnoporus cinnabarinus*	Hoshida et al. (2005)
amy	α-Amylase	*A. nidulans*	Téllez-Jurado et al. (2006)	*A. niger*	Laccase	*Trametes versicolor*	Téllez-Jurado et al. (2006)
bgaS	β-Galactosidase	*Penicillium canescens*	Nikolaev et al. (1992)	*P. canescens*	Laccase	*Trametes hirsuta*	Abianova et al. (2010)
cbh1	Cellobiohydrolase	*T. reesei*	Saloheimo et al. (1989)	*T. reesei*	Laccase	*Phlebia radiata*	Saloheimo and Niku-Paavola (1991)
						Pleurotus ostreatus	Dong et al. (2012)
						Melanocarpus albomyces	Kiiskinen et al. (2004)
glaA	Glucoamylase	*A. nidulans*	Pisanelli et al. (2010)	*A. nidulans*	Pyranose dehydrogenase	*Agaricus meleagris*	Pisanelli et al. (2010)
glaA	Glucoamylase	*A. niger*	Pisanelli et al. (2010)	*A. niger*	Pyranose dehydrogenase	*A. meleagris*	Pisanelli et al. (2010)
glaA	Glucoamylase		Conesa et al. (2000)	*A. vadensis*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
				A. niger	Cellobiose dehydrogenase	*Coprinopsis cinerea*	Turbe-Doan et al. (2013)
gpdA	Glyceraldehyde-3-phosphate dehydrogenase	*A. nidulans*	Punt and van den Hondel (1992)	*T. reesei*	Laccase	*Trametes* sp.	Zhang et al. (2012)
				A. niger	Laccase	*P. cinnabarinus*	Record et al. (2002)
							Sigoillot et al. (2004)
						P. eryngii	Rodríguez et al. (2008)
						T. versicolor	Bohlin et al. (2006)
						P. ostreatus	Macellaro et al. (2014)

(continued)

Table 7 (continued)

Promoter	Corresponding gene	Species of origin	Reference	Host species	Expressed gene	Donor species	Reference
GPD	Glyceraldehyde-3-phosphate dehydrogenase	*S. commune*	Alves et al. (2004)	*P. cinnabarinus*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
GPD	Glyceraldehyde-3-phosphate dehydrogenase	*P. chrysosporium*	Li et al. (2001)	*P. chrysopsorium*	Manganese peroxidase	*Dichomitus squalens*	Li et al. (2001)
pgkA	Phosphoglucokinase	*A. oryzae*	Ogawa et al. (1994)	*A. oryzae*	Cellobiohydrolase	*Athelia* (*Corticium*) *rolfsii*	Yasokawa et al. (2003)
SC3	Hydrophobin	*S. commune*	Alves et al. (2004)	*S. commune*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
Taka amylase	α-Amylase	*A. oryzae*	Christensen et al. (1988)	*A. oryzae*	Laccase	*Rhizoctonia solani*	Wahleithner et al. (1996)
					Laccase	*Trametes villosa*	Yaver and Golightly (1996)
					Lignin peroxidase	*P. chrysopsorium*	Stewart et al. (1996)
					Manganese peroxidase	*P. chrysopsorium*	Stewart et al. (1996)
					Lignin peroxidase	*P. chrysopsorium*	Montiel-González et al. (2009)
				A. rouxii	Manganese peroxidase	*P. chrysopsorium*	Montiel-González et al. (2009)
				A. niger	Manganese peroxidase	*P. chrysopsorium*	Cortés-Espinosa et al. (2011)
tanA	Tannase	*A. oryzae*	Hatamoto et al. (1996)	*A. oryzae*	Laccase	*S. commune*	Hatamoto et al. (1999)

[a]In this *A. niger* strain the *alcR* gene from *A. nidulans* has been introduced

4 Heterologous Expression in Zygomycetes

Only one example of heterologous expression of basidiomycete genes in zygomycetes has been described (Montiel-González et al. 2009) (Table 3). *Amylomyces rouxii* was used as a cell factory for heterologous expression of a LiP and a MnP encoding genes from *P. chrysosporium* under control of the Taka amylase promoter of *A. oryzae*. *A. rouxii* is an efficient degrader of organopollutants such as pentachlorophenol (PCP) through its ability to produce phenoloxidases. As no heme peroxidases were found in cultures of this fungus, the introduction of heterologous peroxidases was believed to further improve its ability to degrade PCP, which was confirmed in this study. Interestingly, no exogenous heme or hemoglobin was added to the media to ensure active forms of the heme-containing lignin-modifying peroxidases, as has been used in filamentous ascomycetes.

5 Conclusions and Outlook

Homologous and heterologous overexpression of basidiomycete genes is a challenging research field. The low number of transformable basidiomycete species strongly limits the options for homologous overexpression, as well as heterologous expression in basidiomycete hosts. Development of better transformation systems for a larger number of basidiomycetes will be required to develop them as an enzyme production platform.

In contrast, ascomycete expression systems are well established and cover both yeasts and filamentous fungi as described in this chapter. However, the levels of basidiomycete enzymes produced in these systems are often much lower than those of ascomycete enzymes. No systematic studies into the reasons behind this have been performed, but it is likely that differences in basidiomycete and ascomycete gene models (basidiomycete genes often have more introns with less conserved start and stop sequences) or sensitivity to ascomycete proteases contribute to this. A better understanding of the factors affecting the production of basidiomycete enzymes in ascomycete hosts is required to reach the levels that are suitable for industrial applications.

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