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C5790 **Production and manipulation
of blue copper oxidases for
technological applications**

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Abstract

Fungal laccases are robust multicopper oxidoreductases. Perfectly amenable to synthetic evolution, the fungal laccase scaffold is a potential generic for the production of tailored biocatalysts, which, in principle, can be secreted at substantial levels in industrially relevant organisms. In this chapter, the strategy we have developed for the rapid production of hundreds of milligram of laccase variants is detailed. It is based on the use of two heterologous expression hosts: the yeast *Saccharomyces cerevisiae* for a rapid upstream screening and the fungus *Aspergillus niger* for downstream production.

Methods for screening active and nonactive laccase variants, convenient setups for enzyme production in both organisms as well as a methodology for efficient purification of large amounts of recombinant enzymes are given. The general procedure for developing new materials for artificial catalysis is also described.

no0010 Abbreviations

no0010 AA	amino acid
no0010 Ab-LAC	polyclonal anti-lac3 antibody
no0010 ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
no0010 ABTS⁺	ABTS stable radical cation
no0010 AIP	alkaline phosphatase
no0010 ATA	aurin tricarboxylic acid ammonium salt
no0010 Bp	base pair
no0010 BSA	bovine serum albumin
no0010 DEAE	diethylaminoethanol
no0010 DNA	deoxyribonucleic acid
no0010 DTT	dithiothreitol
no0010 ELISA	enzyme-linked immunosorbent assay
no0010 FPLC	fast protein liquid chromatography
no0010 GRAS	generally regarded as safe
no0010 MWCN	multiwall carbon nanotube
no0010 MWCO	molecular weight cut off
no0010 PBST	phosphate buffer saline with tween 20
no0010 PCR	polymerase chain reaction
no0010 PEG	polyethylene glycol
no0010 PES	polyethersulfone
no0010 pNPP	4-nitrophenyl phosphate disodium salt hexahydrate
no0010 PPD	<i>p</i> -phenylenediamine
no0010 rpm	rotation per minute
no0010 RT	room temperature
no0010 SD	synthetic defined
no0010 SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
no0010 SGZ	syringaldazine
no0010 SN	supernatant
no0010 sp	signal peptide
no0010 ss	single strand
no0010 STC	sodium tris chloride
no0010 TFA	trifluoroacetic acid
no0010 TFF	tangential flow filtration
no0010 TNC	trinuclear cluster
no0010 UF	ultrafiltration
no0010 UNIK	single lysine LAC3 variant
no0010 UV-vis	ultraviolet-visible
no0010 YPD	yeast extract-peptone-dextrose
no0010 <i>e</i>	molar extinction coefficient

s0010

1. Introduction

p0205

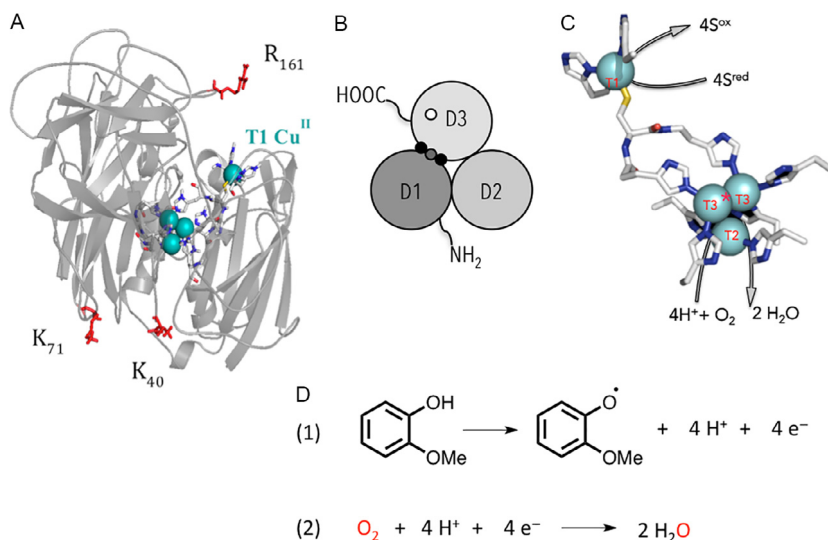
New technologies for a more sustainable society are urgently needed (Kates, 2017). Considering the needed change of paradigm from fossil to renewable feedstock, a selective and efficient conversion of bioresources is crucial and will have a real impact on the sustainability of our developed society (Armstrong & Blundell, 2007). A number of current industrial processes feature catalytic methods for aerobic oxidation. However, their long-term use is condemned as reagents such as metal-oxide or chlorine-based oxidants are often required, generating organic and heavy metal wastes with safety concerns (Anastas & Warner, 1998). As an oxidant, dioxygen (O₂) is available at no cost and produces no environmentally hazardous by-products. Therefore, catalytic systems capable of selective oxidation reactions driven by clean dioxygen reduction with no by-products represent an important asset to progress toward sustainability.

p0210

Nowadays, the principle of using enzymes for large-scale industrial processes is well established (Kirk, Borchert, & Fuglsang, 2002; Meyer et al., 2013; Nestl, Hammer, Nebel, & Hauer, 2014). Limitations to the use of these excellent (enantio) selective and renewable catalysts, such as good operational stability or high production costs remain, but are progressively overcome thanks to the availability of constantly improving molecular evolution, fermentation, and immobilization methodologies.

p0215

Laccase (EC 1.10.3.2 from plant, fungi, or bacteria) is a particularly robust and well-studied biocatalyst. This copper-containing enzyme (four copper ions) couples the oxidation of a wide range of substrates to the neat reduction of one molecule of dioxygen to two water molecules. In the past 2 decades, a significant number of reports have illustrated the potential of this enzyme in technological and bioremediation processes (e.g., bleaching, clarification of beverages, detoxification, sensors) (Zucca, Cocco, Sollai, & Sanjust, 2016), focusing on its stability and efficiency (Alcalde, Ferrer, Plou, & Ballesteros, 2006; Couto & Toca-Herrera, 2006, 2007), as well as on its adaptability (plasticity) to different reaction conditions (e.g., tolerance to different organic solvents) (Kunamneni et al., 2008). Recent reviews address the use of laccases in these different fields (Cannatelli & Ragauskas, 2017; Mate & Alcalde, 2017) as well as in organic synthesis (Cracknell, Vincent, & Armstrong, 2008; Mikolasch & Schauer, 2009; Riva, 2006; Witayakran & Ragauskas, 2009) and in biofuel cells, in which they are wired



fo010 **Fig. 1** Structure and function of laccase. (A) Structural model (*ribbon*) of LAC3 from *Trametes* sp. C30 with amino acids subjected to mutagenesis colored in *red*; Cu(II) ions are depicted as *blue spheres*. (B) Cartoon of the three cupredoxin domains (D1, D2, and D3) in the laccase structure; *white circle*, T1 copper ion; *gray circle*, T2 copper ion, *black circle*, T3 copper ions. (C) Close-up of the copper-active sites; *blue spheres*: copper ions annotated T1, T2, and T3. * This sign stands for the presence of a hidden histidine residue coordinated to the T3 copper site. (D) Overall outcome of the catalytic reaction.

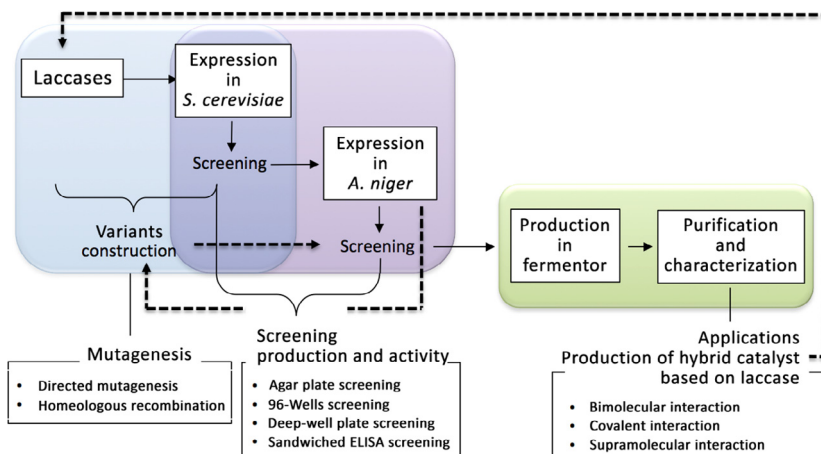
on solid support and serve as the cathodic catalyst reducing dioxygen to water (Fernández-Fernández, Sanromán, & Moldes, 2013; Gutiérrez-Sánchez, Pita, Vaz-Domínguez, Shleev, & De Lacey, 2012; Jones & Solomon, 2015; Lalaoui et al., 2016; Le Goff, Holzinger, & Cosnier, 2015; Milton & Minter, 2017; Pistone et al., 2016; Pita et al., 2011, 2014; Qiu et al., 2009; Shleev et al., 2005; Shleev, Pita, Yaropolov, Ruzgas, & Gorton, 2006). Recently, the potential of laccases for the development of molecular hybrid catalysts has been demonstrated (Lazarides et al., 2013; Mekmouche et al., 2015; Schneider et al., 2015; Simaan et al., 2011; Tarrago et al., 2018).

p0220 Genes encoding laccases from different origins have been heterologously expressed with various successes in bacteria, yeasts, filamentous ascomycetes, basidiomycetes, or plants reaching expression levels of up to a gram per liter (Alves et al., 2004; Kiiskinen et al., 2004; Record et al., 2002). Both variation of the cultivation conditions and mutagenesis in a given host can be used to tune production (Bulter et al., 2003; Mate et al., 2010; Rodgers et al., 2010). In addition, well-established protein engineering methodologies are available for modulating enzyme properties such as redox potential, pH tolerance, and thermostability (Mate & Alcade, 2015). However, it is

noteworthy that a complex posttranslational maturation of the enzyme is influencing expression of laccases genes as well as the diversity obtainable among synthetic variants. The two copper-containing redox centers of laccases—a blue copper site located near the surface (oxidation of substrates) and an embedded trinuclear copper center (O_2 reduction) (Fig. 1) (Jones & Solomon, 2015; Riva, 2006; Solomon et al., 2014; Solomon, Szilagyi, DeBeer George, & Basumallick, 2004; Tron, 2013)—need to be precisely inserted within the three cupredoxin domains that are typical of proteins from the multicopper oxidase family. Moreover, fungal laccases are secreted, a process involving both enzymatic decorations of the enzyme surface with sugar moieties (glycosylation) and of the signal sequence by specific peptidase(s) during secretion. Thus, maximizing the production of fungal laccase variants requires a careful selection of the initial sequences, highly secreting hosts and a simple and straightforward selection procedure. Since the “perfect” host (allowing both selection and production at the same time) does not exist, best performances are obtained by splitting the process into two steps: (1) selection of variants (from sequences initially propagated in *Escherichia coli*) and (2) production of selected variants, involving each a specialized heterologous expression host (e.g., yeasts for step 1 and filamentous fungi for step 2).

p0225 We have forged our own experience in large-scale production of recombinant laccases from the study of isoenzymes originating from the fungus *Trametes* sp. C30 (Robert, Mekmouche, Rousselot-Pailley, & Tron, 2011). The isoenzyme LAC3, which is the enzyme pivotal to all of our studies since 2005, has been selected from a functional screen of the yeast *Saccharomyces cerevisiae* transformants. LAC3, and several of its variants are produced in the yeast *S. cerevisiae* in the milligram per liter range (Balland et al., 2008; Cusano, Mekmouche, Meglecz, & Tron, 2009; Klonowska et al., 2005; Liu et al., 2014). Further, recombinant laccase production has recently been scaled up to levels of nearly 1 gL^{-1} , through the transformation and subsequent optimization of fermentation conditions in the GRAS ascomycete *Aspergillus niger* (Mekmouche et al., 2014). Altogether, these works highlight the potential of combining a generic laccase sequence with yeast and fungal expression systems for the generation and large-scale production of variants. Demonstrations of the practical use of this combination can be found in our most recent works on the production and use of robust laccase-based hybrids catalysts (Lalaoui et al., 2016; Mekmouche et al., 2015; Robert et al., 2017; Schneider et al., 2015).

p0230 In this chapter, we will detail our methodologies to produce large-scale synthetic laccases with desired properties for the purpose of developing new



f0015 **Fig. 2** General strategy for large-scale production of laccase synthetic variants covering the construction of variants (mutagenesis), the transformation and screening of laccase secreting transformants (screening production and activity) and finally the large-scale production, purification and targeted chemical functionalization (applications, production of hybrid catalyst based on laccase).

molecular hybrids for catalysis. The general strategy is depicted in Fig. 2. We will cover: (i) *the construction of variants* based on rational (structure guided) or random (molecular evolution) available mutagenesis methodologies; (ii) *the transformation procedures*; (iii) *the screening of laccase secreting transformants* directly on transformation plates containing a chromogenic substrate (e.g., guaiacol) or in various plate formats and, for nonfunctional variants (i.e., copper-depleted variants), with a sandwiched ELISA test; (iv) *the large-scale production* of laccase variants from reactor-optimized fermentation of *A. niger*; and (v) *the downstream purification* of laccase variants. We will then describe *selective chemical functionalization of the laccase surface* with synthetic effectors (transition metal complexes, macrocycles, organic probes, materials) via robust protein chemistry methodologies. We will discuss these different steps in depth by exploring the heterologous production of fungal laccases and their purification and characterization. Finally, we will provide an overview of possible applications.



s0015 **2. Heterologous production of fungal laccases**

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Engineering of recombinant laccases for targeted oxidative transformations at desired conditions is a challenging issue.

p0240

Despite high production capabilities of fungal strains naturally producing laccases, heterologous expression of fungal laccase genes is popular because it

offers possibilities: (i) to overcome the production of several isoenzymes resulting from the coexpression of multiple laccase-encoding genes usually found in parental organisms; (ii) to produce laccases from genes poorly expressed in parental organisms; (iii) to engineer a laccase by random or rational mutagenesis to improve its properties (e.g., stability vs pH, $T^{\circ}\text{C}$, solvents). Recent reviews covering laccase engineering are available (Alcalde, 2015; Mate & Alcade, 2015; Pardo & Camarero, 2015; Rodgers et al., 2010).

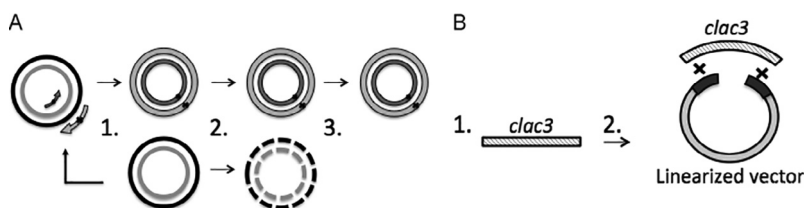
p0245 As mentioned, our reference enzyme is LAC3, a laccase from *Trametes* sp. C 30. Cloning and heterologous expression of laccase *Trametes* sp. C 30 cDNAs has been undertaken to select enzymes with high oxidative capacities. In particular, *lac3* (GenBank accession number AY397783), which encodes a laccase isoform previously undetected in fungal cultures, has been isolated and successfully expressed in the yeast *S. cerevisiae* (Klonowska et al., 2005).

s0020 2.1 Cloning

p0250 Laccase-encoding sequences (cDNA, synthetic genes) are fused to bacteria/yeast shuttle vectors that are either commercially available (ex. Gateway™ pYES-DEST52 Vector) or custom (ex. pAK145, a constitutive yeast expression vector the construction of which is detailed in Klonowska et al., 2005) and are propagated in *E. coli* strain DH5 α (F^{-} Δ 80lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk $^{-}$, mk $^{+}$) phoA supE44 thi-1gyrA96 relA1 λ^{-}) using standard techniques (Sambrook, Fritsch, & Maniatis, 1989). Note that the secretion signal (encoding the signal peptide) to be fused to the sequence encoding the mature peptide is a matter of tuning. Heterologous peptides like the yeast alpha factor sp (Camarero et al., 2012; Mate et al., 2010) or that of the yeast invertase (Klonowska et al., 2005) are often chosen to replace the native laccase sp although in our hand the latter was found to lead to LAC3 secretion as well (Klonowska, unpublished results). In addition, improvements in the secretion (and therefore the production) of laccases have been linked to the accumulation of mutations in the sp during molecular evolution rounds (Bulter et al., 2003; Camarero et al., 2012; Mate et al., 2010). We sought both cloning methods to produce recombinant laccases and variants, i.e., a rational and a random method (Fig. 3).

s0025 2.1.1 Site-directed mutagenesis

p0255 Site-directed mutagenesis (Fig. 3A) is the basis for structure and function studies (Mate & Alcade, 2015). In this view, a 3D structure of the favorite



0020 **Fig. 3** Selected methods for producing variants of laccase. (A) Rational-directed mutagenesis: (1) mutant strand synthesis containing the targeted mutation; (2) digestion of template; and (3) transformation of mutated molecule into competent cells. (B) Gap repair technique: (1) gene of interest and (2) yeast transformed with vector and insert and homologous recombination to repair the gap.

enzyme or, failing that, a robust model (easily obtainable from web-based protein modeling platforms like SwissProt, PMP, Modeller, etc.) is highly recommended. Through site-directed mutagenesis, we have challenged the plasticity of our favorite enzyme (Klonowska et al., 2005; Liu et al., 2014), as well as its robustness in electrocatalysis (Balland et al., 2008; Lalaoui et al., 2016) or in chemo-enzymatic catalysis involving electron transfer from a transition metal catalyst (Mekmouche et al., 2015; Schneider et al., 2015) or from photosensitizers (Robert et al., 2017) (see Table 1). Replacement of selected sequences or codons in the *lac3* original coding sequence was obtained using a commercial kit (QuikChange[®], Stratagene[™]). Mutated laccase sequences were constructed by designing the appropriate primers and following the recommendation of the manufacturer (Stratagene).

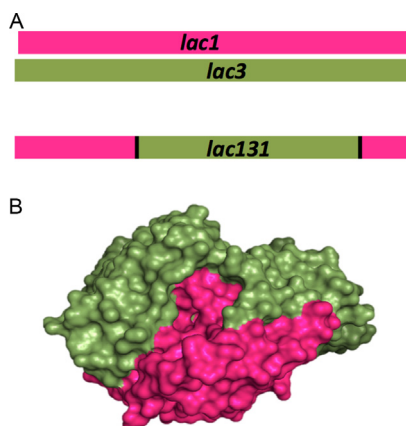
p0260 Recently, we started the construction of single lysine laccase variants of LAC3 with the intention of obtaining genetically defined, single surface-located, primary amino group variants amenable for posttranslationally site-directed chemical modification. A three-step semiempirical strategy was used in order to achieve the following aims: (i) To replace the only two lysine residues (K₄₀ and K₇₁) naturally present in the mature sequence of LAC3 (Fig. 1A). Libraries of sequences coding K₄₀Q, M or I and K₇₁H, A, R, E (representing natural variations found at positions 40 and 71) were constructed from which the variant K₀ (K₄₀M, K₇₁H) devoid of lysine was selected: (ii) To replace a chosen surface accessible residue by a lysine, for example, in the UNIK₁₆₁ (R₁₆₁K) variant (Lalaoui et al., 2016; Robert et al., 2017).

s0030 2.1.2 Yeast recombination

p0265 In vivo gene cloning or gap repair is a very efficient and fast method by which to assemble recombinant DNA in the yeast *S. cerevisiae* (Fig. 3B). This method produces, in a single step, propagatable circular DNA by homologous recombination between a linearized vector (gap) and a linear

10010 **Table 1** Variants of LAC3 engineered by site-directed mutagenesis

Study	Characterization	Main Results	References
Functional expression in yeast	E° T1; EPR spectroscopy; activity	Characterization of recombinant enzyme and comparison with homologous LAC1 and LAC2 from <i>Trametes</i> sp. C30	Klonowska et al. (2005)
Immobilization of LACCASE on electrode	Electrocatalysis; activity; spectroscopy	Characterization of a self-assembled monolayer immobilized laccase. Redox-mediated electrocatalysis	Balland et al. (2008)
Functional expression of C- and N-terminus modified laccases	Activity	Characterization and comparison of C-terminal modified laccases; depleted or/and His-tagged. Effect of glycosylation pattern on activity	Liu et al. (2014)
Oriented immobilization of LAC on electrodes to maximize direct electron transfer	Molecular biology; spectroscopy; activity; electrocatalysis	Efficient direct electron transfer for reduction of dioxygen using nanostructured electrodes modified with a site-specific surface modification laccase (single covalently bound pyrene group close to the T1 center)	Lalaoui et al. (2016)
Controlled orientation of a graft at the surface of a fungal laccase	Spectroscopy; activity; molecular biology; covalent docking; molecular dynamics simulations	Engineering of laccase variants with unique surface accessible lysine residues and orientated covalent grafting of a photosensitizer to promote photocatalysis. Simulation studies fit well with observed reactivity	Robert et al. (2017)



f0025 **Fig. 4** Construction of laccase chimeras through yeast homologous recombination of *Trametes* sp. strain C30 laccase cDNAs sharing 65%–71% identity. (A) Schematic view of intermolecular recombination events between the *lac1* and *lac3* parental genes and the resulting hybrid *lac131* gene. Genes are represented by rectangles of variable lengths and recombinant junctions are represented by vertical black bars. (B) Molecular models of LAC131 hybrids constructed using the structure of the laccase 2HRG from *Trametes trogii* as template. The surface of LAC3 is colored in green; that of LAC1 is colored in pink. Adapted from Cusano, A. M., Mekmouche, Y., Meglecz, E., & Tron, T. (2009). Plasticity of laccase generated by homeologous recombination in yeast. *FEBS Journal*, 276, 5471–5480.

overlapping DNA fragment (insert) cotransformed in yeast (Oldenburg, Vo, Michaelis, & Paddon, 1997; Orr-Weaver, Szostak, & Rothstein, 1983). The recombination occurs within short homologous sequences between the vector and the insert. Laccase-encoding sequences sharing 65%–71% identity (*lac1*, *lac2*, *lac3*, and *lac5*) have been shuffled in vivo by homologous recombination in yeast using a *lac3* PCR fragment as the repair sequence. In recombinant sequences, junctions have been found to map within short stretches of identity varying from 5 to 45 base pairs. Three chimeric laccases (LAC131, LAC232, and LAC535) have been isolated and characterized (Fig. 4). This single random mutagenesis step has led to the generation of variants having extended pH tolerance toward alkaline conditions (Cusano et al., 2009).

s0035 2.2 Transformations

s0040 2.2.1 *S. cerevisiae*

p0270 The yeast *S. cerevisiae* W303-1A (*MATa* {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*}) is transformed with recombinant DNA materials using either a lithium acetate/ss DNA carrier/PEG protocol (Gietz & Woods,

2002) or electroporation (Becker & Guarente, 1991). After transformation, cells are spread on selective media and plates are incubated at 28°C for 4 days. Below is a brief description of: (i) the preparation of electrocompetent cells; (ii) electroporation; and (iii) plating.

p0275 *Equipment*

- o0010 1. Temperature-controlled orbital shaker set at 30°C.
- o0015 2. Stationary incubator at 30°C.
- o0020 3. Electroporation cuvette (sterile).
- o0025 4. Electroporator (e.g., Eppendorf Eporator[®]).
- o0030 5. Floor centrifuge.
- o0035 6. UV-vis spectrometer.

p0310 *Buffers and reagents*

p0315 Sterilization is performed by autoclaving unless otherwise specified. Standard recipes can be found in *Methods in Enzymology*.

- o0040 1. YPD (1% yeast extract, 2% peptone, 2% dextrose) medium.
- o0045 2. 1 M sorbitol in sterile distilled water.
- o0050 3. Yeast strain (e.g., *S. cerevisiae* W303-1A).
- o0055 4. Synthetic dextrose (SD) medium agar.

p0340 Example: YNB without AA, 0.67%; dextrose, 2%; adenine, 30 mgL⁻¹; casein hydrolysate, 0.5%; tryptophan, 40 mgL⁻¹; CuSO₄, 100 μM; 50 mM succinate buffer pH 5.3; 15 gL⁻¹ Agar. Sterile (filtered through Ø0.2 μm filter) adenine, tryptophan, and guaiacol solutions are added postautoclave. Note that the composition of this medium has been optimized for the selection of uracyl prototrophs (*URA3* marker as part of the expression plasmid) secreting laccase.

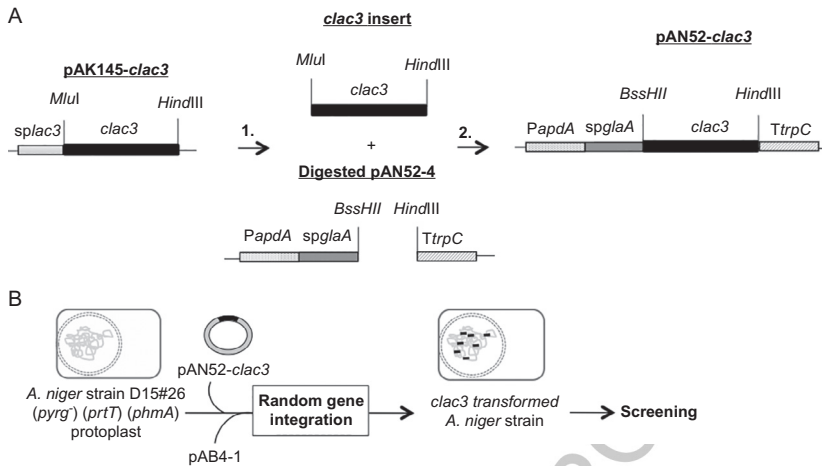
p0345 *Procedure*

- u0010 • Electrocompetent cells
- o0060 1. A fresh culture is prepared by growing 5 mL of *S. cerevisiae* strain in YPD in a 50 mL conical tube at 30°C, at 160 rpm overnight in order to reach an OD₆₀₀ = 1.3–1.5. The preparation can be started directly from a plate.
 - o0065 2. The cells are centrifuged at 1500 × g for 5 min at 4°C in a precooled rotor, the pellet is retained and the supernatant is discarded. This step allows for concentrating the cells.
 - o0070 3. The pellet is resuspended with 5 mL of *ice-cold* sterile water, vortexing the cells if necessary.
 - o0075 4. The cells are centrifuged as in step 2, the pellet is resuspended with 2.5 mL of *ice-cold* sterile water.
 - o0080 5. The cells are centrifuged as in step 2, the pellet is resuspended in 1.5 mL of *ice-cold* 1 M sorbitol.

- o0085 **6.** The cells are centrifuged as in step 2, then the pellet is resuspended in
 0.1 mL of *ice-cold* 1 M sorbitol to a final volume of approximately
 0.15 mL. *The cells are kept on ice* for immediate use, not stored.
- u0015 • Electroporation
- o0090 **7.** Yeast suspension (50 μ L) from step 6 is mixed with 2 μ g of plasmid
 (in 5 μ L sterile water) in a sterile Eppendorf tube.
- o0095 **8.** The mixture is transferred to an ice-cold 0.2 cm sterile electropo-
 ration cuvette.
- o0100 **9.** The cuvette is placed on ice for 5 min.
- o0105 **10.** The cells are pulsed according to the parameters for *S. cerevisiae* as
 suggested by the manufacturer of the specific electroporation
 device used.
- o0110 **11.** Ice-cold 1 M sorbitol (1 mL) is added immediately to the cuvette
 using a sterile pipette. After mixing gently, the cuvette contents
 are transferred to a 15 mL sterile tube.
- o0115 **12.** The tube is incubated at 30°C without shaking for 1 h.
- u0020 • Plating
- o0120 **13.** Aliquots (200 μ L) of the transformation (either LiAc or electropo-
 ration) are plated on SD plates. Transformants are selected for the
 appropriate prototrophy resulting from the complementation of
 one of the auxotrophies of *S. cerevisiae* W303-1A after transforma-
 tion (corresponding to the selection marker present on the plasmid).
- o0125 **14.** Plates are incubated for 2–3 days at 30°C until colonies form.
- o0130 **15.** 10–20 colonies are chosen and streaked onto fresh SD plates. It is
 possible to screen for laccase activity/extracellular production by
 plating on an activity plate (i.e., containing guaiacol or ABTS),
 as will be further discussed in [Section 2.3.2](#).

s0045 **2.2.2 A. niger**

p0440 Efficient expression in *A. niger* requires multiple integrations of the heterol-
ogous sequence to the genome. We are routinely using the strain D15#26
[(*pyrG*⁻) (*priT*) (*phm4*)] ([Gordon et al., 2000](#)) and plasmids pAN52-4
(EMBL accession number Z32750) for laccase sequence subcloning and
pAB4-1 harboring *pyrG* as selection marker ([van Hartingsveldt, Mattern,
van Zeijl, Pouwels, & van den Hondel, 1987](#)) all obtained from Peter Punt's
laboratory. The following procedures have been adapted from previously
published works ([Mekmouche et al., 2014](#); [Punt & van den Hondel,
1992](#); [Record et al., 2002](#)).



f0030 **Fig. 5** (A) Description of the gene expression vectors for LAC3 plasmid pAK145-*clac3* for *S. cerevisiae* expression and plasmid pAN52-*clac3* for *A. niger* expression. The cloning restriction sites are annotated and signal peptides are indicated as sp (*splac3* for pAK145-*clac3* and *spglaA* for pAN52-*clac3*). The constitutive promoter is the untranslated region *PgdpA*. The terminator gene is *trpC*. (B) Schematic description of pAN52-*clac3* and pAB4-1 transformation of *A. niger* [strain D15#26 (*pyrG*⁻) (*prtT*) (*phmA*)] protoplast. (i) The host strain is sensitive to the antibiotic; (ii) preparation of protoplasts competent for DNA integration and subsequent regeneration; and (iii) selection procedures for optimal transformation frequencies. From Punt, P. J., & van den Hondel, C.A. (1992). Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods in Enzymology*, 216, 447–457.

o0135 **a.** Construction of a pAN52-laccase expression plasmid

p0450 The sequence encoding a laccase of interest (a *MluI/HindIII* PCR fragment encompassing the sequence coding for the mature LAC3 in our case) is easily inserted at the *BssHIII/HindIII* sites of the pAN52-4 vector and is known as pAN52-*clac3* (Fig. 5). In the recombinant molecule, the sequence encoding the mature laccase is fused to a synthetic signal sequence (from the glucoamylase gene) and flanked by the 5' untranslated region of the glyceraldehyde-3-phosphate dehydrogenase gene (constitutive promoter) and the terminator region of the *trpC* gene from *A. nidulans*. The plasmid is propagated in *E. coli* (ampicillin resistance) (Fig. 5).

p0455 Site-directed mutagenesis can be directly performed on this expression vector. *K*₀, a variant of LAC3 devoid of lysine was constructed starting from pAN52-*clac3* expression plasmid (unpublished).

o0140 **b.** Transformation of *A. niger*

p0465 *A. niger* D15#26 is transformed by the pAN52-*lac* plasmid along with the pAB4-1 plasmid that houses the auxotrophic marker *pyrG* (cotransformation) as described by Punt and van den Hondel (Punt & van den Hondel, 1992; van Hartingsveldt et al., 1987). Stable *pyrG*⁺ transformants resulting from recombination at the *pyrG* locus are selected for uridine prototrophy. Cotransformants with multiple random integration of the laccase-encoding sequence in the *A. niger* genome are selected for laccase activity.

p0470 *Equipment*

- o0145 **1.** Malassez cell counting chamber.
- o0150 **2.** Microscope.
- o0155 **3.** Temperature-controlled orbital shaker.
- o0160 **4.** Ø90 mm Petri dish plates.

p0495 *Buffers and reagents*

- o0165 **1.** *A. niger* [strain D15#26 (*pyrG*⁻) (*prtT*) (*phmA*)].
- o0170 **2.** Trace elements (1000×) solution: 76 mM ZnSO₄, 178 mM H₃BO₃, 25 mM MnCl₂, 18 mM FeSO₄, 7.1 mM CoCl₂, 6.4 mM CuSO₄, 6.2 mM Na₂MoO₄, 174 mM EDTA.
- o0175 **3.** Complete medium: 70 mM NaNO₃; 3.7 mM KCl, 11 mM KH₂HPO₄, 2 mM MgSO₄, glucose 1% (w/v), trace elements (1×), 10 mM uridine; 2 gL⁻¹ casaminoacid; 5 gL⁻¹ yeast extract.
- o0180 **4.** Solid minimum medium: 70 mM NaNO₂, 3.7 mM KCl, 11 mM KH₂HPO₄, 2 mM MgSO₄, glucose 1% (w/v), trace elements (1×) 15 gL⁻¹ agar. Make the final solution 50 mgL⁻¹ CuSO₄, 5H₂O, and 0.2 mM ABTS from sterile stock solutions prior to gelification (hardening) if a laccase activity detection is desired.
- o0185 **5.** Vector pAB4-1 containing the *pyrG* selection marker.
- o0190 **6.** STC buffer: 1.2 M sorbitol; 10 mM Tris-HCl, pH 7.5, 50 mM CaCl₂·2H₂O, 35 mM NaCl.
- o0195 **7.** CaCl₂ buffer: 0.27 M CaCl₂·2H₂O; 0.6 M NaCl.
- o0200 **8.** PEG buffer: 60% PEG 4000; 10 mM Tris-HCl, pH 7.5; 50 mM CaCl₂·2H₂O.
- o0205 **9.** pAN-52-*lac*.
- o0210 **10.** Glucanex from Sigma (L1412).
- o0215 **11.** ATA (aurin tricarboxylic acid ammonium salt, 10–20 mM, MERCK).
- p0555 Trace elements stock solution: 76 mM ZnSO₄, 178 mM H₃BO₃, 25 mM MnCl₂, 18 mM FeSO₄, 7.1 mM CoCl₂, 6.4 mM CuSO₄, 6.2 mM Na₂MoO₄, 174 mM EDTA.

- o0220 **12.** Minimum medium: 70 mM NaNO₃, 7 mM KCl, 200 mM Na₂HPO₄, 2 mM MgSO₄, 50 gL⁻¹ glucose, 50 mgL⁻¹ CuSO₄, 5H₂O. Then add 1‰ of trace element stock solution and adjusted to pH 5 with a 1 M citric acid solution.
- o0225 **13.** Tween 80–water: 10 drops of tween 80 in 10 mL of water.
- o0230 **14.** Sterile 1 M citric acid.
- p0575 *Procedure*
- u0025 • Generation of protoplast
- p0585 Protoplasts generation is generally the basis for fungal transformation: it is followed by a PEG/CaCl₂-mediated DNA uptake.
- o0235 **1.** 250 mL of complete medium is inoculated in a 1 L conical flask at a final concentration of 2 × 10⁶ spores mL⁻¹ (described in [Section 2.2.2](#), Mass production of spores) and incubated with shaking at 150 rpm (24h) at 30°C.
- o0240 **2.** The spent medium is filtered through sterile miracloth. The collected mycelium is washed with CaCl₂ buffer, resuspended in CaCl₂ buffer (5 g mycelium in 50 mL buffer) containing Glucanex (500 mg)^a and incubated at 30°C for 2 h in an orbital shaker (80 rpm) ([Li, Tang, Lin, & Cai, 2017](#)).
- o0245 **3.** The protoplast suspension is then filtered through sterile miracloth, gently resuspended in the same volume of cold STC buffer and centrifuged at 1026 × g at 4°C for 15 min. Protoplasts should be visualized as a small white strip at the bottom of the tube.
- o0250 **4.** The supernatant is carefully decanted and the protoplasts are resuspended in 25 mL of cold STC buffer. The suspension is then centrifuged at 1478 × g at 4°C for 10 min.
- o0255 **5.** After removing the supernatant the protoplasts are resuspended in 1 mL of cold STC buffer, and kept on ice. At this stage, the number of protoplasts (with an appropriate dilution) is estimated by counting them with a Malassez cell counting chamber under a microscope. The count should be between 10 × 10⁷ and 10 × 10⁸ protoplast mL⁻¹.
- u0030 • Transformation of protoplasts
- o0260 **6.** In a cell culture tube, 1 μL ATA, 10 μg of pAN-52-lac, 2 μg of pAB4-1 and 150 μL of protoplasts are mixed well and incubated for 30 min at RT.

np0010^a The enzyme mix used for cell wall digestion originally used in the reference publication is no longer available. Satisfactory results have been obtained with the Glucanex preparation from Sigma that is therefore used as replacement.

- o0265 7. PEG buffer (850 μ L) is added, mixed well, and the suspension is incubated at RT for 20 min.
- o0270 8. After adding 10 mL STC buffer, the suspension is centrifuged at $1864 \times g$ at 4°C for 10 min and the supernatant is removed.
- o0275 9. The protoplasts are resuspended in 1 mL of STC buffer.
- u0035 • Plating and selection
- o0280 10. About 50 μ L of solution from step 9 are plated on solid selective minimum medium (\varnothing 90 mm Petri dish plates) and incubated for 4 days at 30°C. Note that it is possible (and easy) to screen for laccase-secreting transformants already at this stage by plating transformants on ABTS- containing plates. See [Section 2.3.2](#) for further details.
- u0040 • Mass production of spores
- p0655 It is necessary to obtain spores (from the parental strain or from transformants) for conservation (storage) as well as for the initiation of further steps of screening and enzyme production.
- p0660 *Equipment*
- o0285 1. Stationary incubator set at 30°C.
- o0290 2. Large (\varnothing 150 mm) Petri dish plates.
- o0295 3. Miracloth filter.
- o0300 4. Malassez cell counting chamber.
- o0305 5. Microscope.
- p0690 *Buffers and reagents*
- o0310 1. Solid minimum medium: the same composition as above is used for transformants selection.
- o0315 2. 500 mL physiological water supplemented with 10 drops of tween 80.
- o0320 3. 1 L of sterile water in a 1 L Erlenmeyer flask.
- p0710 *Procedure*
- o0325 1. Minimum medium agar plates are prepared. About 15 large plates should be necessary for generating enough spores to inoculate 8 L of culture in a bioreactor.
- o0330 2. Plating is carried out, using about 50 μ L of spores stock solution per plate, and these are incubated for 10 days at 30°C to allow vegetative growth and a subsequent sporulation. A 1-L Erlenmeyer flask full of sterile water is placed in a top bench incubator chamber to maintain a humid atmosphere and prevent plates from drying out over time.
- o0335 3. After 10 days, the dark spores are collected with 10 mL of saline/tween 80 solution per plate and filtered through a sterile film (Miracloth) to eliminate traces of agar and mycelium debris.

- o0340 4. A few microliters of a known dilution of the sample is introduced to a Malassez cell counting chamber, and the number of spores is estimated under a microscope.
- o0345 5. The spores can be stored in 15% of glycerol saline solution and kept at -80°C until further use.

s0050 **2.3 Screening for laccase secreting transformants in *S. cerevisiae* and *A. niger***

p0740 Clone-to-clone variations in expression and/or secretion levels are high both in *S. cerevisiae* and in *A. niger*. It is therefore important to reach transformation frequencies allowing screening on a sufficient number of clones (typically a few tens to several hundred in the case of libraries) in order to select the best secretors. Laccase secretors are selected either for their ability to oxidize a laccase substrate (e.g., using an activity test with ABTS or guaiacol) or for their specific reaction with an antilaccase antibody (immunodetection is especially useful in the case of production of nonactive enzyme, such as a T1-depleted enzyme). Screening tests can be performed on activity plates (primary screening) and thereafter on liquid cultures of isolated clones for both organisms. Deep-well plates and microtiter plates are adapted to the cultivation of *A. niger* and *S. cerevisiae* transformants, respectively.

s0055 **2.3.1 Screening of clones from deep-well or microtiter plate cultures**

p0745 This is a convenient method for rapid screening of a large number of parallel liquid cultures of transformants from both *S. cerevisiae* and *A. niger*.

o0350 **a.** Cultivation of *S. cerevisiae* transformants in microtiter plate

p0755 *Equipment*

o0355 1. Floor centrifuge (equipped with microplate holder).

o0360 2. Rotary incubator.

p0770 *Buffers and reagents*

o0365 1. Sterile SD medium (see [Section 2.3.1](#)).

p0780 *Procedure*

o0370 1. A 96-well microtiter plate is filled with 300 μL of SD medium.

o0375 2. Each yeast colony is inoculated with a sterile toothpick.

o0380 3. Cells are grown for 3 days at 30°C .

o0385 4. An aliquot of 10 μL of this cell suspension is taken and inoculated 300 μL of fresh SD medium.

o0390 5. The culture is grown for 24 h at 30°C .

o0395 **6.** Cells are pelleted at 3000 rpm for 2 min, and the supernatant (SN) is retained in a clean microtiter for measuring activity and protein concentration (see [Section 2.3.3](#)).

o0400 **b.** Cultivation of *A. niger* transformants in deep-well plates

p0820 *Equipment*

o0405 **1.** Polypropylene 24 deep-well plates (Whatman INC, Piscataway, USA).

o0410 **2.** Breathable sterile sealing film (Breathseal, Greiner Bio-One).

o0415 **3.** Shaking incubator.

o0420 **4.** Malassez cell counting chamber.

o0425 **5.** Microscope.

p0850 *Buffers and reagents*

o0430 **1.** Trace elements stock solution (see [Section 2.2.2](#), part b).

o0435 **2.** Minimum medium: 70 mM NaNO₃, 7 mM KCl, 200 mM Na₂HPO₄, 2 mM MgSO₄, 50 g L⁻¹ glucose, 50 mg L⁻¹ CuSO₄, 5H₂O. Then add 1% of trace element stock solution adjusted to pH 5 with a 1 M citric acid solution.

o0440 **3.** Tween 80–water: 10 drops of tween 80 in 10 mL of water.

o0445 **4.** Sterile 1 M citric acid.

p0875 *Procedure*

o0450 **1.** Samples of stored spores, prepared according to the protocol of [Section 2.2.2](#) (mass production of spores), are thawed.

o0455 **2.** Each well of a 24 deep-well plate (containing 3 mL of sterile minimum medium) is inoculated at final concentration of 1×10^6 spores mL⁻¹, and 100 μL of sterile tween 80–water is added to each well to avoid adhesion of spores.

o0460 **3.** The deep-well plate is covered with a breathable sterile sealing film.

o0465 **4.** Incubation is carried out for 6 days at 30°C with orbital shaking set at 200 rpm. The pH is adjusted daily to 5.0 with 1 M citric acid. White balls of mycelium start to be visible after 3 days of culture ([Fig. 6](#)).

o0470 **5.** At the end of the culture, 1 mL of culture medium is filtered and analyzed for activity of production.

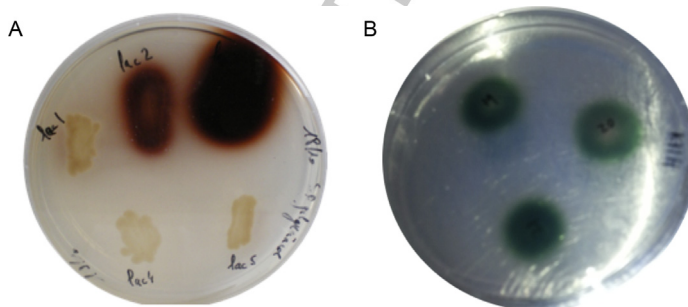
o0475 **6.** All cultures and analysis are carried out in triplicate.

s0060 **2.3.2 Screening of laccase producing transformants on petri dish plates**

p0910 Activity plate screening is based on the oxidation of chromogenic phenolic or nonphenolic laccase substrate. A colored halo (brownish and greenish for guaiacol and ABTS oxidation, respectively) developing with time around isolated colony attests for the secretion of active laccases ([Fig. 7](#)).



f0035 **Fig. 6** Deep-well plate culture of laccase producing *A. niger* transformants. (A) View of the breathable film covering the plate during cultivation. (B) Aspect of a 3 mL culture of *A. niger* in minimum medium supplemented with glucose and oligoelements after 6 days of growth at 30°C. Each well corresponds to the growth of a single transformant. The presence of *white balls* attests for the development of the fungus.



f0040 **Fig. 7** Activity plates: guaiacol oxidation by yeast laccase producing variants (A) and ABTS oxidation by *A. niger* laccase producing variants (B).

This procedure is a particularly powerful primary screen applicable to a large number of clones at the same time directly on transformation plates, as the chromogenic substrates included in the selective media (ABTS or guaiacol) is nontoxic to transformants. Moreover, for colonies of similar size, both the extent of the halo and the color depth are indicative of differences in secretion levels and/or in specific activities (library screening).

p0915 *Equipment*

o0480 **1.** Stationary incubator set at 30°C.

o0485 **2.** Regular Petri dish plates.

p0930 *Buffers and reagents*

o0490 **1.** Solid minimum medium for *A. niger*. Same composition as above for transformants selection. Make the solution 50 mg L⁻¹ CuSO₄, 5 H₂O, and 0.2 mM ABTS final from sterile stock solutions prior to gelification (hardening).

o0495 **2.** Solid SD medium for *S. cerevisiae*: YNB without AA, 0.67%; galactose, 2%; adenine, 30 mg L⁻¹; casein hydrolysate, 0.5%; tryptophan, 40 mg L⁻¹; CuSO₄, 100 μM; 15 g L⁻¹ agar; 50 mM succinate buffer pH 5.3. Add 500 μL L⁻¹ guaiacol prior to gelification (hardening).

p0945 *Procedure*

o0500 **1.** Activity ABTS minimum medium plate is used for the selection of *A. niger* transformants. About 50 μL of transformants solution are applied per plate, followed by incubation for 4 days at 30°C. Development of a green halo around a colony, visible to the eye, attests for active laccase secretion.

o0505 **2.** SD medium plates are used for the selection of *S. cerevisiae* transformants. 200 μL of transformants are spread per plate, which are incubated for 2–3 days at 30°C until colonies form. Development of a green halo around a colony attests for active laccase secretion.

s0065 **2.3.3 Screening of activity on 96-well plate**

p0960 96-Well ABTS plate screening is an easy way to follow laccase activity.

p0965 *Equipment*

o0510 **1.** Three 96-well microtiter plates.

o0515 **2.** Microtiter plate reader.

p0980 *Buffers and reagents*

o0520 **1.** Buffer A: ABTS solution (5 mM final) freshly prepared in any buffer compatible with laccase activity and ABTS stability. We routinely use 100 mM acetate buffer pH 5.7 or Britton and Robinson buffer for extended pH studies.

p0990 *Procedure*

o0525 **1.** The ABTS activity assay is based on the rate of production of the chromophoric radical cation ABTS^{+•} at 414 nm ($\epsilon = 3.5 \times 10^4$ L mol⁻¹ cm⁻¹ at pH 6.0).

o0530 **2.** The enzyme concentration is determined by the method of Bradford colorimetric assay on microtiter plates (protocol from furnisher). Generally, Bradford assays are performed on 150 μL of nondiluted SN. The

laccase protein concentration is estimated from a standard curve obtained from BSA solution (bovine serum albumin from 0 to 80 $\mu\text{g mL}^{-1}$).

- o0535 **3.** Supernatants are diluted with buffer A (dilution factors are SN/20 and SN/40).
- o0540 **4.** 20 μL of SN at an appropriate dilution is added to 180 μL of buffer A solution to initiate the reaction (final volume 200 μL). The specific activity of the laccase enzyme is calculated from the initial rate of the absorbance increase at 414 nm within 5 s of shaking, at 30°C. Kinetics are followed for 1 h within 30 s intervals with a microtiter plate reader (Fig. 8).
- o0545 **5.** The specific activity of laccase toward ABTS is described as the number of micromoles of ABTS oxidized per minute per milligram of enzyme.

p1020 *Note*

- o0550 **1.** This test is perfectly amenable for determining the enzymatic parameters of pure laccase as previously exemplified (Cusano et al., 2009).

s0070 **2.3.4 ELISA screening**

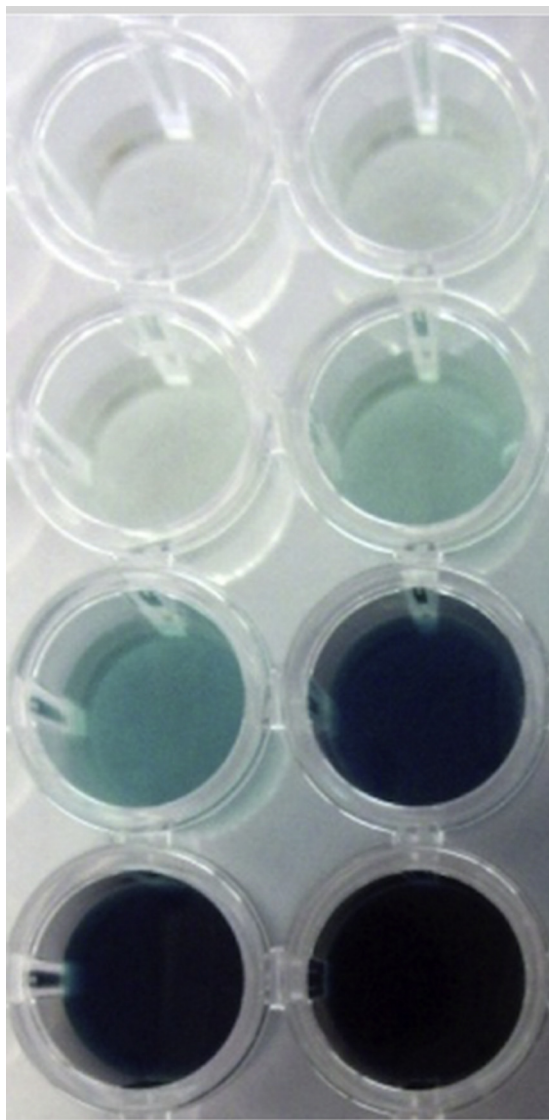
p1030 Sandwich ELISA, a variant of ELISA, is a highly sensitive technique for sample antigen detection. It allows quantification of the antigen between two layers of antibody molecules: the capture layer and the detection layer. An immobilized polyclonal anti-LAC3 antibody (multiepitope recognition) is used to capture the antigen. The same polyclonal biotinylated anti-LAC3 antibody serves for the detection (Fig. 9). This method is particularly suited to the detection of low-activity or inactive laccase variants. The advantage of this method is that the antigen does not need to be purified before analysis.

p1035 *Equipment*

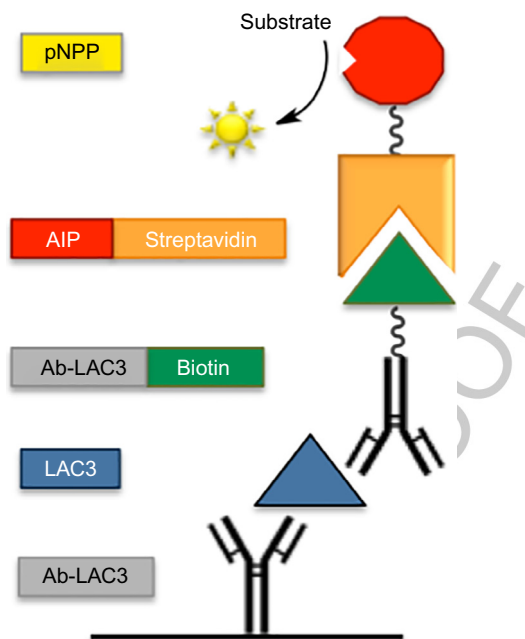
- o0555 **1.** 96-Well microplates coated for ELISA.
- o0560 **2.** Any microplate reader.
- o0565 **3.** Multichannel precision pipettes.

p1055 *Buffers and reagents*

- o0570 **1.** Coating buffer: 0.2 M sodium carbonate/bicarbonate, pH 9.4.
- o0575 **2.** Capture antibody: antilaccase antibody Ab-LAC (Thermo Fisher) diluted in coating buffer.
- o0580 **3.** PBST washing buffer: 0.1 M phosphate, 0.15 M sodium chloride, pH 7.2 containing 0.05% tween 20.
- o0585 **4.** Blocking buffer: 2% (w/v) BSA in PBST washing buffer.
- o0590 **5.** Standard dilution buffer: purified laccase diluted in blocking buffer.
- o0595 **6.** Detection antibody: biotinylated polyclonal Ac-LAC antibody.
- o0600 **7.** Biotin-secondary antibody.



10045 **Fig. 8** Microtiter plate assay for laccase-mediated ABTS oxidation. The green color attests for laccase activity. Adapted from Cusano, A. M., Mekkouchi, Y., Meglecz, E., & Tron, T. (2009). Plasticity of laccase generated by homologous recombination in yeast. *FEBS Journal*, 276, 5471–5480.



o050 **Fig. 9** Scheme of the “Sandwich ELISA” technique. The coated polyclonal antibody anti-LAC3 (Ab-LAC3) captures the antigen (LAC3). The secondary antibody, the same polyclonal biotinylated anti-LAC3 antibody (Ab-LAC3-biotin) serves for detection. The detection is made possible with the enzyme conjugate ALP-Streptavidin. The substrate pNPP (4-nitrophenyl phosphate disodium salt hexahydrate) is hydrolyzed into a colorimetric product detected at 405 nm.

o0605 **8.** Enzyme conjugate: Streptavidin Alkaline Phosphatase (Jackson Immuno Research).

o0610 **9.** Substrate: pNPP (Sigma S0942).

o0615 **10.** Glycine buffer: 0.1 M glycine, pH 10.4, with 1 mM MgCl₂ and 1 mM ZnCl₂.

p1110 *Procedures*

p1115 The steps are as follows:

u0045 • Antibodies preparation (capture and detection)

o0620 **1.** Purification of Ab-LAC: following procedure from manufacturer (Thermo Scientific 89952).

o0625 **2.** Biotinylation of Ab-LAC: following the protocol of a Biotin Protein Labeling Kit (Sigma Aldrich).

o0630 **3.** Dilute to 2 mg mL⁻¹, adding an equal volume of glycerol (final concentration: 50%), and store at -20°C.

u0050 • Surface preparation

o0635 **4.** The capture antibody is diluted (20,000 ×) in coating buffer.

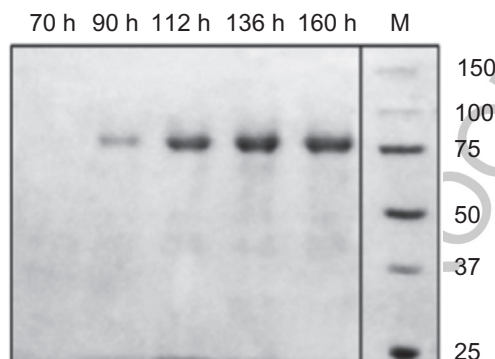
- o0640 5. 100 μL of diluted capture antibody is added to each well; the micro-plates are then covered and incubated overnight at 4°C.
- o0645 6. Discarding the solution, the plates are washed three times with 200 μL per well of PBST washing buffer.
- o0650 7. 200 μL of blocking buffer is added per well, the plates are then covered and incubated for 1 h at RT.
- u0055 • Antigen capture
- o0655 8. The blocking buffer is discarded and 100 μL of sample or standards (100–2000 pg) is added. The plates are incubated for 1 h at RT.
- o0660 9. The solution is discarded and the plate washed three times with 200 μL per well of PBST washing buffer.
- o0665 10. The biotinylated detection antibody is diluted 5000-fold in blocking buffer.
- o0670 11. To each well is added 100 μL of diluted biotinylated detection antibody, the plate is then covered and incubated for 1 h at RT.
- o0675 12. The solution is discarded and the plate is washed five times with 200 μL per well of PBST washing buffer.
- u0060 • Detection and quantification
- o0680 13. The enzyme conjugate ALP-Streptavidin is diluted 1000-fold.
- o0685 14. 100 μL of the diluted ALP-Streptavidin is added to the plate, which is then covered and incubated for 1 h at RT.
- o0690 15. After removing the solution, the plate is washed seven times with 200 μL per well of PBST washing buffer.
- o0695 16. The substrate solution is prepared: one tablet of pNPP (5 mg) is used in 10 mL of glycine buffer.
- o0700 17. The plate is incubated at RT until the desired color intensity is reached. Ideally, a clear gradient is obtained with the variable concentrations of standard used.
- o0705 18. The absorbance at 405 nm is recorded with a plate reader to estimate the amount of bound antigen from the standard curve established from different concentrations of pure LAC3 antigen.

s0075 2.4 Fermentation in *A. niger*

p1230 As stated earlier, the production of laccase and variants is carried out in 5 or 10 L bioreactors following production procedures already described (Mekmouche et al., 2014; Record et al., 2002). Prior to the fermentation, it is necessary to produce a sufficient stock of spore allowing an inoculum of 2×10^6 spores mL^{-1} for 10 L culture: this is described below.

t0015 **Table 2** Typical growth parameters for heterologous expression of fungal laccase in *A. niger*

Parameters	Aeration (VVM)	Inoculum Size		Glucose (g L ⁻¹)	Rotation (rpm)
		Spore (mL ⁻¹)	pH		
	1.2	2 × 10 ⁶	5.5	50	200–600



f0055 **Fig. 10** Time course of laccase production during up-scaled fermentation. SDS-PAGE (7.5%) is stained with Coomassie blue. 10 mL of culture supernatant were loaded per well as a function of cultivation time (from 0 to 184 h); *M*: molecular weight markers in kDa. Adapted from Mekmouche, Y., Zhou, S., Cusano, A. M., Record, E., Lomascolo, A., Robert, V., et al. (2014). Gram-scale production of a basidiomycetous laccase in *Aspergillus niger*. *Journal of Bioscience and Bioengineering*, 117, 25–27 with permission.

s0080 **2.4.1 Preparation of spore inoculum and storage**

p1235 See Section 2.2.2.

p1240 A stock of stored spores is thawed to prepare the inoculum, and as many plates as necessary for a 10 L culture are prepared. Usually, 15 plates are necessary to inoculate a 10 L fermentor.

s0085 **2.4.2 Production in bioreactor and growth parameters**

p1245 The initial growth parameters are presented in Table 2. Parameters such as agitation or addition of antifoam are adjusted daily. Specific activity, O₂ consumption, and protease activity are measured routinely (Mekmouche et al., 2014).

p1250 Growth conditions in a 10 L bioreactor have been described previously (Mekmouche et al., 2014). The recombinant enzyme is the major secretion product: it is therefore possible to follow the time course of production of the enzyme simply by loading an SDS-PAGE with extracellular culture medium (Fig. 10).



s0090

3. General purification procedure and characterization

p1255

Purification of laccase variants can be obtained easily following a protocol adapted from [Cusano et al. \(2009\)](#) based on four steps: (1) filtration to eliminate the mycelium, (2) concentration, (3) capture (anionic exchange), and (4) polishing (gel filtration), altogether allowing for a satisfactory level of homogeneity ([Fig. 11](#)).

s0095

3.1 Purification procedure

s0100

3.1.1 Assay procedures

p1260

Although several substrates are regularly used for laccase activity determination, we will here describe only the syringaldazine (SGZ) colorimetric assay for volumetric activity determination and an electrophoretic colorimetric assay based on phenylenediamine (PPD) oxidation (zymogram).

o0710

a. SGZ assay procedure

p1270

Equipments

o0715

1. UV–vis spectrophotometer.

o0720

2. Disposable cuvettes of 1.5 mL.

p1285

Buffers and reagents

o0725

1. Syringaldazine stock solution: 8 mg mL^{-1} in MeOH.

o0730

2. Buffer A: 100 mM sodium acetate pH 5.7.

o0735

3. Bradford reagent (Millipore).

p1305

Buffers and reagents

o0740

1. The SGZ activity assay is based on the rate of production of the corresponding quinone at 525 nm ($\epsilon = 65,000 \text{ L mol}^{-1} \text{ L cm}^{-1}$) ([Bauer & Rupe, 1971](#)).

o0745

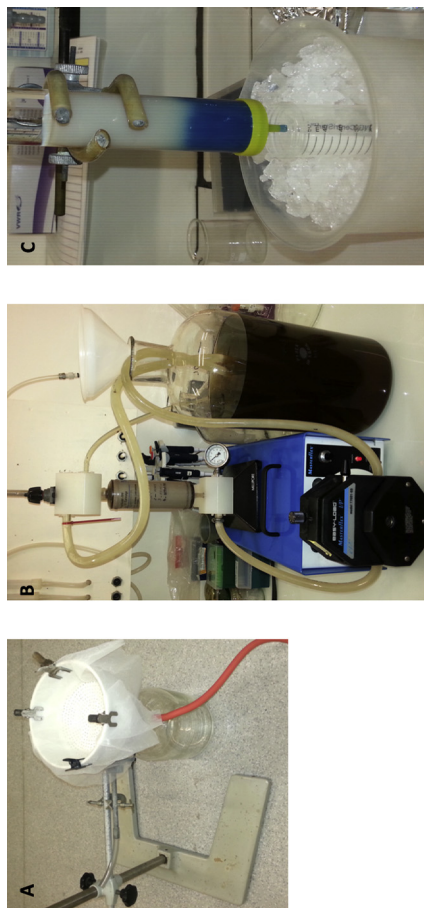
2. The laccase concentration is determined by two colorimetric methods. The first method is the Bradford colorimetric assay ([Bradford, 1976](#)) and the second one is based on oxidized T1 absorption (when enzyme is purified) at 610 nm ($\epsilon = 5600 \text{ L mol}^{-1} \text{ L cm}^{-1}$) ([Simaan et al., 2011](#)).

o0750

3. For routine assay a working concentration of $88 \mu\text{M}$ of syringaldazine is used. Laccase is added at an appropriate concentration to buffer A solution in the disposable cuvette to a final volume of $970 \mu\text{L}$.

o0755

4. The addition of $30 \mu\text{L}$ of SGZ initiates the reaction. The volumetric activity of the laccase enzyme is calculated from the initial rate measurement of the absorbance increase at 525 nm, followed over the time course of 2 min.



10060 **Fig. 11** Initial steps for the purification of recombinant laccases produced in *A. niger*. (A) Setting for the filtration of the cultivation medium through a Separ (SEFAR MEDIFAB 30-170/54) filter allowing for the elimination of the mycellium; (B) tangential ultrafiltration and concentration of 10L of cultivation medium (UF cartridge regenerated cellulose cutoff 30 kDa from Millipore); and (C) NaCl gradient elution of laccase on a DEAE column (2.5 × 25 cm²) loaded with the ultrafiltrate.

o0760 **5.** The specific activity of laccase toward SGZ is described as the number of micromoles of SGZ oxidized per minute per milligram of the laccase.

o0765 **b.** Zymogram assay procedure

p1340 It may be convenient to visualize laccase distribution in protein samples throughout purification. Beside (or in addition to) protein staining and immunodetection, laccase conversion of chromogenic substances to colored compounds is sensitive and quick. Laccase activity is easily detectable *in gel* after protein separation on a regular PAGE-SDS gel, providing any denaturation of the protein sample is avoided prior to loading the gel (i.e., no reducing agent is present in the loading buffer and no heat treatment has been used).

p1345 *Equipment*

o0770 **1.** SDS-PAGE gel (e.g., 7.5%).

o0775 **2.** Electrophoresis apparatus.

p1360 *Buffers and reagents*

o0780 **1.** Modified Laemmli sample buffer: 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH approx. 6.8.

o0785 **2.** Buffer A: 20 mM acetate buffer pH 3.6.

o0790 **3.** *p*-Phenylenediamine.

o0795 **4.** Buffer B: 100 mM phosphate buffer pH 7.5.

p1385 *Procedure*

o0800 **1.** Protein samples are diluted in modified Laemmli sample buffer (no reducing agent) and then loaded onto a PAGE SDS: it is important not to heat the protein sample.

o0805 **2.** The PAGE-SDS gel is run under appropriate conditions.

o0810 **3.** The gel is soaked in buffer A for 10 min.

o0815 **4.** The gel is rinsed three times with buffer A.

o0820 **5.** The gel is soaked in a freshly prepared solution of PPD (2 mg mL⁻¹ in buffer A) until color is developed. The reaction is quenched by soaking the gel in buffer B.

o0830 **6.** Rinse the gel three times with buffer B.

o0835 **7.** Extensively wash with water prior to (short-term) storage.

s0105 **3.1.2 Laccase purification procedure**

p1430 *Equipment*

o0840 **1.** Vacuum filtration setting (e.g., 110 mm Büchner funnel + 5 L flask + vacuum pump).

o0845 **2.** Filters (SEFAR MEDIFAB 30-170/54).

- o0850 **3.** High speed floor centrifuge, centrifuge tube.
- o0855 **4.** Ultrafiltration apparatus (e.g., Prep/Scale Spiral-Wound Ultrafiltration Modules: TFF-2. MWCO 30 kDa 150 mL working volume).
- o0860 **5.** High flow rate peristaltic pump (e.g., Cole Palmer Masterflex I/P Easy-Load Pump).
- o0865 **6.** Empty glass gravity chromatographic column $2.5 \times 25 \text{ cm}^2$ (e.g., Econo-Column[®] Bio-Rad).
- o0870 **7.** Size-exclusion chromatographic column (e.g., HiPrep[™] 26/60 Sephacryl[®] S-300 HR, GE Healthcare).
- o0875 **8.** Centrifugal concentrator 30,000 MWCO PES (e.g., Vivaspin 20 Sartorius).
- o0880 **9.** Stirred-cell ultrafiltration apparatus (e.g., Amicon) with 30,000 MWCO PES membranes.
- o0885 **10.** UV-vis spectrophotometer.
- o0890 **11.** FPLC apparatus equipped with double wavelength detection 280 and 610 nm (e.g., Äkta purifier 10, GE Healthcare).

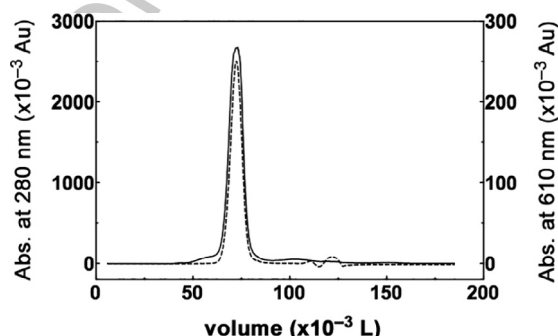
p1490 *Buffers and reagents*

- o0895 **1.** Buffer C: 20 mM phosphate buffer pH 6.0.
- o0900 **2.** Buffer D: 20 mM phosphate buffer pH 6.0, 300 mM NaCl.
- o0905 **3.** Buffer E: 20 mM phosphate buffer pH 6.0, 200 mM NaCl.
- o0910 **4.** Syringaldazine stock solution: 8 mg mL^{-1} in MeOH.
- o0915 **5.** ABTS solution: buffer containing 5 mM ABTS, freshly prepared. It is possible to use any buffer compatible with laccase activity and ABTS stability. We generally use 100 mM acetate buffer pH 5.7 or Britton and Robinson buffer for pH studies.

p1520 *Procedure*

- o0920 **1.** Filter the *A. niger* dark brown fermentation medium (typically 10 L) on a Büchner funnel equipped with a low porosity filter (SEFAR MED-IFAB 30-170/54) under vacuum. Wash the mycelium several times (three to five times) with a minimum volume of buffer A (for a total volume representing 10% of the initial cultivation volume).
- o0925 **2.** Concentrate the filtered medium using an ultrafiltration apparatus equipped with a MWCO 30 kDa membrane down to the minimal volume (depends on apparatus and membrane specifications, usually $\approx 200 \text{ mL}$ for a 2.3 sq. ft. membrane) at a flow rate compatible with the maximum operated pressure recommended by the membrane manufacturer (flow rate of $\approx 3 \text{ L min}^{-1}$). At this stage, most of the dark brown material goes to the permeate tank and the intensity of the blue-green color of the retentate visually translates laccase production.

- 00930 3. Add 50 mL of DEAE-Sepharose resin previously equilibrated with buffer C to the retentate (batch process). Allow binding under slow magnetic stirring for 1 h at 4°C.
- 00935 4. Pour the DEAE slurry into an empty glass chromatographic column $2.5 \times 25 \text{ cm}^2$ and pack it by gravity.
- 00940 5. Wash the resin with 250 mL buffer C. Depending on the laccase variant the resin should be more or less intensely blue colored.
- 00945 6. Connect the column to a low flow peristaltic pump and elute laccase by sharply raising the ionic strength of the buffer. Laccases derived from LAC3 produced in our laboratory elute usually with 50–75 mL of buffer D (containing 300 mM NaCl) at a flow rate of 2 mL min^{-1} . The presence of colorless laccase variants (e.g., delta T1) may be confirmed by immunodetection.
- 00950 7. Protein concentration is evaluated by Bradford assay and laccase concentration is determined by UV-vis spectroscopy ($\epsilon_{610\text{nm}} = 5600 \text{ L mol}^{-1} \text{ cm}^{-1}$).
- 00955 8. If necessary the protein concentration of the sample is adjusted to maximize loading of size-exclusion chromatography.
- 00960 9. An appropriate volume of sample is applied to the top of a preequilibrated (buffer E) $2.6 \times 60 \text{ cm}^2$ Sephacryl S-300 HR column (or equivalent) connected to a FPLC system. Gel filtration is carried out at a flow rate of 1 mL min^{-1} (buffer E) collecting 5 mL fractions. Progress is followed with detectors set at 280 and 610 nm. Repeated injections are necessary given the size of the sample obtained after DEAE. Fractions with a ratio between $14 < \lambda_{280\text{nm}}/\lambda_{610\text{nm}} < 16$ and an activity within 20% of the peak fraction are pooled (Fig. 12).



0065 Fig. 12 Typical S300 chromatogram obtained for LAC3. Two wavelengths (280 nm *solid line*, 610 nm *dashed line*) are selected to follow the elution.

- o0965 **10.** Pooled fractions are exchanged with buffer A to remove NaCl using a 30-kDa cutoff ultrafiltration membrane in a stirred-cell filtration unit (e.g., Amicon). The sample is concentrated to 10 mg mL^{-1} and 10% of glycerol (v/v) is added to the solution.
- o0970 **11.** The sample is filtered under a sterile atmosphere using a $0.2\text{ }\mu\text{m}$ filter; $500\text{ }\mu\text{L}$ is then dispensed to 1 mL aliquots in cryotubes which are then stored at -20°C for long-term storage.
- p1580 Representative yields for several laccase variants are presented in [Table 3](#). Yields and catalytic properties for LAC3 and UNIK variants (unique lysine variants) are twofold higher than those of the K_0 variant (no lysine). Eventually, yields are markedly higher than those obtained in *S. cerevisiae* (about 40 times for LAC3 heterologous production) ([Table 3](#)).

t0020 **Table 3** Typical purification yields obtained for various LAC variants

	LAC3	UNIK ₁₆₁	UNIK ₁₅₇	K_0
Production	8 L	8 L	8 L	8 L
	$40,000\text{ U L}^{-1}$	$28,000\text{ U L}^{-1}$	$25,000\text{ U L}^{-1}$	8900 U L^{-1}
Purification	900 mg	700 mg	600 mg	400 mg
	170 U mg^{-1}	160 U mg^{-1}	160 U mg^{-1}	90 U mg^{-1}

p1585 *Notes*

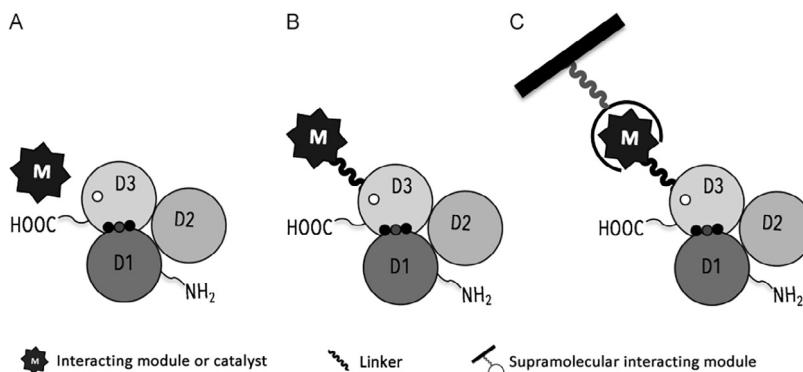
- o0975 **1.** Filtration of the cultivation medium may lead to a rapid browning of the solution. This is probably a manifestation of laccase activity consecutive to a reoxygenation of the solution during the elimination of the O_2 -consuming *A. niger* mycelium.
- o0980 **2.** After ultrafiltration (step 2), the volume of the retentate is the minimal one that can be obtained, as it is constrained by the volume of the cartridge and tubings. It is usually necessary to rinse the system one or two times with a minimal volume of 200 mL . This variation of the volume does not affect the capture of the enzyme on an anionic exchanger.
- o0985 **3.** At each step of the purification process, a small aliquot is taken out to load on SDS-PAGE, measure the protein concentration (Bradford assay and T1 absorbance for the last step) and the specific activity.

s0110

4. Applications

p1605

Over the past few years, efforts have been made to investigate alternative routes for the oxidation of organic compounds and to perform selective catalytic chemical transformations. To date, these reactions are highly



0070 **Fig. 13** General strategy for producing hybrid catalysts. Three cupredoxin domains (D1, D2, and D3) represent the laccase structure. *White circle*, T1 copper atom; *gray circle*, T2 copper atom; and *black circle*, T3 copper atoms. (A) Bimolecular interaction; (B) oriented covalent interaction; and (C) oriented supramolecular interaction between laccase and heterogeneous material.

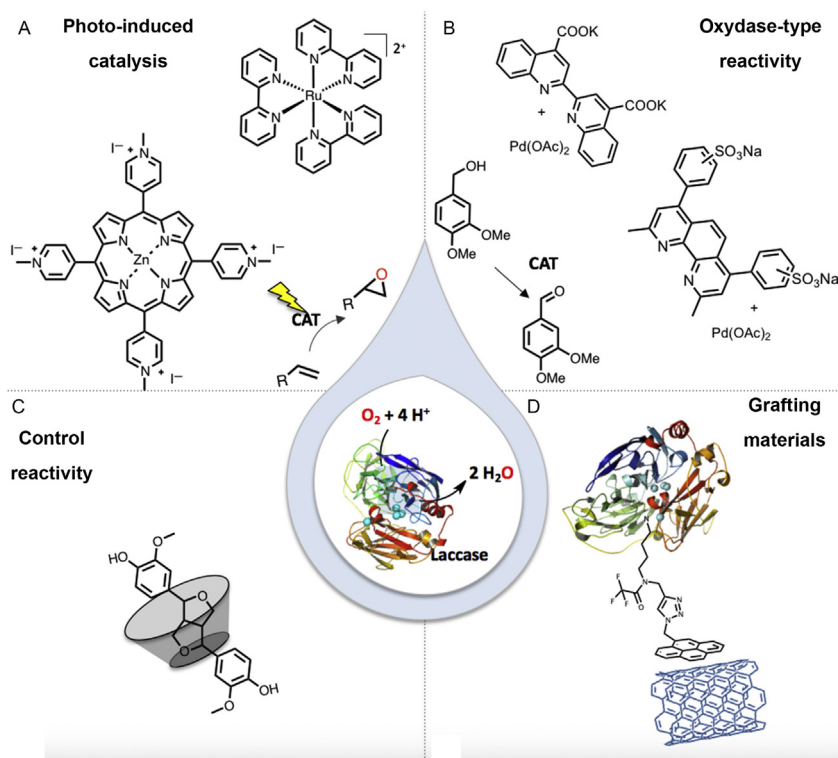
important at an industrial level and the current processes are among the most polluting ones. Appearing as highly promising sustainable alternatives to traditional catalysts, artificial enzymes often combine a transition metal catalyst moiety with a protein environment (Diéguez, Bäckvall, & Pàmies, 2018; Schwizer et al., 2018). Inspired from this new field, we recently engaged ourselves in studying interactions between laccase and devices or materials. We study three types of interactions: (i) bimolecular; (ii) oriented covalent; and (iii) oriented supramolecular (Fig. 13).

p1610 We have recently described the first examples of catalytic systems combining the O₂-reducing power of a laccase with the catalytic power of inorganic moieties represented either by a palladium-based water-soluble catalyst (aerobic oxidation of veratryl alcohol) or a ruthenium-based complex (photo-induced oxidation of styrene compounds) (Fig. 14). In both cases, substrate is oxidized under mild conditions by the inorganic moiety probably redox cycling, via the enzyme, with the four-electron reduction of dioxygen into water. In these hybrid systems, the enzyme and the inorganic catalysts form functional bimolecular systems and O₂ acts as a renewable, cheap, and clean oxidant.

s0115 4.1 General procedure for an oriented covalent grafting of the laccase surface

s0120 4.1.1 Preparation of laccase variants for single-site anchoring

p1615 The well-known topography of laccases makes them easy material for surface-targeted modification. The design of variants for surface-targeted

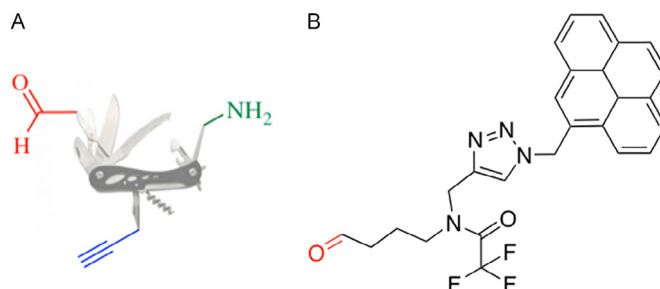


f0075 **Fig. 14** Hybrid catalysts combining inorganic or organic moieties with the powerful oxidoreductase laccase. (A) Interaction of small photosensitizers with laccase to promote photo-induced oxidation of alkenes to epoxides; (B) laccase as a palladium oxidase. Primary alcohol oxidation mediated by the Pd/laccase/ O_2 system under mild conditions; (C) controlled polymerization of coniferyl alcohol mediated by β -cyclodextrin with fungal laccase; and (D) supramolecular interaction of a pyrene-grafted laccase and MWCNT electrode to promote oriented direct electron transfer.

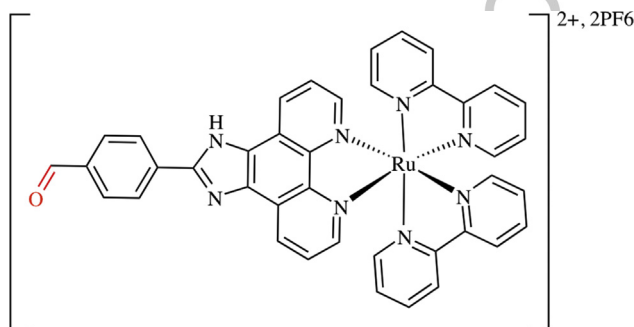
AA functionalization is addressed in [Section 2.2.1](#). Briefly, a single lysine (K) residue is precisely positioned at the surface of a lysine-less variant through site-directed mutagenesis. The variants created this way are designed UNIK.

s0125 **4.1.2 Reductive amination of lysine**

p1620 Highly selective modifications of primary amino groups present at the surface of proteins can be obtained under mild conditions. The chemical modification of UNIK variants is adapted from the procedure described by [McFarland and Francis \(2005\)](#). The primary amino group of lysine present



f0080 **Fig. 15** (A) Artistic view of a multifunctional molecule as a graft for laccase. (B) Structure of the 2,2,2-trifluoro-*N*-(4-oxo-butyl)-*N*-(1-pyren-4-ylmethyl)-1*H*-[1,2,3]triazol-4-ylmethyl-acetamide reactant (compound **1**).



f0085 **Fig. 16** Structure of compound **2** [(bpy)₂Ru(fimbzl)][PF₆]₂·2H₂O (fimbzl=4 ([1,10]phenanthroline[5,6-*d*]imidazol-2-yl)benzaldehyde).

at the surface of UNIK is reacted with an aldehyde-containing molecule under reducing conditions. Two recent publications from our group describe a strategy for achieving a controlled orientation in the grafting of the surface of a fungal laccase (Lalaoui et al., 2016; Robert et al., 2017). The grafting procedure of two molecules is detailed hereafter. The first molecule is an original 2,2,2-trifluoro-*N*-(4-oxo-butyl)-*N*-(1-pyren-4-ylmethyl)-1*H*-[1,2,3]triazol-4-ylmethyl-acetamide reactant (compound **1**, Fig. 15) synthesized to favor the anchoring of laccase variants on carbon nanotubes containing electrodes through supramolecular interaction. This molecule features: (i) an aldehyde moiety for reductive amination and (ii) a pyrene moiety favoring interaction with the electrode material. Fig. 14A summarizes a schematic view of this multifunctional platform. The second molecule is an original heteroleptic ruthenium(II)-polypyridine-type complex with one of the bipyridine ligands containing a fused imidazole motif and a benzaldehyde (compound **2**, Fig. 16).

p1625 This complex has been selected for the following reasons: (i) its ability to photoreduce laccases upon visible light excitation (Schneider et al., 2015; Simaan et al., 2011); (ii) its intense absorption band in the visible spectrum at $\lambda_{\max} = 450 \text{ nm}$ ($\epsilon_{450\text{nm}} = 14,500 \text{ L cm}^{-1} \text{ mol}^{-1}$) (Kalyanasundaram, 1982) which does not overlap the $\lambda_{\max} = 605 \text{ nm}$ of the T1 Cu(II) site of laccases; and (iii) its fluorescence properties ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 600 \text{ nm}$).

p1630 *Equipment*

- o0990 1. $1.5 \times 150 \text{ cm}^2$ glass chromatography column.
- o0995 2. Sephadex[®] G-25 fine (GE Healthcare).
- o1000 3. Peristaltic pump (e.g., Gilson).
- o1005 4. Centrifugeable ultrafiltration devices, 30,000 MWCO PES (e.g., Vivaspin 20, Sartorius).
- o1010 5. Floor centrifuge and centrifuge tubes.
- o1015 6. UV-vis spectrometer.

p1665 *Buffers and reagents*

- o1020 1. Buffer A: 20 mM sodium phosphate buffer pH 6.0 containing 10% glycerol.
- o1025 2. Buffer B: 25 mM sodium phosphate buffer pH 7.4 containing 100 mM sodium formate.
- o1030 3. Buffer C: 50 mM sodium acetate buffer pH 5.5, 100 mM NaCl.
- o1035 4. Buffer D: 50 mM sodium acetate buffer pH 5.5.
- o1040 5. Custom aldehyde-donor molecule (e.g., compound 1 or compound 2). Prepare a 3 mM stock solution in buffer A. This solution can contain up to 30% DMSO to solubilize the molecule of interest.
- o1045 6. Water-stable $[\text{Cp}^*\text{Ir}(\text{bipy})(\text{H}_2\text{O})]\text{SO}_4$ complex (Ir catalyst) synthesized as previously described (McFarland & Francis, 2005). Prepare a 14 mM stock solution in deionized water, aliquot, and store at -20°C .

p1700 *Procedure*

- o1050 1. A 200 μM stock solution of laccase is prepared in buffer B, the concentration being based on the oxidized T1 absorption at 610 nm.
- o1055 2. In a 5-mL round bottom flask equipped with a Teflon magnetic stirring bar, 625 μL of laccase, 417 μL of the custom aldehyde-donor molecule, and 90 μL of Ir catalyst are combined, adjusting to a final volume of 2.5 mL with buffer A. The reaction is carried out for 72 h with permanent stirring at RT. The solution is then concentrated to 1 mL with a centrifugeable ultrafiltration device.
- o1060 3. The 1 mL mixture is loaded onto a G-25 gel filtration column preequilibrated with buffer C (5 CV) for desalting and buffer exchange. Elution is carried out with a linear flow of 1 mL min^{-1} of buffer C,

collecting 500 μL fractions. Fractions containing laccase are identified with a colorimetric assay (ABTS or SGZ oxidation), pooled, and concentrated to 250 μL with an ultracentrifugation device (Vivaspin 20, 4500 rpm).

- o1065 **4.** The concentrated protein solution is exchanged with buffer D (to remove NaCl) prior to analysis.

p1725 *Notes*

- o1070 **1.** During the grafting procedure, laccase is solubilized at neutral to basic pH. In these conditions, laccase is reversibly inactivated and easily reduced by the formate-containing solution as testified by the loss of the characteristic blue color of the oxidized T1 copper site. The G25 column allows for the elimination of the excess of unreacted aldehyde and reduction catalyst and thus a progressive reoxidation of laccase (reapparition of the blue color) can be observed as elution progresses.

- o1075 **2.** For photosensitive molecules, the desalting column (G25) and the 5-mL balloon are covered with a piece of black plastic to avoid any light-induced processes.

s0130 **4.1.3 Evaluation of the grafting**

p1740 The efficiency of grafting can be evaluated simply by use of UV-vis spectrophotometry, providing the graft absorbs at a wavelength clearly distinct from the transition at $\lambda_{\text{max}}=605\text{ nm}$ characteristic of the T1 Cu(II) site of laccases. For example, compounds **1** and **2** have each an intense absorption band at, respectively, $\lambda_{\text{max}}=345\text{ nm}$ ($\epsilon_{350\text{nm}}=37,500\text{ L mol}^{-1}\text{ cm}^{-1}$) and $\lambda_{\text{max}}=450\text{ nm}$ ($\epsilon_{450\text{nm}}=14,500\text{ L mol}^{-1}\text{ cm}^{-1}$). Therefore, the $A_{\lambda_{\text{max}}\text{graft}}/A_{\lambda_{\text{max}}\text{laccase}}$ ratio reflects well the grafting efficiency. With transition metal-containing complexes (like compound **2**) the metal/Cu ratio in the grafted enzymes (therefore the efficiency of the grafting) can be deduced from inductively coupled plasma mass spectrometry (ICP-MS) analysis. Compounds **1** and **2** being also fluorescent molecules, their grafting to the laccase surface can be visualized after electrophoresis on an SDS-PAGE gel under appropriate irradiation. However, this method is not quantitative. Ultimately, confirmation of the grafting location can be obtained by mass spectrometry analysis of peptides produced by trypsin digestion of the grafted laccase and separated by HPLC (UV-vis or fluorescence detection). A potential grafting at the amino terminus can be evaluated by AA sequencing (Edman degradation).

p1745 *Equipment*

- o1080 **1.** RP-C18 HPLC column (150/4) (e.g., Nucleosil Macherey Nagel).
o1085 **2.** Desalting columns (e.g., PD-10 Amersham).
o1090 **3.** HPLC equipped with UV/fluorescence detector.
o1095 **4.** UV-vis spectrometer.
o1100 **5.** 7.5% SDS-PAGE gel.
o1105 **6.** Electrophoresis apparatus.
o1110 **7.** Thermomixer (e.g., Eppendorf).
o1115 **8.** Digital gel imaging system (e.g., UVITEC Cambridge).

p1790 *Buffers and reagents*

- o1120 **1.** Buffer A: Britton and Robinson buffer pH 5.0.
o1125 **2.** Buffer B: 250 mM Tris-HCl, 4 mM EDTA, 6 M guanidine, pH 8.5.
o1130 **3.** Dithiothreitol (60-fold molar excess over disulfide bridges) in buffer F.
o1135 **4.** 2-Iodoacetamide.
o1140 **5.** Buffer C: 125 mM of NH_4HCO_3 pH 8.0.
o1145 **6.** Trypsin.
o1150 **7.** HPLC solvent A: water 0.1% TFA.
o1155 **8.** HPLC solvent B: acetonitrile 0.1% TFA.

p1835 *Procedure*

- u0065 • UV-vis spectrometry analysis
- o1160 **1.** Solutions of both unreacted and functionalized laccases are prepared in buffer A at a convenient concentration ($\approx 50 \mu\text{M}$). The spectra of both laccase solutions (Fig. 17) are recorded. The number of grafted molecules per laccase can be deduced from the $A_{345\text{nm}}/A_{605\text{nm}}$ and $A_{450\text{nm}}/A_{605\text{nm}}$ ratios for compounds **1** (Fig. 15) and **2** (Fig. 16), respectively.
- u0070 • SDS-PAGE analysis
- p1855 This method is qualitative and highlights the grafting of a fluorescent probe at the surface of the enzyme. Under the conditions of electrophoretic migration, unbound molecules will not follow the same profile as laccase because compound **1** is uncharged and compound **2** is positively charged.
- o1165 **1.** A 7% SDS-PAGE gel is prepared.
o1170 **2.** Samples are heat denatured according to standard SDS-PAGE electrophoresis protocol.
o1175 **3.** The gel is loaded with unreacted enzyme and grafted laccase samples ($5 \mu\text{g}/\text{well}$).
o1180 **4.** After electrophoresis, the gel is examined under irradiation at the appropriate wavelength to excite the fluorophore. Thereafter, the

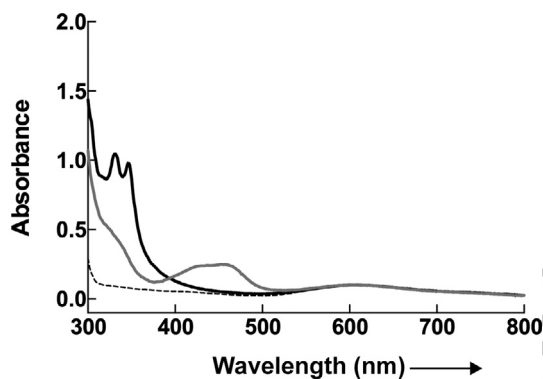


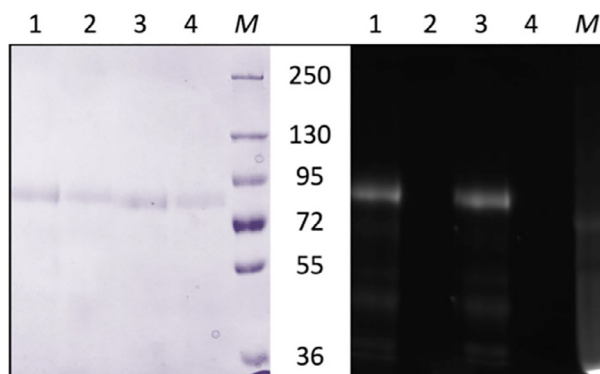
Fig. 17 UV-vis spectra of unreacted and grafted laccases. *Dashed line*, unreacted LAC3; *solid dark line*, UNIK grafted with compound **1**; and *solid gray line*, UNIK grafted with compound **2**. Adapted from Lalaoui, N., Rousselot-Pailley, P., Robert, V., Mekmouche, Y., Villalonga, R., Holzinger, M., et al. (2016). Direct electron transfer between a site-specific pyrene-modified laccase and carbon nanotube/gold nanoparticle supramolecular assemblies for bioelectrocatalytic dioxygen reduction. *ACS Catalysis*, 6, 1894–1900; Robert, V., Monza, E., Tarrago, L., Sancho, F., De Falco, A., Schneider, L., et al. (2017). Probing the surface of a laccase for clues towards the design of chemo-enzymatic catalysts. *ChemPlusChem*, 82, 607–614.

gel is stained with Coomassie Blue and examined under white light irradiation. An example of analysis of samples subjected to reductive alkylation with compound **2** is given in Fig. 18.

• Mass spectrometry analysis

In case the graft is a transition metal complex the exogenous metal (graft)-to-laccase ratios can be confirmed by analyzing the metal content of the grafted enzymes by ICP-MS. Eventually, the precise location of the graft at the surface of enzymes can be confirmed by MALDI-TOF-MS analysis of trypsin peptides. Samples are prepared according to a procedure adapted from Kharrat et al. (1998).

1. The modified enzyme solution at a desired concentration is placed in a 1-mL microtube.
2. Prior to digestion, the protein is reduced with an excess of dithiothreitol (DTT) (60-fold molar excess over disulfide bridges) in buffer B for 20 h at 40°C, under nitrogen and in the dark.
3. S-alkylation of the protein is carried out by adding an excess of 2-iodoacetamide (threefold molar excess over DTT). This mixture is maintained under constant gentle stirring at room temperature for 20 min using a thermomixer.



f0095 **Fig. 18** Representative separation of grafted enzymes on a 7% SDS-PAGE gel. *Left panel:* white light irradiation (Coomassie blue staining); *right panel:* UV irradiation (360 nm). *M:* molecular weight markers. Apparent masses are expressed in kDa. Lane 1: 1-UNIK161 (5 mg); lane 2: UNIK161 (5 mg); lane 3: 1-LAC3 (5 mg); and lane 4: LAC3 (5 mg). *Adapted from Robert, V., Monza, E., Tarrago, L., Sancho, F., De Falco, A., Schneider, L., et al. (2017). Probing the surface of a laccase for clues towards the design of chemo-enzymatic catalysts. ChemPlusChem, 82, 607–614.*

- o1200 4. The S-alkylated protein is desalted on a PD-10 column preequilibrated with buffer C.
- o1205 5. The alkylated protein is digested with trypsin (5%, w/w) for 20 h in buffer A at 37°C.
- o1210 6. The resulting peptides are purified by HPLC equipped with a RP-C18 column.
- o1215 7. Example of conditions; injection of 50–100 μL of sample; elution is monitored both by UV-vis ($\lambda = 280$ and 450 nm) and by fluorescence ($\lambda_{\text{ex}} = 450$ nm) with the following elution program (Table 4). Peaks displaying fluorescence are collected and submitted to MALDI-TOF analysis (Microflex II (2008) Bruker).

t0025 **Table 4** Elution conditions for the separation of peptides digested from modified laccase

Time (min)	Solvent A	Solvent B	Flow Rate (mL min^{-1})
0	100	0	1
45	50	50	1
50	50	50	1
55	0	100	1
60	0	100	1

s0135 4.2 Hybridization with materials

p1925 The development of new processes for a sustainable chemistry lies at the heart of the great challenges for tomorrow. In heterogeneous catalysis or supported catalysis, major breakthroughs are expected in terms of functionalization and surface modification as well as in the development of nanocatalysts. Along with these developments, immobilization of enzymes on solid supports for various applications (biosensors, medical fields, biocatalyst carriers, biofuel cells, etc.) has gained a spectacular growing interest as judged by the number of reviews published in this field (Bernal, Rodríguez, & Martínez, 2018; Bezerra, de Farias Lemos, de Sousa, & Gonçalves, 2015; Górecka & Jastrzębska, 2011; Hartmann & Dirk Jung, 2010; Milton & Minteer, 2017; Singh, Tiwari, Singh, & Lee, 2013; Zhou & Hartmann, 2013). As for synthetic catalysts, many advantages arise from immobilization, such as improved stability, reusability, and localization. Additionally, the architecture, the chemical nature, and mechanical properties of the solid support can be tuned to improve enzyme efficiency. Indeed, there is growing interest in achieving a comprehensive view of processes that control all mechanistic aspects related to stability, efficiency, and selectivity of reactions catalyzed by these systems that are often much less controlled than in homogeneous catalysis. Molecular biology has also facilitated strategies of enzyme immobilization (Bernal et al., 2018), techniques that include entrapment, encapsulation, physical adsorption, ionic binding, affinity and, most recently, covalent attachment. Covalent binding of an enzyme to appropriately functionalized surfaces can prevent leaching. The ultimate aim is to understand fundamental synthesis and processing concepts in order to tailor materials fully controlling all involved mechanistic aspects.

p1930 We aim at developing such materials. Enzyme electrodes for example represent promising electrocatalytic biomaterials for bioenergy conversion. By combining methodologies described above (i.e., large production of a site-directed variant of laccase followed by an oriented postfunctionalization of the enzyme surface), we have obtained a homogeneous population of pyrene-functionalized laccase π -stacked at a MWCNT material. The resulting biocathode is robust and operates at high catalytic current densities (up to 3 mA cm^{-2}). Compared to our previous attempt based on His-tagged laccases (Balland et al., 2008), the precise control of the orientation of our fungal laccase during its immobilization on an electroactive surface has helped to maximize the rate of direct electron transfer between the electrode

and the enzyme (Lalaoui et al., 2016). We are also studying the effect of the orientation of the enzyme grafted at the surface of superparamagnetic particles on particles activity (Lu Ren, unpublished data).

s0140

5. Conclusions

p1935

Fungal laccases are known to be particularly robust catalysts. A significant number of reports have focused on the application of this eco-friendly enzyme in many applications. Improving the productivity of heterologous systems is a challenging task from fundamental to practical points of views. In this chapter, an overview of the large-scale production of laccase variants, their purification, and their use in different applications has been given. Exploring the plasticity of laccase through mutagenesis and expression in the yeast *S. cerevisiae*, it is efficient to use the GRAS organism *A. niger* for mass production of chosen variants. Precisely controlled chemical postfunctionalizations of the enzyme surface are leading to new biohybrid catalysts or materials with unprecedented performances regarding laccase function.

st0160 Acknowledgment

p1940

We thank the Plateforme Analyse et Valorisation de la Biodiversité (AVB-AMU) for help in producing the recombinant enzyme. This study was supported by grants from the Agence Nationale de la Recherche (ANR-09-BLANC-0176 and ANR-15-CE07-0021-01).

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Non-Print Items

Keywords: Laccase heterologous expression, *Aspergillus niger*, *Saccharomyces cerevisiae*, Homeologous recombination hybrid catalyst, Directed mutagenesis, Hybrid catalyst, Surface modification

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