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## NOTE

Gram-scale production of a basidiomycetous laccase in *Aspergillus niger*Yasmina Mekmouche,<sup>1,\*</sup> Simeng Zhou,<sup>1,2</sup> Angela M. Cusano,<sup>1,§</sup> Eric Record,<sup>2</sup> Anne Lomascolo,<sup>2</sup> Viviane Robert,<sup>1</sup> A. Jalila Simaan,<sup>1</sup> Pierre Rousselot-Pailley,<sup>1</sup> Sana Ullah,<sup>1</sup> Florence Chaspoul,<sup>3</sup> and Thierry Tron<sup>1</sup>Aix Marseille Université, CNRS, iSm2 UMR 7313, 13397 Marseille, France,<sup>1</sup> Aix-Marseille Université, INRA, UMR 1163, Biotechnologie des Champignons Filamenteux, Faculté des Sciences de Luminy-Polytech, Case 925, 13009 Marseille, France,<sup>2</sup> and Aix-Marseille Université, CNRS, IMBE, UMR 7263, Laboratoire Chimie Physique et Prévention des Risques et Nuisances Technologiques, FR3098 ECCOREV, Faculté de Pharmacie, F-13005 Marseille, France<sup>3</sup>**We report on the expression in *Aspergillus niger* of a laccase gene we used to produce variants in *Saccharomyces cerevisiae*. Grams of recombinant enzyme can be easily obtained. This highlights the potential of combining this generic laccase sequence to the yeast and fungal expression systems for large-scale productions of variants.****[Key words:** Multicopper enzyme; Bioreactor; *Aspergillus niger*; *Saccharomyces cerevisiae*; Glycosylation]

Achievements in biocatalyzed reactions require robust methods of production for enzymes coming from natural biodiversity and/or for evolved enzymes. The multicopper oxidase laccase (*p*-diphenol: O<sub>2</sub> oxidoreductase, EC 1.10.3.2) is a popular biocatalyst for potential technological and bioremediation processes in addition to its use in organic synthesis (1). So far, laccases from plant, bacteria, ascomycetes and basidiomycetous fungi have been expressed in bacteria, yeasts, filamentous ascomycetes, basidiomycetes or plant with various success reaching occasionally expression levels close to a gram per liter in complex cellular contexts (2,3). Improvement in the production of single laccase variants in a given host may be obtained through variations of cultivation conditions, induction or mutagenesis (mainly through directed evolution) (4,5). For practical reasons, prior to any rationalization, it may appear crucial first to select a generic laccase encoding sequence that is generously expressed in heterologous expression hosts. Having previously used the yeast *Saccharomyces cerevisiae* to produce LAC3 (LAC3<sup>Sc</sup>) and several variants with yields in the mg/L range (6–8), we report here on the production of grams of LAC3 (LAC3<sup>An</sup>) in the industrially relevant generally recognized as safe (GRAS) ascomycete *Aspergillus niger*.

We successfully integrated the *lac3* cDNA to the genome of *A. niger* [strain D15#26 (*pyrg*<sup>-</sup>) (*prtI*) (*phmA*) (9)] and selected the LAC3 highest producing transformants for their ability to oxidize ABTS (volumetric activity). We followed a construction strategy and selection procedures identical to those previously published (10). Prior to any adjustments, the laccase titer obtained in flask culture was multiplied by 10 comparing *S. cerevisiae* to *A. niger* recombinants

(data not shown). In *A. niger* it is known that both the pH regulation and the type of agitation of automated bioreactor systems have a marked influence on heterologous expression. So, scouting for fermentation parameters was conducted on 0.5 L cultures with a BIOSTAT-Q bioreactor. Initial fermentation conditions (20 g/L glucose, pH 5.5, aeration of 0.6 VVM (volume of air/volume of culture medium/min), 2 × 10<sup>6</sup> spore/mL inoculum) allowed us to reach a maximum of 6000 U/L (~120 mg/L, using syringaldazine as substrate) laccase in 7 days before the enzyme started to degrade. From this initial fermentation we varied individually four cultivation parameters susceptible to influence the laccase production and established step-by-step the conditions for a routine fermentation (Fig. S1). Briefly, varying the glucose concentration from 20 g/L to 50 g/L laccase activity increased from 18,000 U/L to 24,000 U/L; doubling, then tripling the aeration rate laccase activity increase substantially from 18,000 U/L to 42,000 U/L; on the other hand, either lowering the temperature from 28°C to 26°C or reducing the inoculum size by 2 orders of magnitude led to a significant decrease of laccase activity. Adjusting these four parameters resulted in a substantial increase in LAC3 titer to a 42,000 U/L corresponding to 840 mg/L of recombinant enzyme produced in a 0.5 L fermentor. In the subsequent up-scaled fermentation (Fig. 1A), owing to the fact that LAC3 is the major secretion product (Fig. 1B), a simple concentration and one chromatographic purification step (anionic exchanger, 50% final yield) allowed us to recover 2.3 g of LAC3 with a specific activity of 50 U/mg and a satisfactory level of homogeneity as controlled by UV/VIS spectroscopy ( $A_{280}/A_{610} = 15$ ) and denaturing gel electrophoresis (SDS-PAGE, Fig. 2A).

All together, this represents a substantial 200-fold increase in laccase production as compared to our previous heterologous production in *S. cerevisiae* (6,8). The yield reported here is comparable to that reported for an ascomycetous laccase produced in

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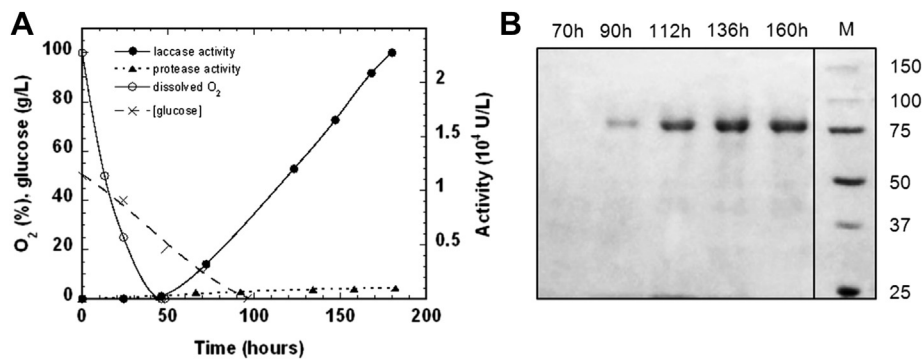


FIG. 1. Time course of laccase production during up-scaled fermentation. (A) Evolution of glucose (g/L), dissolved dioxygen (%), proteases and laccase (U/L) parameters in the 10 L tank. Data obtained in a Bioflo3000 10 L bioreactor with 50 g/L glucose, pH 5.5, aeration of 1.2 VVM,  $2 \times 10^6$  spore/mL inoculum. (B) SDS-PAGE (7.5%) stained with Coomassie blue; 10  $\mu$ L of culture supernatant were loaded per well as a function of cultivation time (from 0 h to 184 h). h: hours; M: molecular weight markers in kDa. Extracellular protease activity was assessed by quantification of free tyrosine with phenol Folin with a protocol adapted from Ref. (11) to microtiter plate scales. Glucose concentration was determined by the phenol sulphuric acid method (12).

the ascomycete *Trichoderma reesei* (2) and is routinely 3–4 times higher than that reported for basidiomycetous enzymes (13,14) expressed in the ascomycete *Aspergillus oryzae*. It is also noteworthy that, strictly using here the same *A. niger* strain, the same vectors and the same basic medium, our laccase production is 10 times higher than that previously achieved expressing a *Pycnoporus cinnabarinus* gene (14). Beyond differences in fermentation strategies, this last case highlights the influence on the final yield of the nature of the primary sequence to express. This is of particular importance for the production of variants where the choice of the primary sequence to evolve might be determinant to the final yield of expensive enzymes.

The native LAC3 has never been purified from the fungus *Trametes* sp. C30 but produced as a recombinant enzyme in *S. cerevisiae* (7) to which the form purified in *A. niger* was compared. The electrophoretic mobility of LAC3<sup>An</sup> is substantially different from that of the same enzyme previously produced in *S. cerevisiae* as judged on gels ran either under denaturing or native conditions (Fig. 2A and B). Fungal laccases being glycoproteins, the discrepancy between the apparent molecular weight of LAC3<sup>An</sup> (84 kDa) and that of LAC3<sup>Sc</sup> (95 kDa) is linked to the specificity of the glycosylation machineries of the respective hosts as supported by a shift towards the same apparent molecular weight ( $\approx 60$  kDa) observed after an extended treatment of the two forms with an endoglycosidase (Fig. 2C and D).

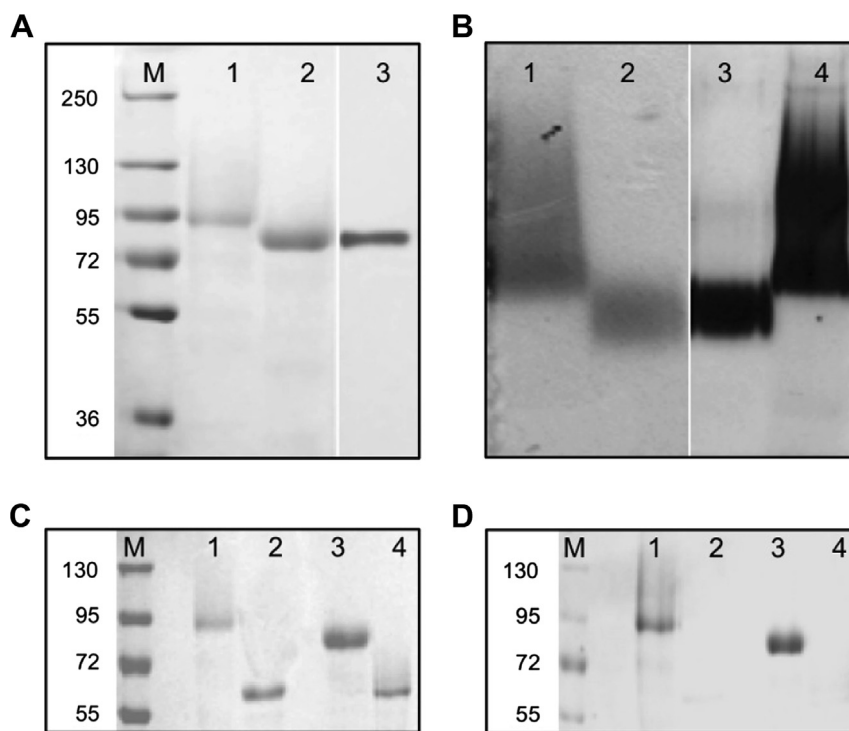


FIG. 2. Comparison of the electrophoretic mobility of the purified LAC3<sup>An</sup> and LAC3<sup>Sc</sup>. (A) SDS-PAGE lane 1: 5  $\mu$ g of LAC3<sup>Sc</sup> stained with Coomassie blue; lane 2: 5  $\mu$ g of LAC3<sup>An</sup> stained with Coomassie blue; lane 3: 0.2 U of LAC3<sup>An</sup> (western blot, standard conditions with an anti-LAC3 rabbit polyclonal antibody). (B) Native-PAGE (4  $\mu$ g/well); lanes 1 and 4: LAC3<sup>Sc</sup>; lanes 2 and 3: LAC3<sup>An</sup>; lanes 1 and 2: activity staining (*p*-phenylene diamine); lanes 3 and 4: silver staining. (C) SDS-PAGE, Coomassie blue staining; lane 1: LAC3<sup>Sc</sup>; lane 2: endoglycosidase treated LAC3<sup>Sc</sup>; lane 3: LAC3<sup>An</sup>; lane 4: endoglycosidase treated LAC3<sup>An</sup>. (D) Idem to C but stained with fuchsin acid. M: molecular weight markers in kDa. Protein deglycosylation was performed by incubating pure laccase (0.5  $\mu$ g/ $\mu$ L) denatured 10 min at 100°C, 500 U/ $\mu$ L of endoglycosidase H (New England Biolabs Inc.) in 0.5 M sodium citrate pH 5.5 for 45 h at 37°C. (Auteur de correspondance), Zhou, S., Cusano, A. M., Record, E., Lomascolo, A., Robert V., Simaan, A. J., Rousselot-Pailley, P., Ullah, S., Chaspoul, F., Tron, T. (2014).

**TABLE 1.** Apparent kinetic parameters for LAC3<sup>An</sup> and LAC3<sup>Sc</sup> in acetate buffer (100 mM, pH 5.7) at 30°C.

Apparent kinetic parameters <sup>a</sup>	ABTS ( $\epsilon_{420 \text{ nm}} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$ )		Syringaldazine ( $\epsilon_{525 \text{ nm}} = 65000 \text{ M}^{-1} \text{ cm}^{-1}$ )		Guaiacol ( $\epsilon_{465 \text{ nm}} = 12100 \text{ M}^{-1} \text{ cm}^{-1}$ )	
	LAC3 <sup>An</sup>	LAC3 <sup>Sc</sup>	LAC3 <sup>An</sup>	LAC3 <sup>Sc</sup>	LAC3 <sup>An</sup>	LAC3 <sup>Sc</sup>
$k_{\text{cat}}$ (min <sup>-1</sup> )	8378 ± 126	13955 ± 300	4756 ± 157	11038 ± 271	3087 ± 79	5278 ± 511
$K_M$ (μM)	734 ± 54	875 ± 87	16.2 ± 1.5	22.1 ± 1.2	4743 ± 375	4457 ± 272
$k_{\text{cat}}/K_M$ (min <sup>-1</sup> μM <sup>-1</sup> )	11.4	16.0	282.4	499.5	0.65	1.2

<sup>a</sup> Apparent  $K_M$  and  $k_{\text{cat}}$  values were obtained from the initial rate ( $v$ ) enzyme concentration ( $E$ ) and substrate concentration ( $S$ ) according to Michaelis–Menten equation [ $v = k_{\text{cat}} \times E \times S / (K_M + S)$ ] (non-linear regression fitting using Prism program, Graphpad, San Diego, CA, USA). Laccase concentration was determined by the Bradford colorimetric assay using a lyophilized sample of pure laccase as the standard (1–50 μg/mL) (15).

Likewise, host specificities in post-translational processes probably account for the differences in catalytic efficiency observed for the enzyme purified from the two hosts (i.e., LAC3<sup>An</sup> is globally 55–70% less efficient than LAC3<sup>Sc</sup>, see Table 1).

LAC3<sup>An</sup> pH tolerance (Fig. S2), copper ions content (4 atoms of copper), spectroscopic signatures and overall structure are features markedly close or even indistinguishable to those of LAC3<sup>Sc</sup> (Table S1). The general understanding on glycosylation fit a general non-specific effect on protein properties such as stabilization of conformation, protection against proteases and secretion of proteins (16). Accordingly, effect of glycans is likely related to local variations in the structure of the protein rather than to a global change in the enzyme structure (17). Therefore, despite the specificity of their respective glycosylation machineries, the *S. cerevisiae* and the *A. niger* expression systems can be profitably used in combination for protein engineering as shown recently with the expression of an evolved laccase mutant in *A. niger* (18). Moreover, the amount of laccase produced in *A. niger* opens the way for a valorization of laccases properties in the construction of artificial metalloenzymes (7,19,20).

In conclusion, we have described the heterologous expression of a basidiomycetous laccase gene in a GRAS organism which secretes the recombinant enzyme at a level close to the g/L. Simple adjustments of fermentation operations in a bioreactor allow the production of grams of the recombinant laccase. As the recombinant enzyme is the main secretion product of the host its subsequent purification is largely facilitated. This enzyme is representative of the laccase family. The form produced in *A. niger* is functionally similar to the one previously produced in *S. cerevisiae*. Perfectly amenable to synthetic evolution, secreted at substantial levels in industrially relevant organisms, this multicopper oxidase is a potential generic for the development of low cost means of production of tailored biocatalysts.

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jbiosc.2013.06.013>.

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