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Role of a thermostable laccase produced by *Streptomyces ipomoeae* in the degradation of wheat straw lignin in solid state fermentation

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ABSTRACT

Streptomycetes are actually considered one of the main groups of ligninolytic microoganisms producing a lignin-carbohydate complex named APPL (acid precipitable polymeric lignin) when growing on lignocellulosic materials. Although in these conditions the production of hemicellulolytic and oxidative extracellular enzymes were reported the specific role of laccases in lignin degradation is poorly understood. SilA, a thermostable salt-resistant and pH-versatile laccase produced by *Streptomyces ipomoeae* CECT 3341 was recently discovered and their particular characteristics make attractive to deep in its knowledge for biotechnological and environmental purposes. Pyrolysis/GC–MS was used to analyse the behaviour of the laccase-producing strain (SilA strain) and a laccase-negative mutant (SilA$^-$ strain) when growing on wheat straw in solid-state fermentation (SSF). Quantitative yields of APPL and the relative abundance of lignin-derived compounds were much higher for SilA strain than for SilA$^-$ showing a higher solubilizing activity of *S. ipomoeae* wild-type on lignocellulosic residues. Nonetheless the patterns of lignin derived compounds found in the APPL pyrograms were similar for both strains and distinct from the control showing a shortening of lignin propyl side-chains. Our results demonstrate that SilA laccase is a key enzyme in the lignin solubilization by *S. ipomoeae* and also points to the involvement of other oxidative enzymatic activities distinct to laccase in this process.

1. Introduction

Biomass is regarded as a promising alternative to fossil fuels due to its wide distribution and renewability. Among grass lignocellulose residues wheat straw represents one of the most abundant worldwide and its potential use for several biotechnological applications such as pulp and paper production [1–4] and for biofuels and bio products [5] has attracted significant attention in recent years.

The use of lignocellulosic residues or its derived compounds would bring about socio-economic and environmental benefits in our transition to a biobased economy. However, for an efficient use of these materials a previous steep of delignification is usually required to get access to the carbohydrates from the biomass [6].

Lignin -one of the main components of lignocellulosic biomass- can be totally or partially eliminated through the action of ligninolytic microorganisms or their oxidoreductive enzymes. In addition, lignin and its depolymerization derived compounds are raw material for the production of high value added products for the food and flavour industry and for fine chemicals and materials synthesis [7].

Lignin forms a three-dimensional network with an aromatic backbone composed of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units. These phenylpropanoid units are linked by C—C and aryl-ether linkages [8]. Due to its high complexity their degradation through eco-friendly enzymatic strategies represents a major challenge for biotechnologists.

Streptomycetes are a group of ligninolytic microorganisms contributing to plant biomass degradation and nutrient recycling in natural terrestrial environments. The solubilizing activity of these filamentous bacteria on lignocellulose residues produces a lignin-carbohydrate complex named APPL (acid-precipitable polymeric lignin) [9–11]. Several oxidative enzymes such as laccases

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[2,3,11,12] and peroxidases [13,14] have been detected during the growth of different streptomycete strains on lignocellulosic substrates. Those enzymes are known for their action on lignin degradation but their specific role in lignin degradation has yet to be demonstrated for streptomycetes. In fact, although some studies have analysed the effect of streptomycetes on whole lignocellulosic substrates, the study of lignin degradation by ligninolytic enzymes has mainly been carried out using model lignin compounds, industrial lignins or lignin derivatives [15–19], making it difficult to elucidate the role of specific oxidative enzymes such as laccases on native lignin.

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are a group of enzymes widely distributed in nature,being described in fungi, plants, insects and more recently in bacteria [20,21]. These enzymes are well-known biocatalysts able to oxidize various substituted phenolic compounds using molecular oxygen as the electron acceptor. Small laccases characterised by the presence of two cupredoxine domains were recently described in streptomycetes [22–26]. Their particular resistance to harsh conditions including high temperatures and wide pH range make them very attractive for biotechnological purposes.

The efficiency of delignification pre-treatments are known to depends on the type of organism, nature of substrate and also on cultivation conditions. Solid-State Fermentation (SSF) culture is an optimal solution for cultivating fungi and filamentous bacteria [27]. The advantages of SSF have been discussed in detail by Tian et al. [6]. Using a SSF system we are able to ferment more substrate per volume unit while favouring the fixation of enzymes and oxygen diffusion in the substrate which ultimately leads to a more efficient lignin depolymerization.

Analytical pyrolysis combined with gas chromatography–mass spectrometry is often used to characterize the main structural components of lignocellulosic materials. The suitability of this analytical technique to detect chemical modifications produced by the bio degradative activity of streptomycetes on woody and grass residues has already been demonstrated [11,28,29].

The purpose of this present study was to analyse by Py/GC–MS the composition of APPL extracted with water from wheat straw SSF by the wild-type laccase-producer strain *Streptomyces ipomoeae* CECT 3341 (SilA) and by a laccase-negative mutant (SilA$^-$) compared to that obtained from uninoculated control. Comparative analysis of the main components of the APPL produced by both strains on the lignocellulosic substrate will elucidate the importance of SilA laccase in lignin solubilization of wheat straw.

2. Material and methods

2.1. Bacterial strains, mutant obtention and growth conditions

For this study the actinobacteria *Streptomyces ipomoeae* CECT 3341, capable of producing the laccase SilA previously characterised [24] and a laccase-negative mutant from this strain (SilA$^-$) were used. To obtain the mutant strain, mutagenesis of the gene encoding the *S. ipmoeae* laccase was performed by gene disruption according to the method previously reported [30]. A 500 bp fragment was amplified from the gen SilA with the primers Mut58-5′ (GAATTCCTGATCGAGCTGAACGAGGGC) and Mut58-3′ (GAATTCGGTGATCTTGTTGTCGATTAC). The amplified product (Sipo500) was cloned into the plasmid pOJ260, conferring resistance to apramycin and subsequently an electrocompetent strain from *E. coli* ET12567 (pUB307) was electroporated with the construction pOJ260-Sipo500. An electroporated colony was conjugated with spores of *Streptomyces ipomoeae* and the selection of apramycin resistant spores (SilA$^-$) was performed. For experiments, spores were harvested from Mannitol-Soy flour agar (MS) plates (mannitol, 20 g L^{-1}; soy flour, 20 g L^{-1}; agar–agar 20 g L^{-1}) with distilled water containing Tween 80 (0.01% v/v). The suspensions were kept at −20 °C in 20% (wt/vol) glycerol. A standard spore suspension (10^7 cfu mL^{-1}) was used as initial inocula in all assays.

2.2. Substrate preparation and solid-state fermentation conditions

Wheat straw (*Triticum aestivum* var. maestro) was ground in a Janke and Kunkel A-10 mill to pass through a 40-mesh screen and air-dried for 24 h at 50 °C. To facilitate the colonization of the substrate unplugged 2 L flasks containing 10 g wheat straw were steamed for 1 h. The flasks were plugged with cotton stoppers and autoclaved for 20 min at 120 °C. Pre-inocula of wild and mutant strains were obtained by growing 2 mL of standardized spore suspensions (10^7 cfu mL^{-1}) in 1 L flasks containing 200 mL of Mineral Basal Medium (MBM) supplemented with 0.6% (w/v) yeast extract and 1 M sucrose [2]. All cultures were inoculated under sterile condition in a laminar flow-cabinet (Telestar SMH-100). For growing the mutant strain 25 μg mL^{-1} apramicin was also added. The cultures were incubated at 28 °C for 36 h with shaking (200 r.p.m.) and the mycelia harvested by centrifugation (10000*g*, 15 min). To facilitate the colonization of wheat straw, inocula were prepared by resuspending the mycelia in 50 mL MBM medium supplemented with 3.5% (w/v) NaCl and 0.1% (w/v) yeast extract. In the case of SilA$^-$ strain an apramicin solution (25 μg mL^{-1}) was also added. Cultures were incubated statically for 7 d. Uninoculated controls were incubated at the same conditions.

2.3. Production of acid precipitable polymeric lignin (APPL)

APPL was extracted from solid-substrate fermentatin cultures of the wild-type (SilA) and the laccase-negative mutant (SilA$^-$) strains after 7 d growth using distilled water. Samples were then steamed at 100 °C for 1 h, filtered through Whatman no. 54 filter paper and washed with distilled water at 60 °C. Supernatants were acidified with HC1 (12 M) to pH 1–2 and the APPL harvested by centrifugation (12000*g*, 10 min). Finally, the APPL was washed twice with distilled water (pH 5) and freeze-dried. The yield of APPL was expressed as the percentage of the freeze-dried APPL per g wheat straw.

2.4. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

APPL (1 mg) extracted from control and fermented wheat straw by wild type (SilA) and laccase-negative mutant (SilA$^-$) strains were analysed by Py-GC/MS as previously described [31] using a double-shot pyrolyzer (Model 2020, Frontier Laboratories) directly connected to a GC–MS system (Agilent 6890) equipped with a fused silica capillary column HP 5MS (30 m × 250 μm × 0.25 μm inner diameter). The detector consisted of an Agilent 5973 mass selective detector (EI at 70 eV). Pyrolysis was performed at 350 °C for 1 min. The final temperature was achieved at a rate of increase of 20 °C min^{-1}. The GC–MS conditions were as follows: oven temperature was held at 50 °C for 1 min and then increased up to 100 °C at 30 °C min^{-1}, from 100 to 300 °C at 10 °C min^{-1} and isothermal at 300 °C for 10 min using a heating rate of 20 °C min^{-1} in the scan modus. The carrier gas used was helium with a controlled flow of 1 mL min^{-1}.

Pyrolysis products were identified on the basis of the Wiley and Nist computer libraries and on the retention times and spectra reported in the literature for lignocellulosic material. For comparative purposes the pyrograms were normalized to the