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The hydrophobicity of the support in solid state culture affected the production of hydrophobins from *Lecanicillium lecanii*

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ABSTRACT

Lecanicillium lecanii has been successfully employed to produce hydrophobin-like proteins (HfbL) in solid state and submerged cultures varying the type of solid inert support. This study shows the results on the effect of hydrophilic Perlite and hydrophobic Polyurethane inert supports in solid state cultures for production of HfbLs by *L. lecanii.* The hydrophobicity of the support employed in solid state cultures influenced the yields and surface activities of the class I and class II HfbLs produced by *L. lecanii*. Class I HfbL was only produced using the hydrophobic polyurethane foam support, showing high surface activity that reduced ca. 50% hydrophobicity of Teflon, whereas class II HfbLs were produced on both polyurethane foam and perlite supports, and they reduced ca. 50% the surface tension of water and ca. 25% reduction of the hydrophobicity of Teflon.

1. Introduction

Hydrophobins (Hfbs) are amphipathic proteins produced by fungi with eight conserved cysteine residues forming disulfide bonds [1]. These proteins are classified according to their biophysical and hydropathic properties; Class I Hfbs are soluble in formic acid and self-assemble like amyloid proteins forming monolayers, named rodlets during their interaction at hydrophilic and hydrophobic interfaces. The rodlets are highly stable layers which are dissolved in trifluroacetic acid (TFA) and they have been observed at surfaces of aerial structures such as conidia, conidiophores and fruiting bodies [2]. Class II Hfbs are soluble in sodium dodecyl sulfate (SDS) or ethanol (60%) solutions and present coat aerial structures as well as yeast-like cells but they assemble as flexible layers without rodlets [3]. The amphipathic properties and self-assemble of Hfb are related to their biological roles in fungal development by enabling the hyphae to migrate from submerged condition to air. In addition, Hfbs have been involved in pathogenic activity acting as toxins and allowing attachment of fungal structures, such as conidia or hyphae, to the host surface [4,5]. Hfb layers also cover fungal aerial structures, which confer hydrophobicity, wetting resistance thus facilitating their dispersal in air, hyphae protection against desiccation and gas exchange [1,6]. The Hfb were also reported as mediators of adhesion on hydrophobic surfaces [7,8]. The regulation mechanism for production of Hfbs is complex and differentially expressed in entomopathogenic fungi. Sevim and co-workers reported three genes of Hfb of *Metarhizium brunneum* which presented several roles in conidiation, pigmentation, hydrophobicity and virulence [5]. In *Beauveria bassiana*, Hfbs are related to the developmental stage, their attachment to surfaces and the virulence of the fungi [4]. In phytopathogenic fungi such as *Verticillium dahliae*, the VDH1 gene is involved in microsclerotial development and sporulation [9]. Other examples are the Hfbs class II from *Trichoderma reesei*, which are responsible of sporulation of the fungi in solid cultures [7]. In another work, the culture conditions affected Hfbs regulation with *M. brunneum*, which expressed classes I and II Hfbs in mycelia growth in solid cultures with added glucose and during the infection of *Spodoptera exigua larvae*, but were not expressed in liquid culture with the addition of the same carbon source [5]. Solid

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state culture (SSC) of fungi mimic living conditions in terrestrial habitats on wet solid substrates. Thus SSC has been proposed as the cultivation technique for filamentous fungi when morphological and metabolic differentiation into substrate-penetrating, aerial hyphae and production of conidiospores is required [10]. Conidia produced by SSC displayed more stable, drying resistance with higher germination rates and more hydrophobic conidia than that from submerged cultures (SmC). The explanation of higher hydrophobicity of conidiospores might be ascribed to the presence of Hfb-like proteins [11], which were also detected only with aerial conidia of entomopathogenic fungus *Beauveria bassiana* but not in blastospores and submerged conidia [8]. Another entomopathogenic fungus used as a biopesticide is *L. lecanii*, which is able to degrade n-hexane or toluene in SmC, producing Hfbs-like proteins with surface activity at the presence of these hydrophobic solvents [12]. The reported comparison between the types of culture using this fungus displayed higher production of Hfbs class I in SSC with added chitin or fructose as carbon source than in SmC with these substrates. Interestingly, the SSC-mediated Hfb reduced ca. 50% the hydrophobicity of Teflon unlike those obtained from SSC with added fructose, which showed no surface activity [13]. However, despite these reports, there are no reported studies on the use of inert supports in the production of these proteins. In the search for adequate supports, Perlite (P) and Polyurethane foam (PUF) attracted our attention for they have been employed as packed beds in SSC. The former is a silicaceous material of volcanic origin while the latter is a synthetic polymer based on isocyanate polymers, which consist of polar urethane groups and soft non-polar segments. Both materials are considered as inert and did not contribute nutritionally to fungal growth; other advantages are the extraction of clean products avoiding contamination from the support and the direct determination and reuse of immobilized biomass. P and PUF can hold several times their weight in water owing to their high porosity and they do not present cation exchange capacity [14–16]. This study is the first to report the use of PUF and P as solid porous matrices to support the growth of *L. lecanii* in SSC for Hfb production. The effect of the solid support on the type of Hfbs produced and their surface activities are described.

2. Materials and methods

2.1. Microorganism and culture conditions

Lecanicillium lecanii 2149 strain was provided by USDA-ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungi, USA) collection. *L. lecanii* was grown in SSC on two inert supports: (i) PUF with a particle size ca. 0.125 cm^3 within nutrient ratio of 1:15 (w v^{-1}); (ii) P with a particle diameter ca. 3.3 mm within nutrient ratio of 1:2 (w v^{-1}). Culture conditions were carried out according to Rocha-Pino et al. [13], the supports added with mineral medium pH 6, colloidal chitin (30 g L^{-1}) and inoculum of 5×10^7 spores g^{-1} of substrate were packed into glass columns and incubated at 25 °C. Aeration of 1.4 mL air min^{-1} per g of moist material was provided during 6 and 15 days. All the materials were sterilized at 121 °C for 15 min before inoculation.

Solids (biomass and matrices) and supernatant (soluble matter in water) were separated by mixing the solids with water (50 wt%), then pressurized to 1000 psi for PUF SSC, while the mixture was pulverized in a mortar for P SSC and centrifuged at 12,700 × *g* at 4 °C for 10 min. Supernatants from both supports were separated using Whatman filter paper No. 40. 0.2 g (wet weight) of support with biomass was mixed with 5 mL of phosphoric acid (0.15 M) and heated in a water bath for 7 min. After centrifugation (12,700 × *g*) the supernatant was used to determine the total protein from biomass [12]. Total soluble protein was determined from biomass and supernatant by Bradford [17].

2.2. Colloidal chitin preparation and characterization

Chitin was obtained from lactic acid fermentation of shrimp wastes and then purified by the following treatments: (i) chitin was washed with distilled water (Ch1); (ii) Ch1 was treated with HCl 0.5 N and NaOH 0.4 M (Ch2). Furthermore, Ch1 and Ch2 were treated with HCl 10 N and neutralized with water to obtain colloidal solutions. Ch1 and Ch2 were characterized on residual protein (%) by Kjeldahl (K-435 BÜCHI, Switzerland) [18]. Degree of acetylation (DA) was determined by proton nuclear magnetic resonance (1H NMR) spectroscopy in a Bruker Advance III 500 (Germany) at 200 MHz using DCl/D_2O and 3-(trimethylsilyl) propionic acid as internal reference [19].

2.3. HfbLs extraction

Samples of class I and class II HfbLs were obtained from the mycelia of *L. lecanii* after 6 days of SSC. HfbLs were extracted from the biomass and the supernatant of *L. lecanii* in SSC. Class II HfbLs were extracted from mycelia following the methodology reported by Askolin et al. [3]. Supports with mycelia were washed with distilled water and incubated with SDS 2% (w v^{-1}) in 100 mM Tris–HCl buffer at pH 9.0 for 2 h in cold water bath with stirring then, mycelia and support were separated by compressing and centrifugation for PUFSSC and PSSC, respectively. SDS extract was precipitated with KCl (2 M) and centrifuged (12,700 × *g*) at 6 °C for 20 min. Subsequently, supports were washed with water and Class I HfbLs were extracted with formic acid, followed by electrobubbling at 300 mA during 3 h. The obtained foam was centrifuged and the pellet solubilized with TFA, which was then evaporated with dry air stream [13]. Class I HfbLs from supernatant were extracted by the following procedure: protein from supernatant was precipitated with trichloroacetic acid (TCA) 5% (w v^{-1}) at 4 °C for 2 h and centrifuged (12,700 × *g*) at 4 °C for 20 min. Pellet was washed with acetone and solubilized with performic acid at 4 °C during 4 h, subsequently the acid was evaporated with air flow [20]. Protein precipitated (without addition of performic acid) was treated as class II HfbLs [20]. Protein was determined by Bradford for each step of the HfbLs extraction [17]. Protein yield with regard to biomass ($Y_{HfbL/biomass}$) was calculated considering protein concentration of each step of purification and the total protein of either biomass or supernatant. Classes I and II HfbLs fractions were analyzed by denaturing electrophoresis SDS-PAGE according to Laemmli [21] using resolving PAA gel in concentrations of 17 and 15% for class I and class II HfbLs, respectively. Gels were stained with coomassie blue or silver nitrate (Bio-Rad, USA) and analyzed with the image processing software (ImageJ 1.41o National Institutes of Health, USA).

2.4. Determination of surface activities of HfbLs by contact angle measurements

Surface activity of HfbLs at air-solid hydrophobic interface was measured by contact angle (θ) of a water drop (1 μL) on Teflon surface previously treated with a HfbL solution of 50 μg mL^{-1} [13]. Teflon treated with class I HfbL was washed with 2% SDS at 100 °C and then with deionized water, while class II HfbLs were washed only with deionized water at room temperature. In addition, class II HfbLs were subjected to a molecular weight cut off membrane of 30 kDa (Amicon Millipore, USA). Digital images of the water droplets were obtained in a horizontal light microscope Q×3 Intel with image processor (Intel Corporation, USA). Contact angles were measured in duplicate from randomly selected six areas per sample and images analyzed with ImageJ 1.41o software.