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- Paola Cicatiello[a],
- Alfredo Maria Gravagnuolo[a],
- Giorgio Gnavi[b],
- Giovanna Cristina Varese[b],
- Paola Giardina[a, ,]

[a] Department of Chemical Sciences, University of Naples Federico II, via Cintia 4, I-80126 Naples, Italy

[b] Department of Life Sciences and Systems Biology, University of Turin, viale P.A. Mattioli 25, I-10125 Turin, Italy

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Highlights

23 Marine fungi have been analyzed to identify hydrophobin producers.

6 New hydrophobins have been isolated.

4 Proteins belong to class I and 2 to class II hydrophobins.

One of the new class I hydrophobins stably changes the wettability of a crystalline silicon chip.
One of the new class II hydrophobin is endowed with remarkable emulsification capacity.

Abstract

Hydrophobins have been described as the most powerful surface-active proteins known. They are produced by filamentous fungi and exhibit a distinct amphiphilic structure determining their self-assembly at hydrophilic-hydrophobic interfaces and surfactant properties which have been demonstrated to be useful for several biotechnological applications. The marine environment represents a vast natural resource of new molecules produced by organisms growing in various stressful conditions. This study was focused on the screening of 100 marine fungi from *Mycoteca Universitatis Taurinensis* (MUT) for the identification of new hydrophobins. Four different methods were set up to extract hydrophobins of class I and II, from the mycelium or the culture broth of fungi. Six fungi were selected as the best producers of hydrophobins endowed with different characteristics. Their ability to form stable amphiphilic films and their emulsification capacity in the presence of olive oil was evaluated.

Graphical abstract

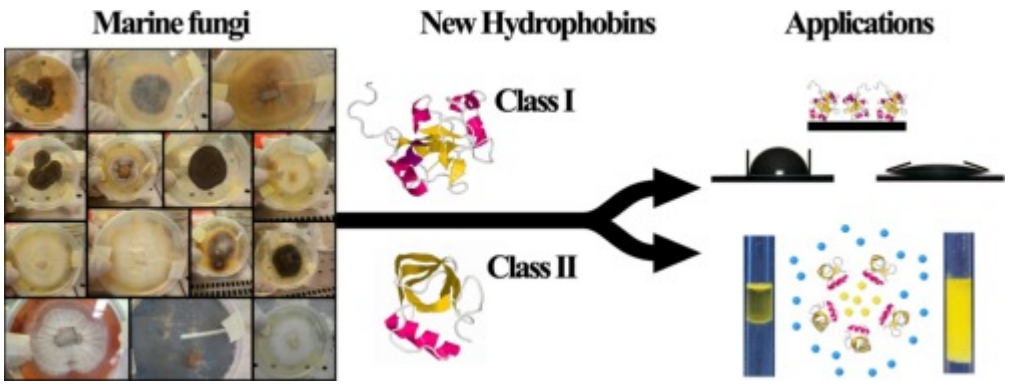

Figure options

Keywords

- Biosurfactants;
- Marine microorganisms;
- Self-assembly;
- Surface wettability;
- Emulsion;
- Protein coating

1. Introduction

Hydrophobins (HFBs), small proteins (about 100 amino acids) typical of filamentous fungi, have been described as the most powerful surface-active proteins known and their activity is intrinsic to the proteins themselves [1] and [2]. Indeed, on one side of their molecular surface, some exposed hydrophobic aliphatic side chains form a flat hydrophobic patch, whilst polar or charged residues are confined to the other side [3] and [4]. The hydrophobic patch could be involved in interaction with an identical protein partner that obscures the hydrophobic region. This allows miscibility with the bulk water phase until reaching an air–water interface or other non-polar surface, where they probably dissociate and re-orient with the hydrophobic surface exposed to the interface [5]. Several features of fungal development have been attributed to these proteins [6], e.g. coating of spores, aerial hyphae and fruiting bodies with a water-repellent and adhesive layer allowing the fungus to escape from the liquid medium and to live in adhesion on different surfaces [7]. HFBs show very little conservation of their sequence, apart from the pattern of eight Cys residues implicated in the formation of four disulfide bridges (Cys1–Cys6, Cys2–Cys5, Cys3–Cys4, Cys7– Cys8) [8]. They have been split in two groups, class I and class II, based on structural differences and properties of the aggregates they form [9]. Class I HFBs form highly insoluble aggregates that have the appearance of distinct rodlets and, similarly to amyloid fibrils, are characterized by cross β-structure [10]. These assemblies show outstanding stability and can be depolymerized in 100% trifluoroacetic acid (TFA) whereas class II HFBs form less stable polymers that are soluble in some organic solvents or SDS aqueous solution, and lack the rodlet appearance of class I HFBs [11]. Both types of HFBs have been used for several biotechnological applications, such as dispersion of hydrophobic materials, foam stabilization in food products, surface coating and modification of the

surface wettability, immobilization of enzymes, peptides, antibodies and nanomaterials on various surfaces [12], [13], [14] and [15].The marine environment host a huge biodiversity of (micro) organisms, and fungi make up a large part of them [16]. Marine fungi form an ecological, and not a taxonomic group. These species live in a stressful habitat, under cold, lightless, high-pressure conditions or other types of mechanical stress. Their capability to survive in different environmental conditions makes them attractive to isolate new molecules [17], [18] and [19]. The aim of the present study is to identify marine fungi as source of HFBs.

2. Material and methods

2.1. Marine fungi

Marine fungi were isolated and identified from the seagrass *Posidonia oceanica*, the green alga *Flabellia petiolata* and the brown alga *Padina pavonica* collected nearby Elba Island in the Mediterranean Sea as previously described [20], [21] and [22] and are preserved at the *Mycoteca Universitatis Taurinensis* (MUT).

2.2. Culture conditions

Each marine fungal strain was maintained through periodic transfer on agar plate at 20 °C. The strains were grown in multiwell plates (9.5 cm^2 cell growth area) in 3 different liquid media XNST30 (malt extract 3 g/L; yeast extract 3 g/L; NaCl 30 g/L; 10 g/L glucose and 5 g/L peptone), PDY15 (24 g/L potato dextrose; 5 g/L yeast extract and 15 g/L NaCl) and WM (10 g/L glucose; 2 g/L peptone; 1 g/L $(NH_4)_2SO_4$; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.875 g/L KH_2PO_4; 0.125 g/L K_2HPO_4; 0.1 g/L $CaCl_2 \cdot 2H_2O$; 0.1 g/L NaCl; 0.05 g/L $MnCl_2$; 0.001 g/L $FeSO_4$) at 2 different temperatures (20–28 °C) in triplicate for 10 days. The inoculum was carried out by addition of mycelium disks (5 mm diameter) taken from actively growing cultures on agar plates in 7.5 mL of medium or by adding 0.5 mL of a suspension in 0.9% NaCl, 0.1% Tween 80 of mycelium mechanically broken by glass beads. In order to extract sufficient amounts of HFBs, marine fungi were grown in 100 mL conical flasks containing 50 mL of the selected medium. Fungi were grown in the dark at 200 rpm for 10 days.

2.3. Extraction of class I and class II HFBs from the culture broth

Proteins were aggregated by air bubbling, using a Waring blender. Foam was collected and treated with 20% trichloroacetic acid (TCA) and was centrifuged after 12 h incubation at 4 °C in static. In order to extract class I HFB candidates, the obtained precipitate was treated by 100% TFA in a bath sonicator, dried using a stream of nitrogen and then dissolved in a 60% ethanol solution, while it was directly treated by 60% ethanol to dissolve class II HFBs.

2.4. Extraction of class I and class II HFBs from the mycelium

To extract Class II HFBs, mycelia were washed by water and proteins were extracted using 60% ethanol in a bath sonicator and, after centrifugation, the supernatant was collected.

To extract class I HFBs, mycelia were firstly washed by 2% SDS, several times by water and once by 60% ethanol to remove soluble proteins, contaminants and the detergent. The residue was freeze-dried, treated by TFA using a bath sonicator to extract the protein from the wall of the mycelium. After centrifugation of the TFA extract, the supernatant was dried using a stream of nitrogen, dissolved in 60% ethanol and centrifuged again. The new supernatant was lyophilized and proteins

were extracted at the interphase using a chloroform:methanol:water mixture 1:2:2 v/v in a bath sonicator. After centrifugation, the precipitate was freeze-dried, treated by TFA in a bath sonicator, dried in a stream of nitrogen and dissolved in 60% ethanol.

2.5. Analysis of the purified proteins

Protein concentrations were evaluated using the PIERCE 660 nm Protein Assay kit using bovine serum albumin as standard. The purity and the molecular weight of the extracted samples were evaluated by SDS-PAGE (15% acrylamide), and silver stained.

2.6. Water contact angle analysis of the functionalized surfaces

Contact angle measurements were performed on a KSV Instruments LTD CAM 200 Optical Contact Angle Meter coupled with drop shape analysis software. Each contact angle was calculated as the average of two drops of 5 μL, spotted on different points of the crystalline silicon chip.

2.7. Evaluation of emulsifying properties of HFBs

HFBs were dissolved in 50 mM phosphate buffer pH 7 at 0.05 mg ml^{-1}. Solutions were mixed by vortexing for 2 min with olive oil (25% v/v) and left standing for 24 h. To evaluate the emulsification capacity of the samples the height of the emulsion phase was monitored.

3. Results and discussion

3.1. Growth conditions of marine fungi

Twenty-three fungi were selected from a pool of 100 marine fungal strains on the base of the ability to produce foam during the growth in shaken cultures, thus indicating the production of biosurfactants. The list of these strains is reported in Table 1.

Table 1.

List of the screened fungal strains with their MUT code and the original substrate of isolation. The asterisks indicate conditions in which they grew. The grey boxes correspond to the best growth conditions in which proteins were extracted.

Table options

Subsequently, the growth conditions of the selected fungi in liquid medium were optimized in multiwell plates changing parameters such as the temperature, 20 and 28 °C, and the composition of the culture broths. Three cultural broths, commonly used for fungal growth, XNST30, PDY15 and WM, characterized by different C/N ratio and salt concentrations, were tested. The growths were performed at two temperatures, 20 and 28 °C, in triplicate for 10 days. Most of the strains grew in more than one condition, however the best combinations of parameters were selected on the basis of the abundance of the produced mycelia (Table 1). Three strains, MUT 4871, 4857 and 4860, only grew when the culture broths were inoculated by addition of mycelia mechanically broken by glass beads, not by addition of mycelium disks from agar plates. When results were comparable, the WM poor medium was preferentially selected to reduce the presence of contaminant molecules in the purified samples.

3.2. Extraction of class I and class II HFBs

In order to extract sufficient amounts of HFBs, marine fungi were grown in conical flasks for 10 days and separated from the culture broths by filtration. Four different extraction methods were set up to extract HFBs belonging to Class I and Class II from both the mycelium and the culture broth, were set up (Fig. 1). The capability of HFBs to migrate at the water/air interface was exploited to extract the secreted proteins by bubbling the culture broth, while the cell wall associated proteins were isolated from the dried mycelium by solvent extraction. The requirement of the TFA treatment to solubilize the Class I HFBs was exploited to discriminate between the two classes. Since *Coprinellus* sp. MUT 4897 belongs to the basidiomycetes which exclusively produce class I HFBs [9], the extraction methods for this class were only used in this case.

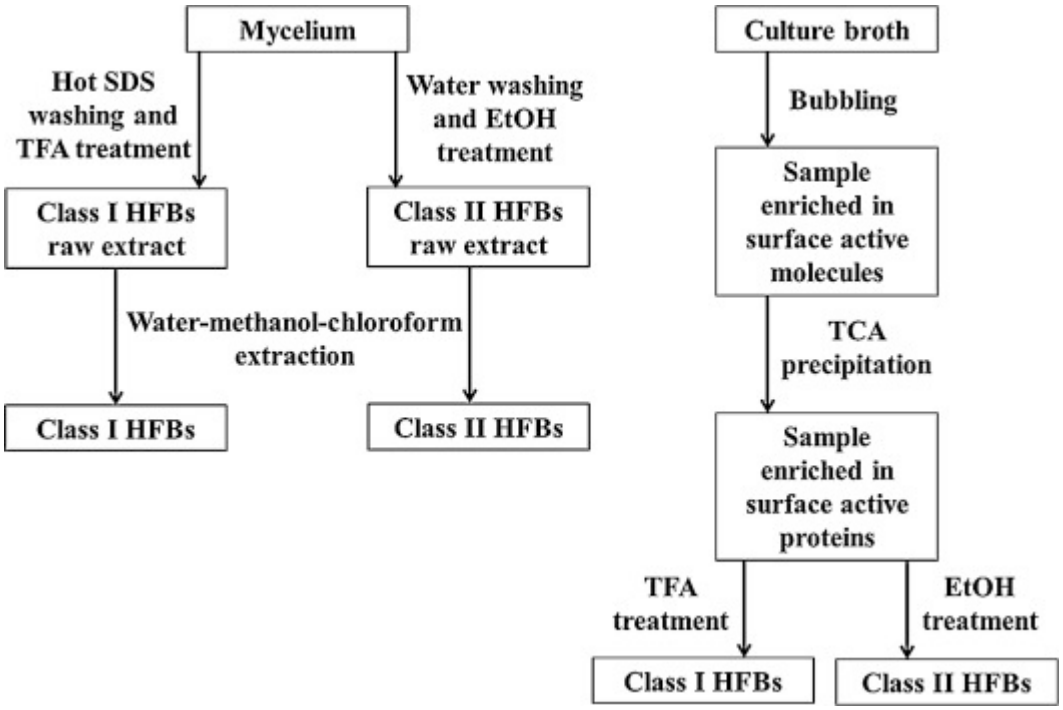

Fig. 1.

Scheme of the procedures set up to extract Class I or Class II HFB, associated to the mycelium wall or secreted in the culture broth.

Figure options

Adequate amounts of putative HFBs were extracted from 6 strains, and analyzed by SDS-PAGE (Fig. 2). The developed procedures allowed us to obtain quite homogeneous protein samples. The molecular weights of the extracted proteins ranged between 11 and 35 kDa, as observed in most of the known HFBs.

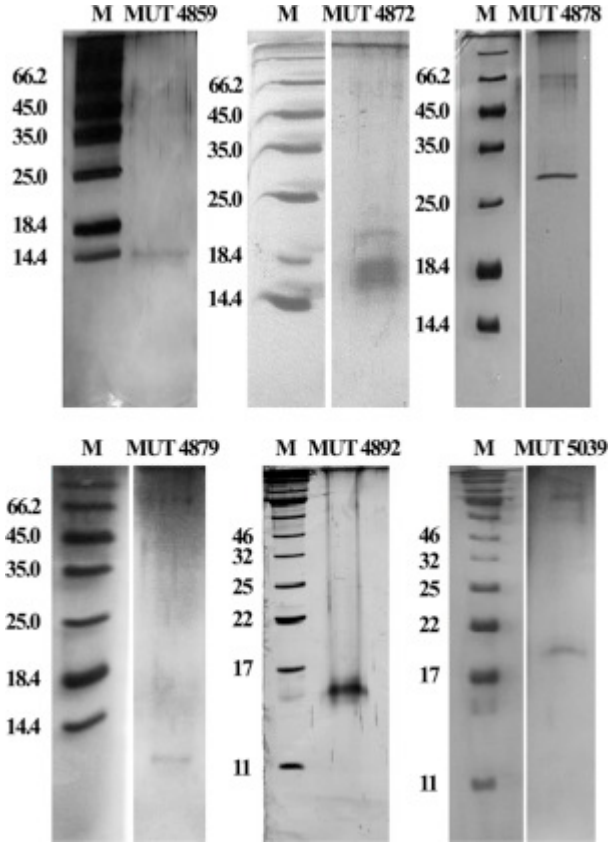

Fig. 2.

SDS-PAGE analysis of the extracted proteins. M is the molecular weight marker, the MUT code of the strain from which the protein was extracted, is reported in each panel.

Figure options

3.3. Analysis of the HFB functions

To test the ability of these putative HFBs to self-assemble into a stable amphiphilic layer and to functionalize a solid surface, the samples were deposited on crystalline silicon chips, dried at 60° C, and washed by a 60% ethanol solution or hot SDS at 2%. The change of the wettability was evaluated by the contact angle of a sessile water drop before and after the functionalization and upon the washing steps (Fig. 3). The 4 putative Class I HFBs formed a stable layer, resistant to 60% ethanol and hot SDS, confirming that they belong to that class. On the other hand the layers of the other two putative HFBs were removed after mild washing in 60% ethanol, confirming that they belong to the Class II HFBs. A list of the selected strains with the corresponding HFB class and production yield is shown in Table 2.

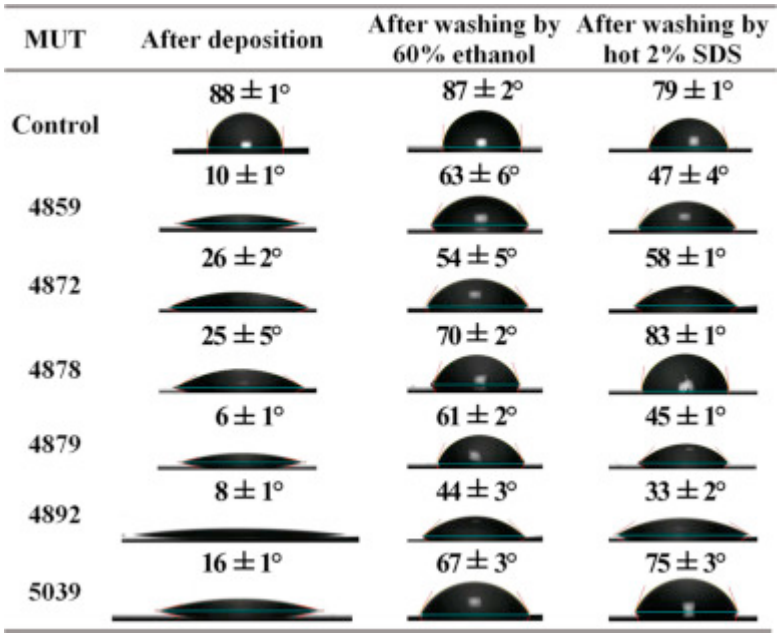

Fig. 3.

WCA analysis of crystalline silicon surface upon functionalization using the extracted HFBs and washings using different solutions.

Figure options

Table 2.

Strains selected as producers of new HFBs, classification and yield of the purified proteins.

MUT accession number and strain	Source	HFB class	Production yield, mg L^{-1}
4859 Roussoellaceae sp. 2	Culture broth	I	2
4872 Acremonium sclerotigenum	Culture broth	I	10
4878 Myceliophthora verrucosa	Culture broth	II	5
4879 Arthopyrenia salicis	Mycelium	II	5
4892 Penicillium roseopurpureum	Culture broth	I	3
5039 Penicillium chrysogenum	Mycelium and culture broth	II	12

Table options

Furthermore, the emulsification capacity of each of the extracted protein was tested in the presence of olive oil upon agitation by vortex. All the six putative HFBs were able to produce water/oil emulsions (Fig. 4). Their stability was followed during one month at room temperature showing that the putative Class II HFB from the *Penicillium chrysogenum* MUT 5039 is the most surface-active protein identified in this study.

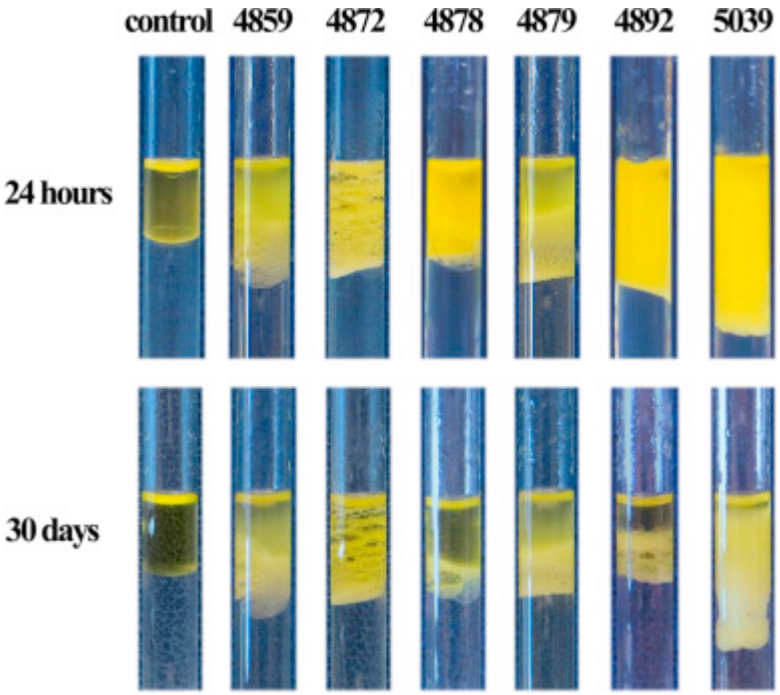

Fig. 4.

Aqueous emulsions of the extracted proteins (0.05 mg mL^{-1}) in the presence of 25% olive oil. Each solution was vortexed for 2 min, left standing and imaged after 24 h and 30 days.

Figure options

4. Conclusions

Twenty three marine fungi, out of 100, have been selected for their ability to produce foam in shaken culture. Afterwards four extraction methods have been set up to isolate secreted or cell wall associated HFBs of Class I or II, allowing the identification of 6 new putative HFBs which show promising properties for biotechnological applications. The purity and the Mw of the proteins have been assessed by SDS-PAGE. On the basis of the stability of the amphiphilic layer that they form on a crystalline silicon chip, it has been confirmed that 4 of them belong to the Class I and 2 proteins to the Class II of the HFB family. One of them was endowed with remarkable emulsification capacity tested on a mixture of water and olive oil.

The use of marine species could enable the large scale and sustainable production of HFBs exploiting the natural resource of widely available seawater for their cultivation.

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Corresponding author at: Department of Chemical Sciences, Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Naples, Italy.