CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PRODUCTION AND CHARACTERIZATION OF HYDROPHOBINS WITH POTENTIAL FOR ORAL MEDICINE APPLICATIONS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to achieve the Master of Science level in Applied Microbiology

by

Catarina Fardilha Fernandes

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RESUMO

Hidrofobinas são proteínas produzidas apenas por fungos (Ascomycetes e Basidiomycetes) que são capazes de se auto-organizar numa membrana anfipática, na interface, alterando assim a natureza das superfícies. Embora as hidrofobinas de diferentes espécies possuam baixa homologia de sequência, apresentam padrões de hidropatia aproximados, são ricos em cisteína e podem ser distinguidos em duas classes: classe I e classe II.

O objectivo geral deste estudo inclui a produção e caracterização de hidrofobinas com potencial uso na medicina oral.

Três estirpes, isolados fúngicos L7 e C1.1 da coleção do laboratório, e *Bjerkandera sp.* BOS55 (L3), também da coleção de laboratório, foram testados a partir da produção de hidrofobinas. A amplificação e sequenciação da zona ITS fúngica permitiram a identificação das espécies L7 e C1.1 isoladas, respectivamente, *Mucor circinelloides* e *Hypocrea lixii (Trichoderma harzianum).*

As hidrofobinas a partir das três estirpes, foram produzidas, extraídas e ainda caracterizadas.

Para a caracterização bioquímica, foi testado o fluido extracelular de todos os isolados para a estabilidade da espuma e o teste de espalhamento de óleo e de tensão superficial. Para todos os isolados a avaliação da hidrofobicidade de conídios foi realizada por suspensão do mesmos e a molhabilidade do micélio foi testada na superfície das hifas.

O fluido extracelular do isolado *Hypocrea lixii (Trichoderma harzianum)* obteve melhor desempenho do que os outros, e também apresentou o micélio mais impermeável, evidenciado no ensaio de molhabilidade da superfície. O *Mucor circinelloides* foi aquele que exibiu os conídios mais hidrofóbicos. Observações no SEM parecem indicar que todos os isolados possuem hidrofobinas de superfície classe I. Todas as misturas de hidrofobinas obtidas apresentaram alguma atividade anti-microbiana, bem como eficácia na inibição da formação de biofilme de *Candida albicans*. A caracterização da mistura de hidrofobinas concentradas por HPLC evidenciou dois picos com características hidrofóbicas os quais serão caracterizados em trabalhos futuros. Assim, foi possível isolar hidrofobinas com eficácia na proteção de

adesão e crescimento de microrganismos, demonstrando potencial de aplicação na saúde oral.

ABSTRACT

Hydrophobins are proteins produced only by fungi (Ascomycetes and Basidiomycetes) that are able to self-assemble into an amphipatic membrane at an interface thus changing the nature of surfaces. Although hydrophobins from different species have low sequence homology, they present approximate hydrophaty patterns, are rich in cysteine and can be distinguished in two classes, class I and class II.

The general objective of this research was the production and characterization of hydrophobins with potential use in oral medicine.

Three strains, fungal isolates L7 and C1.1 from the laboratory collection and *Bjerkandera sp.* BOS55 (L3) also from the laboratory collection were tested for hydrophobin production. Fungal ITS amplification and sequencing allowed the identification to species of L7 and C1.1 isolates, respectively, *Mucor circinelloides* and *Hypocrea lixii (Trichoderma harzianum).*

Hydrophobins from all three strains were produced, extracted and further characterized.

For biochemical characterization of all three isolates, the extracellular fluid of all isolates was tested for foam stability, oil spreading assay and surface tension. The assessment of conidial hydrophobicity was performed for conidial suspension and mycelium wettability was tested in surface hyphae.

Extracellular fluid of isolate Hypocrea lixii (Trichoderma harzianum) showed better performance than the others, and also presented the most impermeable mycelia, as observed by surface wettability assay. *Mucor circinelloides* presented the most hydrophobic conidia. SEM observations seem to indicate that all three isolates possess class I surface hydrophobins. All hydrophobin mixtures obtained presented anti-microbial, as well as efficient inhibition of biofilm formation by *Candida albicans*.

The characterization of concentrated hydrophobin mixtures by HPLC showed two peaks with hydrophobic characteristics, which will be characterized in future studies. So, within this work was possible to isolate hydrophobins with efficacy on the protection of biofilm formation and inhibition of microbial growth, demonstrating a potential for oral medicine applications.

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LIST OF ABBREVIATIONS

ATM	Atomic force microscopy
ATR FT-IR	Attenuated total reflectance Fourier-transform infrared spectroscopy
BSA	Bovine serum albumin
CD	Circular dichroism spectropolarimetry
CDB	Czapek-Dox broth
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FA	Formic acid
MALDI-TOF	Matrix-assisted laser desorption ionization – Time of flight-mass spectrometry
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance spectroscopy
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
SDS	Sodium Dodecyl sulfate
SDS-PAGE	Sodium Dodecy sulfate - Polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TFA	Trifluoroacetic acid
YE	Yeast extract

1. INTRODUCTION

1.1. Hydrophobins

In 1991 Wessels identified, for the first time, a group of proteins, called hydrophobins, which immediately raised great interest due to their ability to change the nature of surfaces (Lutterschmid et al., 2011). In his work, when studying genes highly expressed in filamentous fungi, he found these proteins associated with those genes (Linder, 2009; Sunde et al., 2008).

Hydrophobins are a group of small proteins (10-15 kDa) produced only by fungi (Ascomycetes and Basidiomycetes). Some evidence suggests that they are also found in zygomycetes, however is not clear if they are actually occurring in the chytridiomycetes (Wösten, 2000; Wösten, 2001; Scholtmeijer et al., 2001).

Hydrophobins can be secreted out to the surroundings or into the fungal structures (Linder, 2009; Sarlin et al., 2012) and are found in the surface of fruiting bodies and aerial hyphaes (Linder, 2009). These proteins are characterized by the capacity to self-assemble at hydrophobic-hydrophilic interfaces into an amphipathic membrane (Wösten & Vocht, 2000; Scholtmeijer & Wösten, 2001; Hektor & Scholtmeijer, 2005; Vigueras et al., 2008; Litlejoh et al., 2012). Although they show low sequence homology, hydrophobins have approximate hydrophaty patterns, solubility profiles and are rich in cysteine. These proteins possess eight conserved cysteine residues with conserved spacing that form four disulfide bridges (Sarlin et al., 2012; Kirkland et al., 2011; Janssen et al., 2003). However, their amino acid sequences differ. The length of the N-terminal sequence preceding the first cysteine residue is also variable, and may contain 17 to 158 amino acids (Scholtmeijer et al., 2004).

In accordance with their characteristics of stability and hidrophaty patterns, these proteins can be distinguished in two classes, class I and class II. Both hydrophobin´s classes are both found in Ascomycetes, but class I is only found in Basidiomycetes (Linder, 2009; Wösten, 2001; Akanbi et al., 2010; Sarlin et al., 2012).

Class I integrates very insoluble hydrophobins, that are only dissolved in strong acids such as formic acid and TFA (Mosbach et al., 2011; Lunkenbein et al., 2011; Wohlleben et al., 2010; Scholmeijer et al., 2004). Proteins belonging to this class form the resulting rodlet layer that coats the surface of spores of filamentous fungi. *Schizophyllum commune* is a wood-rotting fungus that secrets a protein named SC3 which is the best studied hydrophobin belonging to the class I hydrophobins (Kirkland et al., 2011; Wösten, 2001, Akanbi et al., 2010; Scholtmeijer et al., 2004; Teertstra, W., 2009).

Class II hydrophobins layers are dissociated in some organic solvents (such as ethanol) and detergents (SDS) and could also be dissociated applying pressure or by lowering the temperature (Kirkland & Keyhani, 2011; Winterburn et al., 2011; Mosbach et al., 2011; Lunkenbein et al., 2011; Scholmeijer et al., 2004). The most studied hydrophobins in class II are HFBI and HFBII from *Trichoderma reesei* (Kirkland & Keyhani, 2011).

The main attribute of hydrophobins is the reduction of the surface tension of water, helping the fungi to escape from an aqueous environment and allowing spores to be hydrophobic for easier dispersion of conidiospores. So, the hydrophobicity of the individual hyphae is modified through the coating of these proteins allowing them to grow through from the wet substrate into the air. Another important function is its pathogenic role in wall structure, such as controlling evasion of immune responses in pathogenic species (Litlejohn et al., 2012).

Certain fungi have more than one hydrophobin connected to different stages of fungal development (Hakanpää et al., 2006; Wohlleben et al., 2010). *Schizophillum commune, Coprinus cinerus, Aspergillus nidulans, Agaricus bisporus* and *Plerotus ostreatus* are a few examples of these (Wohlleben et al., 2010).

In the GenBank sequence database (www.ncbi.nlm.nih.gov) around 140 hydrophobins can be found (Sarlin et al., 2012). Tables 1.1, 1.2 and 1.3 systematize the list of hydrophobins that have been identified and published till present.

Table 1.1. Class I hydrophobins identified in Basidiomycetes.

Hydrophobi	Strain	Gene	References
Class I- From Basidiomycetes	*H. jecorina (Schizophyllum commune)*	SC1	Dons et al., 1984; Ohn, et al., 2010;
		SC3	Schuren & Wessels, 1990; De Vocht et al., 2000; Klimes et al., 2008; Cox & Hooley, 2009; Akanbi et al., 2010; Vejnovic et al., 2010; Wohlleben et al., 2010; Wang et al., 2010; Kirkland & Keyhani, 2011; Pedersen et al., 2011
		SC4	Schuren & Wessels, 1990; Klimes et al., 2008
		SC6	Mulder et al., 1988
	Agaricus bisporus	ABH1/HYPA	De Groot et al., 1996; Lugones, et al., 1996; Cox & Hooley, 2009; Lunkenbein et al., 2011
		ABH2/HYPC	De Groot et al., 1996; Lugones, et al., 1996
		ABH3	Lugones et al., 1998; Lunkenbein et al., 2011
		HypB	De Groot et al., 1999; Lunkenbein et al., 2011
	Pleurotus ostreatus	POH1	Asgeirsdóttir et al., 1998; Wessels, 2000; Lunkenbein et al., 2011
		POH2	Asgeirsdóttir et al., 1998; Lunkenbein et al., 2011

		POH3	Asgeirsdóttir et al., 1998; Vejnovic et al., 2010; Lunkenbein et al., 2011
		VMHI	Larraya et al., 1999
		VMH3	Larraya et al., 1999
		FBH1	Peñas et al., 1998;
	Coprinus cinereus	COH1	Lunkenbein et al., 2011
		COH2	Asgeirsdóttir, et al., 1997; Wessels, 2000; Lunkenbein et al., 2011
	Lentinula edodes	Le.Hyd1	Lunkenbein et al., 2011
		Le.Hyd2	Ng et al., 2000
	Agrocybe aegerita	Aa-Pri2	Lunkenbein et al., 2011
	Pisolithus tinctorius	Hyd-Pt1	Tagu et al., 1996
		Hyd-Pt2	Tagu et al., 1996
		Hyd-Pt3	Wessels, 1997
	Ustigalo maydis	Hum1	Wessels, 1997
		Hum2	Wessels, 1997
	Dictyonema glabratum	DGH1	Trembley et al., 2002
		DGH2	Trembley et al., 2002
		DGH3	Trembley et al., 2002
	Flammulina velutipes	FVH1	Lunkenbein et al., 2011
		FvHID1	Linder et al., 2005
	Pholiota nameko	PNH1	Tasaki et al., 2004
		PNH2	Tasaki et al., 2004
		PNH3	Tasaki et al., 2004
	Grifola frondosa	HGFI	Yu et al., 2008; Wang et al., 2010; Pedersen et al., 2011
	Dictyonema glabratum	DICH1	Lunkenbein et al., 2011
		DICH2	Lunkenbein et al., 2011
		DICH3	Lunkenbein et al., 2011

Table 1.2. Class I hydrophobins identified in Ascomycetes

Hydrophobins	Strain	Gene	References
Class I- from Ascomycetes	*Magnaporthe grisea*	MPG1	Martin et al., 1999; Kershaw et al., 2005; Lunkenbein et al., 2011; Mosbach et al., 2011
	Aspergillus nidulans	RodA	Aimanianda et al., 2009; Seidl-Seiboth et al., 2011; Lunkenbein et al., 2011; Pedersen et al., 2011
		DewA	Seidl-Seiboth et al., 2011; Lunkenbein et al., 2011; Pedersen et al., 2011
	Aspergillus fumigatus	Hyp1	Lunkenbein et al., 2011
		RODB	Seidl-Seiboth et al., 2011
	Metarhizium anisopliae	SsgA	Seidl-Seiboth et al., 2011; Lunkenbein et al., 2011
	Xanthoria estaneoides	XEH1	Scherrer et al., 2000
	Xanthoria parietina	XPH1	Scherrer et al., 2000
	Cladosporium fulvum	Hcf1	Lunkenbein et al., 2011
		Hcf2	Lunkenbein et al., 2011
		Hcf3	Lunkenbein et al., 2011; Mosbach et al., 2011
		Hcf6	Nielsen et al., 2001; Lunkenbein et al., 2011
	Neurospora crassa	EAS	Seidl-Seiboth et al., 2011; Kirkland & Keyhani, 2011; Lunkenbein et al., 2011; Litlejohn et al., 2012
	Cladosporium herbarum	HCH1	Weichel et al., 2003
	Aspergillus orysae	ROLA	Wessels, 1997
		HYPB	Linder et al., 2005
	Paracoccidioides brasiliensis	PbHYD1	Albuquerque et al, 2004
		PbHYD2	Albuquerque et al, 2004
	Gibberella moniliformis	HYD1	Fuchs et al., 2004
		HYD2	Fuchs et al., 2004
		HYD3	Fuchs et al., 2004; Seidl-Seiboth et al., 2011
	Beauveria bassiana	Hyd2	Kirkland & Keyhani, 2011
	Talaromyces thermophilus	TT1	Vejnovic et al., 2010
	Paecilomyces farinosus	PfaH1	Lunkenbein et al., 2011

Table 1.3. Class II hydrophobins identified in Ascomycetes

Hydrophobins	Strain	Gene	Reference
Class II- From Ascomycetes	*Claviceps fusiformis*	CFTH1_I	Lunkenbein et al., 2011
		CFTH1_II	De Vrie et al., 1999
		CFTH1_III	De Vrie et al., 1999
	Trichoderma harzianum	TRI1	Hakanpää et al., 2006
		TRI2	Hakanpää et al., 2006
		TRI3	Hakanpää et al., 2006
		Qid3	Lora et al., 1994; Hakanpää et al., 2006
		SrH1	Hakanpää et al., 2006
	Cryphonectria parasítica	Cry	Hakanpää et al., 2006
	Trichoderma reesei	HFBI	Nakari-SetäLä et al., 1996; Hakanpää et al., 2006; Wang et al., 2010; Valo et al., 2011; Kirkland & Keyhani, 2011; Pedersen et al., 2011; Litlejohn et al., 2012
		HFBII	Hakanpää et al., 2006; Valo et al., 2011; Vejnovic et al., 2010; Wohlleben et al., 2010; Kirkland & Keyhani, 2011; Winterburn et al., 2011; Lutterschmid et al., 2011; Litlejohn et al., 2012
		HFBIII	Hakanpää et al., 2006; Valo et al., 2011
	Ophistoma novo-ulmin and O.ulmin	CU	Bowden et al., 1994; Hakanpää et al., 2006; Lunkenbein et al., 2011
	Cryphonectria parasitica	CRP	Zhang et al., 1994
	Magnaporthe grisea	Mag	Hakanpää et al., 2006
		MPH1	Lunkenbein et al., 2011
		MGP	Sunde et al., 2008
	Verticillium dahlia	VDH1	Klimes et al., 2008
	Fusarium culmorum	FcHyd5p	Stübner et al., 2010; Lutterschmid et al., 2011; Pedersen et al., 2011;
	Claviceps purpurea	CPPH1_I	Hakanpää et al., 2006
		CPPH1_II	Hakanpää et al., 2006
		CPPH1_III	Hakanpää et al., 2006
		CPPH1_IV	Hakanpää et al., 2006
		CPPH1_V	Hakanpää et al., 2006
	Gibberella moniliformis (Fusarium verticillioides)	HYD4	Sunde et al., 2008
		HYD5	Mosbach et al., 2011
	Neurospora crassa	NCU08192.1	Galagan et al., 2003

1.2. Molecular Structure

Different techniques were used in the past to obtain a complete description of the molecular structure of hydrophobins and their assembly. Circular dichroism spectropolarimetry (CD), attenuated total reflectance Fourier-transform infrared spectroscopy (ATR FT-IR), peptide digestion and hydrogen/deuterium exchange, X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) were the principal experimental techniques (Sunde et al., 2008). Although of low resolution results, the techniques indicated above gave insights of certain aspects of the structure of hydrophobins. A better resolution of the biophysical studies has been difficult because of a peculiar physical property of these proteins: their natural ability to aggregate and self-polymerize, as well as the presence of disorder in the soluble state, is an obstacle to obtain crystals suitable for X-ray crystallography (Sunde et al., 2008).

In 2004, Hakanpää and coworkers obtained the crystalized protein of HFBII from *T.reesei* by careful screening of crystallization conditions. In molecular level, the first crystallographic structure of hydrophobins gave a new basis for understanding of how this type of proteins works (Linder et al., 2005; Linder, 2009).

Generally the shape of the molecule is globular (Zhang et al., 2011; Linder, 2009), the molecular weight is approximately 7 kDa (Linder, 2009; Cox & Hooley, 2009) and it has a diameter of about 3nm (Linder, 2009). It has a central β structure comprised of two β-hairpins (Sunde et al, 2008; Linder, 2009). One β-hairpin is located close to the N-terminus and the other one close to the C-terminus. These hairpins connect with each other to form one anti-parallel β-sheet and consequently form a barrel-like structure. In a middle of the two β-hairpins there is one α-helix (Linder, 2005). The α-helix is connected to the outside of the barrel through a disulfide bond, and another one disulfide bond operates to crosslink the two strands of each of the two β-hairpins. Those last two disulfide bonds are restricted by the barrel and are located at opposite ends, offering a high stability to the structure. The four disulfide bonds links the N-terminal loop to the core β-barrel (Sunde et al, 2008; Linder, 2009). This structure is schematically visible in Fig. 1.1 - A.

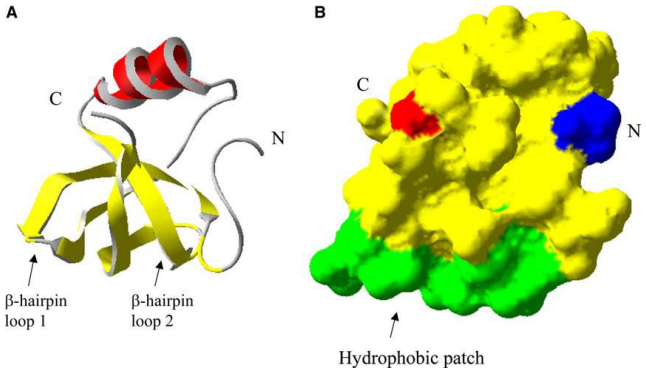

Figure 1.1. Structure of the HFBII hydrophobin from T. reesei. (A) Scheme of the secondary structure of HFBII. A central β sheet structure formed by two β hairpins and the two loops of the hairpins form most of hydrophobic patch are observed and they are indicated with an arrow. (B) A space-filling model of the structure in the same scale and orientation shows the hydrophobic patch in green, the rest of the surface in yellow, except for the N- and C-termini that are indicated in blue and red , respectively (Adapted from Linder et al., 2005).

In the surface of hydrophobin HFBII there is a large patch consisting of hydrophobic aliphatic residues (Sunde et al., 2008; Linder et al., 2005; Hakanpää et al., 2006; Wang et al., 2010). The hydrophobic patch is formed of side-chain residues of leucine, valine, and alanine. The surface is a plane and comprises about 20% of the total surface area of the protein (Sunde et al., 2008; Wang et al., 2010; Zhang et al, 2011). The structure looks like a surfactant with one hydrophobic and one hydrophilic part, but the size and structural details are quite different (Linder, 2009).

Crystallographic studies of hydrophobins were limited for a long time to the HFBII structure and this structure was for long the only one placed in the Protein Data Bank (Hakanpää et al. 2006). More recently, another class II hydrophobin, HFBI from *T. reesei* have been reported, and an NMR study of a class I hydrophobin EAS from *Neurospora crassa* has been described too (Hakanpää et al. 2006; Linder, 2009).

At first, EAS was determined to be largely unstructured in solution but then it was re-examined and found to share a similar fold as that of HFBII (Hakanpää et al., 2006; Linder, 2009). But, in the NMR structure of EAS, the α-helix is missing and to occupy this region it has two short β-strands (Hakanpää et al., 2006). The disulfide bridges are formed by the eight conserved cysteine residues in the same way to that of HFBII (Hakanpää et al., 2006; Linder, 2009). The zone which corresponds to the hydrophobic patch in the HFBII

structure is either disordered or could not be defined in the NMR structure of EAS (Hakanpää et al., 2006; Linder, 2009).

1.3.The interfacial self-assembly

Hydrophobins self-assemble at hydrophilic-hydrophobic interfaces into amphipathic films (Wösten et al., 2000; Wösten, 2001; Scholtmeijer et al., 2001; Scholtmeijer et al., 2004; Stübner et al., 2010; Valo et al., 2011), even under *in vitro* conditions (Wohlleben et al., 2010). They self-assemble between water and air, water and oil, water and hydrophobic solid (Teflon) and can change the nature of surfaces (Fig. 1.2) (Wösten & Vocht, 2000; Cox & Hooley, 2009), resulting in an inversion of the surface polarity (Wohlleben et al., 2010; Valo et al., 2011).

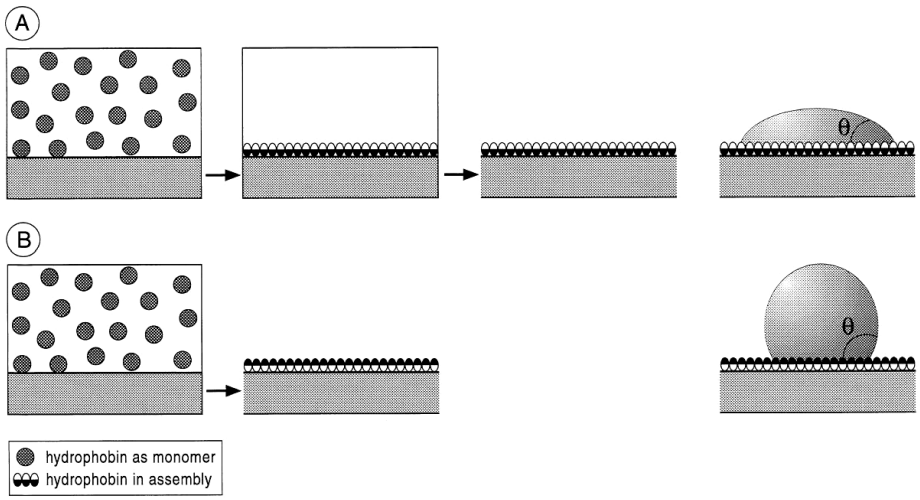

Figure 1.2. Self-assembly of hydrophobins that results in modification of the nature of a surface. **(A)** A hydrophobic solid surface is coated with an approximately 10 nm thick. The coating is achieved by immersing the surface in a hydrophobin solution followed by washing; the surface becomes wettable after coating, with the resulting water contact angle ranging between 22º and 63º. **(B)** A hydrophilic solid surface coated with a hydrophobin film. The coating is achieved by drying down a hydrophobin solution on the surface; the surface becomes hydrophobic after coating, resulting in a water contact angle of 110º (Figure taken from Wang, X., 2004).

Some of the experiments with hydrophobins showed that they form various types of aggregates (Linder, 2005) and that their properties can be solely attributed to their amino acid sequences (Wösten, 2001; Scholtmeijer et al., 2001).

The best characterised class I hydrophobin, SC3 of *Schizophyllum commune*, and the other members of the class I hidrophobins have similar properties. Upon contact with hydrophilic-hidrophobic interfaces, SC3 monomers self-assemble into a 10 nm thick amphipathic film (Scholtmeijer et al., 2004). One side of the hydrophobin membrane is moderately to highly hydrophilic (water contact angles ranging between 22º and 63º), while the other side exposes a surface as hydrophobic as Teflon or paraffin (water contact angle 110º) (Wösten et al., 2000; Wösten, 2001).

Self-assembly of hydrophobins is accompanied by changes in its conformation (Wösten, 2001; Scholtmeijer et al., 2004). Class I and class II hydrophobins, formed only by a polypeptide chain, are rich in β-sheet structure. At the water-air interface, class I hydrophobins are in β-sheet state, achieving more β-sheet structure, while at the interface between water-hydrophobic solid is observed the α-helical state because there is an increase in the α-helix form. The α-helical state seems to be an intermediate of self-assembly, whereas the β-sheet state is the stable end-form. At the water-air interface, monomers of class I hydrophobins obtain the α-helical state, very fast, within seconds, but the conversion to the β-sheet state is much slower and has a delay of minutes to hours. At the water-solid interface, the protein obtained easily reaches the α-helical state, but is believed to be arrested in this intermediate state. The β-sheet end state can only be reached by applying a combination of heat and diluted detergent. Both forms of the assembled hydrophobin have an amphipathic nature and can be dissociated with TFA, which unfolds the protein. After removing the solvent and dissolution in water, class I hydrophobins refold to the same monomeric structure that was observed before purification or TFA treatment and the process of self-assembly can be repeated (Fig. 1.3). It is not yet known which structural changes accompany self-assembly of class II hydrophobins. However, self-assembly and disassembly of class II hydrophobins can also be repeated even after dissociation of the membrane by TFA. This shows that both classes of hydrophobins are highly resilient to this type of treatment (Wösten, 2001).

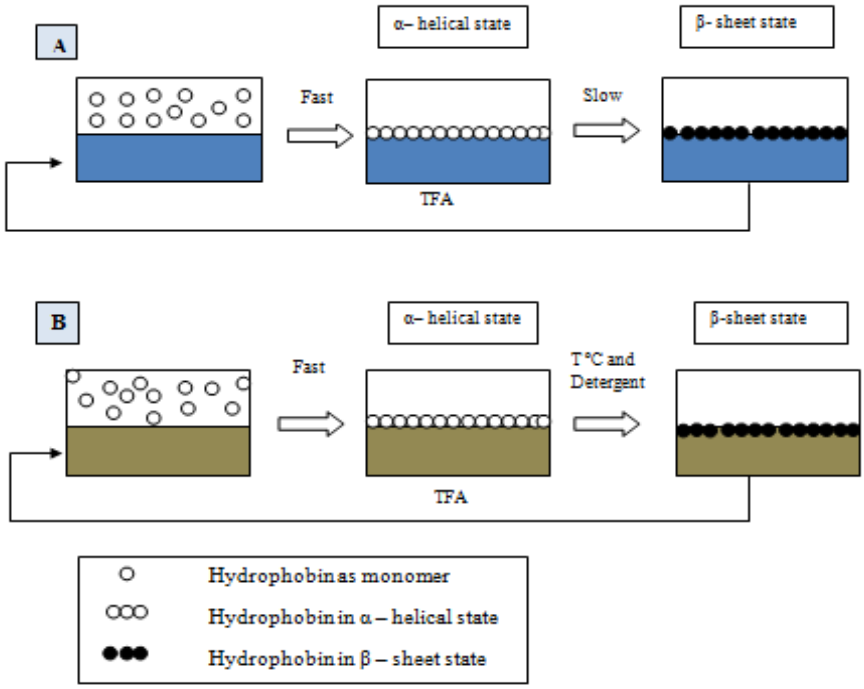

Figure 1.3. Self-assembly of class I hydrophobin and changes in its conformation. (A) Interface air-water. (B) Interface water -hydrophobic solid

1.4.Special properties

Hydrophobins appear to be ubiquitous in the Kingdom Fungi (Wang, X., 2004; Table 1, 2, and 3). They all have special properties that make them unique. Nature has produced several hydrophobins with small differences, but hydrophobins could also be modified by chemical cross-linking or genetic engineering as well (Wosten et al., 2000).

1.4.1. Surface-activity and surface adhesion

Hydrophobins belong to the most surface active molecules group (Linder, 2009; Wösten & Vocht, 2000; Stübner et al., 2010; Rieder et al., 2011). With a maximal lowering of the water surface tension from 72 mJ m^{-2} to 24 mJ m^{-2} at 50µg ml^{-1}, SC3 is the most surface active protein known. Other hydrophobins are also highly surface active (Wösten & Vocht, 2000) and their surface activity is similar to that of traditional biosurfactants such as glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids, substituted fatty acids and lipopolysaccharids used in a wide range of industrial applications such as in emulsions and dispersions. Surface activity of hydrophobins is solely caused by the amino acid sequence and

is thus not dependent on a lipid molecule as in a traditional surfactant (Wösten & Vocht, 2000; Scholmeijer et al., 2004).

Hydrophobins have been extensively studied, due to their surface adhesion capabilities (Szilvay, 2007; Wang et al., 2010). It was found that hydrophobins were capable to recruit enzymes to solid surfaces and thereby accelerate the degradation of a solid substrate (Wang et al., 2010). These proteins have been described as mediators for adhesion of hyphae and spores on host surfaces like an insect cuticle for example (Rocha-Pino et al., 2011) and this type of adhesive attitude may be related to features of hydrophobins as aiding fungal adhesion when pathogenic fungi colonize their hosts (Szilvay, 2007; Wang et al., 2010).

1.4.2. Foam formation

Another property that seems unique to hydrophobins is the tendency to form highly stable foams, which is connected with the high surface elasticity of hydrofobin membranes (Linder, 2009). The foam stability is due essentially to self-assembly of hydrophobins on the bubbles surface, forming a film with a high surface dilatational elasticity delaying the disproportionation, which is the diffusion of gas from small to large bubble due to a difference in Laplace pressure (Winterburn et al., 2011).

The foaming competence of hydrophobins has been known already for some time and the foaming tendency for Class I members may be weaker than class II members. Cox et al. (2008) found that the foams and bubbles of HFBII from *T. reesei* were stable for at least 4 months, and even up to several years in some cases, where the concentrations of protein used was relatively low (0,1 % wt.). HFBII confers a special stability to colloidal dispersions such as foams and emulsions because to properties of the protein surface (Winterburn et al., 2011).

A different concept that has raised considerable attention is the relation between gushing of beer and hydrophobins. The gushing foam consists in foaming formation very effectively and instantly, however is not exceptionally stable. Gushing is likely to be related with hydrophobins who act as nucleation sites for the formation of CO_2 bubbles (Linder, 2009).

Sarlin et al. (2005) showed that there is a clear connection between the phenomenon and the presence of hydrophobins (Linder, 2009; Sarlin et al., 2012). A small amount of this protein is sufficient for gushing to occur and the gushing approached by Sarlin isn't the same thing as the foam stability described by Cox (Linder, 2009).

Zapf et al. (2006) cloned hydrophobin genes from *Fusarium culmorum*, that are widespread in causing gushing problems and found that hydrophobins in gushing foam belong

to class II, which may indicate that gushing is also more related to class II than class I members (Linder, 2009)

1.4.3. Rodlet formation

A thin layer of rodlets coats surface conidia. The proteins existent in the cell wall of fungal aerial structures responsible for this rodlets are hydrophobins (Paris et al., 2003). This was observed on the surface of class I hydrophobin assemblages but not of their class II (Linder, 2009; Kudo et al., 2011).

The rodlets of class I hydrophobins have a characteristic appearance (Fig. 1.4). They are very similar to the fibrils that compose amyloid proteins (Wösten & Vocht, 2000; Wang, W., 2004; Linder, 2009; Wang et al., 2010).

Several aggregates of class II hydrophobins have been also described as needles or fibrils, but their size and solubility are different (Sunde et al., 2008). The formation of those classes is done differently. Class II aggregates are much larger than class I and found in the solution, the class I rodlets form when a surface membrane gets mature. The aggregates of class II may originate from the surface of the membrane which is ruptured by shear and rearranged (Linder, 2009).

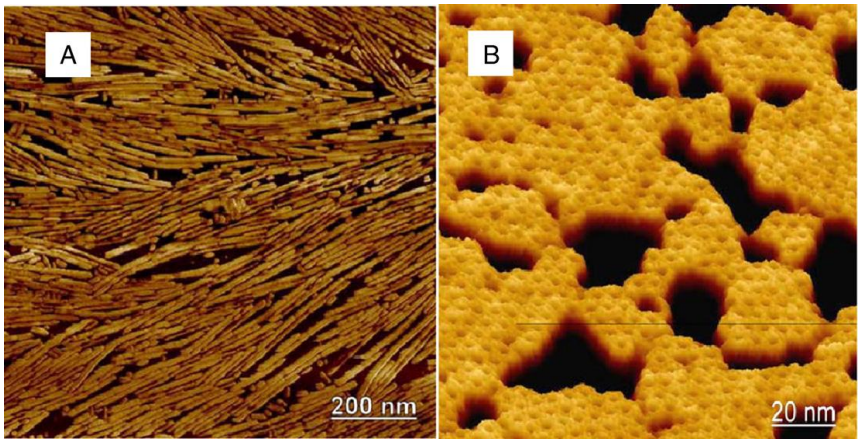

Figure 1.4. Atomic force microscopy (AFM) & A surface membrane of HFBI (A) Atomic force microscopy (AFM) image of rodlets formed by the HGFI hydrophobin from *Grifola frondosa*. Rodlet formation is characteristic of class I hydrophobins. The rodlets were formed at the air–water interface by multiple compressions and lifted on a solid support for imaging. (B) A surface membrane of HFBI imaged by AFM showing an organized structure. The film was formed at the air–water interface and lifted onto a mica support (Figure taken from Linder, 2009).

Rodlets of class I hydrophobins are made up of 4-6 protofilaments with similar diameters, have a large number of β-sheet structures, are protease resistant, self-assemble by means of intermediates, and just aggregate above a critical concentration.

The rodlets typically are formed in the air-water interface when a solution of hydrophobins is dried over a solid surface (Linder, 2009). Class I form self-assemble rodlets soluble in trifluoroaceticacid (TFA) and formic acid (FA) (Linder, 2009; Rocha-Pino et al., 2011), whereas class II are easily dissolved in ethanol or sodium dodecyl sulfate (SDS) (Rocha-Pino et al., 2011).

1.5. Applications

The several specific surface related properties of hydrophobins make them of high interest in biotechnology, having a huge potential in various practical applications (Hektor and Scholtmeijer, 2005).

Considering all their characteristics, hydrophobins have several possible applications (Schmol et al., 2010; Pedersen et al., 2011) in different areas of study such as anti-foulings (Hektor & Scholtmeijer, 2005; Hakanpää et al., 2006; Bimbo et al., 2011; Rieder at al., 2011), biomaterials and medical applications (Stübner et al., 2010; Kirkland & Keyhani, 2011; Rieder et al., 2011), personal care (Vic, G., 2003; Hakanpää et al., 2006) and emulsions (Linder et al., 2005; Winterburn et al., 2011; Pedersen et al., 2011; Kirkland & Keyhani, 2011), biosensors (Akambi et al., 2010; Pedersen et al., 2011) and electrodes (Hektor & Scholtmeijer, 2005), separation technologies (Kirkland & Keyhani, 2011) and gushing factor detection (Linder et al., 2005; Stübner et al., 2010).

As already mentioned before, hydrophobins can modify the surface binding properties, making them of interest in anti-foulings application (Bimbo et al., 2011). The window panes and cars suffer from fouling and the growth of undesirable microorganisms in ships represents a problem. Coating with hydrophobins could be the solution, thus increasing the hydrophobicity of the surfaces in contact. Coatings may also be used to immobilize growth-inhibiting compounds (Hektor & Scholtmeijer, 2005).

The antifouling capability of hydrophobins can be exploited in the field of biomaterials reducing non-specific protein binding and due to better hydrophilicity of the surface can also improve cell adhesion (medical implants biocompatibility is an important factor by using hydrophobins) (Janssen et al., 2002; Bimbo et al., 2011).

Hydrophobins can also be used to prevent growth in medical applications by lowering the hydrophobicity of surfaces. A problem with catheters, for example, is the occurrence of bacterial infections (Scholtmeijer et al., 2001).

Personal care has a common problem where products intended to treat hair don't adhere or absorb well enough and are easily removed upon shampooing. Hydrophobins can prolong the residence time and may therefore be a good additive to hair care products, surviving several shampoo washes (Vic, G., 2003). Alternatively, hydrophobins can be used to stabilize emulsions (Wösten et al., 1994; Linder, 2009) in creams and ointments. It is known that the assembly of hydrophobins at the interface between hydrophobic and hydrophilic liquids can stabilize emulsions. This property might also be convenient for pharmaceutical and food industry, which both required stable emulsions for certain formulations and ingredients (Hektor & Scholtmeijer, 2005).

Hydrophobins can be used to prevent denaturation when immobilizing enzymes and to improve the quality of the biosensor and activity is also maintained for extended periods of time (Chakarova & Carlson, 2004).

Hydrophobins are also useful in electrodes without enzymes. It has been demonstrated that hydrophobins can control the access of compounds from the solution to the electrode surface, therefore increasing the specificity and sensitivity of the electrode (Bilewicz et al., 2001).

These proteins can also be exploited in separation technologies. They can be extracted from the detergent phase by the addition of ethylene oxide-propylene oxide, resulting in hydrophobin purification. A protein of interest can be fused to the hydrophobin and will be co-purified (Linder et al., 2001).

The stabilization of foam can be used in certain food applications but it can also cause problems (Talbon, N.J., 2001).

All of these mentioned applications require the production and purification of large amounts of hydrophobin, which has proved difficult so far (Askolin et al., 2001; Schmoll et al., 2010). Hydrophobins are available from natural resources only in milligram amounts (Wohleben et al., 2010). It would be necessary to increase the levels of production of hydrophobins significantly, particularly in the food industry, before the application of hydrophobins. If the production levels and price become acceptable to the food industry, the advantage of hydrophobins is that several are considered to be food-grade surfactants (Hektor & Scholtmeijer, 2005).

A summary of the many applications of hydrophobins is tabulated in Table 1.4.

Table 1.4. Possible applications of hydrophobins in Biotechnology

Applications	**Examples**	**References**
Anti-fouling	Prevent fouling in window panes and cars	Linder et al., 2005; Hektor & Scholtemeijer 2005; Hakanpää et al., 2006;Rieder et al., 2011;
	Prevent growth of unwanted organisms on ships	Hektor & Scholtemeijer, 2005;
Biomaterials and medical applications	Prevent the occurrence of bacterial infections in catheters;	Scholtemeijer et al., 2001; Hakanpää et al., 2006; Linder, 2009; Stübner et al., 2010; Rieder et al., 2011; Kirkland & Keyhani, 2011;
	Improvement of the biocompatibility of implants;	Janssen et al., 2002; Bimbo et al., 2011
	Improve bioavailability of hydrophobic drugs	Bimbo et al., 2011
Emulsions	Stabilize emulsions in creams and ointments for certain formulations and ingredients in pharmaceutical and food industry applications	Scholtmeijer et al., 2001; Hektor & Scholtemeijer 2005; Kirkland & Keyhani, 2011;
	Use as emulsifying agents;	Scholtmeijer et al., 2001; Talbon, N.J., 2001; Kirkland & Keyhani, 2011, Linder, 2009
Biosensors	Prevent the denaturation of the proteins in enzimatic immobilization	Chakarova & Carlson, 2004; Linder et al., 2005; Hakanpää et al., 2006; Kirkland & Keyhani, 2011;
Eletrodes	Control the access of compounds from the solution to the electrode surface	Hektor & Scholtemeijer, 2005; Linder, 2009;
Separation technologies	Efficient purification system	Hektor & Scholtemeijer, 2005;
Gushing factor detection	Stabilization of foam in food industry	Hektor & Scholtemeijer 2005; Hakanpää et al., 2006;
Personal care	Prolong the residence time and additive to hair care products	Vic, G., 2003; Hankanpää et al., 2006; Stübner et bal., 2010
Nanotechnology	Pattern different molecules on a surface with nanometre accuracy	Scholtmeijer et al., 2001;

No hydrophobin applications were found for oral medicine in scientific literature.

The search for hydrophobin patentes syielded a high number of hydrophobin application but, none-related to oral medicine were found (Appendix I, Table 1).

The use of hydrophobins in oral medicine could have many benefits once these proteins are capable of coating and changing surfaces. Furthermore, many surface-

active molecules are toxic due to interaction with cell membranes. But hydrophobins are non-toxic proteins because in their assembly is not expected to diffuse through the cell wall neither interacts with the plasma membrane (Wösten, 2001). They are generally considered safe for human consumption due to their natural presence in widely consumed foods such as the common button mushroom, *Agaricus bisporus* (Winterburn et al., 2011).

The potential application of hydrophobins in dentistry and the lack of extensive research on this topic make this study interesting and innovative.

1.6. Objectives

The general objective of this research effort are the production and characterisation of hydrophobins with potential use for hydrophobic coating in oral medicine, namely in intracanal formulations. The specific objectives of this work were:

- Hydrophobin production at laboratory scale from selected fungal strains;
- Hydrophobin purification through bubble production, trifluoroacetic acid treatment and diverse chromatography, electrophoreses and filtration techniques;
- Scanning electron microscopy for preliminary characterization of isolated or polymerized hydrophobins;
- Characterization of superficial tension changes of hydrophobin solutions;
- Biochemical characterization of the hydrophobins obtained, namely, molecular weight and amino-acid sequence analysis by MALDI-TOF.

2. MATERIAL AND METHODS

2.1. Microorganisms isolation and identification

Previously to this work, carphosphores were collected from decaying wood of hybrid black poplar trees from Escola Superior de Biotecnologia gardens (41°10'37''N; 8°36'23''W), located in Porto, Portugal. The filamentous fungi growing in that substrate were selected using traditional methodologies for fungal growth on solid medium. Their differentiation was accomplished based on color and texture of the colony, growth rate and aerial hyphae. Isolates were selected for taxonomic identification and evaluation of potential production of hydrophobins and the selection criteria were based on morphology, growth, production of aerial hyphae and sample origin. All isolates obtained were kept in the laboratory collection. For this research effort two strains referred as C1.1 and L7 from the aforementioned isolation procedure were used.

The strain identified as *Bjerkandera sp.* BOS55 from the laboratory collection was also tested, due to lack of information on the production of hydrophobins in this genus.

Isolates C1.1, L7 and *Bjerkandera* sp. BOS55 were further selected as potential producers of hydrophobins and tested for their specific characteristic.

2.2.Culture media

Czapek-Dox broth (CDB, Oxoid) supplemented with 5.0 $g.l^{-1}$ of yeast extract (YE, Bacto™) was used as liquid medium (CDBYE) for production of fungal mycelia as well as extra cellular fluid for hydrophobin isolation.

Potato Dextrose Agar (PDA, Difco) supplemented with 4.0 $g.l^{-1}$ of agar (Agar, Liofilchem) was used as solid medium for mycelia maintenance and production for the 3 selected strains.

2.3. Hydrophobins production and extraction

The 3 selected strains were inoculated in 250 ml of CDBYE medium with 6 plugs of mycelia grown with approx.1 cm of diameter during 21 days at 30 ºC with or without agitation (80 rpm).

The extracellular fluid was separated from the mycelia by gravity filtration with Whatman Nº1 filters followed by vacuum filtration with 0.45 µm or 0.22 µm filters

(Millipore).

For the extraction of hydrophobins, the extracellular fluid filtrate was subjected to stirring with an ultraturrax homogeniser (Ultraturrax ® T18 basic IKA®) at maximum speed for 5 min. The foam obtained was collected, its volume was measured after liquefaction and immediately frozen at -80 ºC and further lyophilized.

For every 5 ml of sample, 20 ml of methanol, 5 ml chloroform and 15 ml of sterile water were added. Between each addition, samples were stirred and centrifuged at 9000 rpm for 30 min. The aqueous phase was removed and an extra 5 ml of methanol in proportion to the sample volume was added. After centrifugation at 9000 rpm for 20 min the supernatant was discharged. The resulting pellet was dried in a Rotational-Vacuum-Concentrator RVC 2-18 CD (CHRIST) with a chemical resistant Diaphragm Vacuum Pump (Vacuumbrand) and then, 20 µl of Trifluoroacetic acid (TFA) was added. After evaporation with a nitrogen gas flow, the sample was redissolved in 50 mM phosphate buffer pH 7.0.

2.4. Total fungal DNA extraction

Full identification of L7 and C1.1 isolates required total fungal DNA. The mycelium was collected by gravity filtration with Whatman Nº1 filters, washed with sterilised water, quickly frozen with liquid nitrogen and stored at – 80 ºC till further use. For DNA extraction, it was first grounded to powder with a sterile mortar and pestle at 4 ºC.

The total DNA extraction followed the method of Pitcher, Saunders and Owen (1989) with small modifications. The quality and purity of the DNA was evaluated spectrophotometrically at 260 and 280 nm, and by electrophoresis in TAE buffer on 1% agarose gel.

Total fungal DNA was amplified with primers ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'- GGA AGT AAA AGT CGT AAC AAG G- 3').

PCRs were performed using 1 µl of each primer (ITS4 and ITS5), 12.5 µl of NZYTaq 2x Green Master Mix (Nzytech), 2.5 µl of total fungal DNA and 8 µl of nuclease free H_2O, up to a total volume of 25 µl. Amplification in a TECHNE TC512 thermocycler was performed for 35 cycles, with a 5 min of pre-denaturation step at 95 ºC, followed by cycles of 1 min denaturation at 95 ºC, 1 min annealing at 50 ºC, and 1 min extension at 72 ºC and with a final extension at 72 ºC for 5 min. DNA sequencing

was performed at Macrogen Inc. (Korea). Identification of strains was performed with analysis of BLASTn results obtained in the NCBI server http://www.ncbi.nlm.nih.gov/.

2.5. Hydrophobin biochemical characterization

2.5.1. Foam stability

For determination of the foam stability of unpurified hydrophobins mixtures, the method of Lutterchmid, Muranyi, Stübner, Vogel and Niessen (2011) was followed with some modifications. Mycelium was removed from the growth medium by filtration as described before. Two 150 ml aliquots of extracellular fluid were placed in sterile flasks. One of the aliquots was heat treated by incubation in boiling water for 1 h, while the other one remained untreated. After cooling down to room temperature, both treated and untreated aliquots were foamed up by mixing with an ultra turrax disperser (Ultraturrax® IKA® T18 basic) at 24000 rpm for 1 min. Foam formation and subsequent degradation was observed and the time it took to total foam fading was recorded.

2.5.2. Oil spreading assay

Surface activity of unpurified hydrophobin mixtures was measured with the oil spreading assay method (Rivardo, F. et al., 2009). Assay consisted of the formation of a thin membrane with 20 µl of Motor Oil 10 W-40 (GM- General motors) deposited onto the surface of 20 ml of distilled water in a Petri dish (90 mm in diameter). Twenty µl of extracellular fluid (as obtained from section 2.3) in 50 mM phosphate buffer (pH 7.0) were gently put onto the center of the oil membrane. Diameters of clearly formed oil displaced circles were measured to determine the presence of biosurfactant activity.

2.5.3. Test for mycelium wettability

Test for mycelium wettability was performed according to Mosbach, Leroch, Mendgen and Hahn (2011), using PDA culture medium for mycelia growth. A spore suspension was obtained adding 4 ml of 50 mM phosphate buffer (pH 7.0) to the surface of 7 days of growth PDA petri dish, stirred gently and collected - No spore counting was performed. PDA plates were inoculated with 4 ml of the spore suspension and incubated during 12 days at ambient light and room temperature, obtaining

sporulating mycelium.

To produce non sporulating mycelium PDA medium were incubated during 4 days in the dark. Aerial mycelia were covered with 20 µl droplets evenly distributed containing 50 mM EDTA and 2% (v/v) SDS, and incubated for up to 24h. Tests were performed in duplicate.

2.5.4. Assessment of conidial hydrophobicity

Conidial hydrophobicity of each selected isolate was assessed using the method of Shan, Wang, Ying and Feng (2010) trough suspension in 10 mM phosphate buffer (pH 7.0). Conidia were suspended in 50 mM phosphate buffer (pH 7.0) containing 0.02% (v/v) Tween 80 and a standardized the spore suspension of 2×10^7 conidia.ml^{-1}. Liquid paraffin was added to spore suspension in proportion to 40 µl in 40 ml. The solution was stirred for 2 min in a decanting funnel. When the solution was separated into two phases, three samples from the aqueous phase were pipetted to Neubauer chambers and conidia were counted in a microscope. Tests were performed in duplicate.

2.5.5. Surface tension

For surface tension measurements between hydrophobin mixture samples and air, a protein solution was prepared in 50 mM phosphate buffer (pH 7.0). The same phosphate buffer was used for calibration. One point five milliliters of hydrophobin mixtures were used for each measurement at ambient temperature on a Kruss Model K6 apparatus. The ring was placed just below the surface of the solution, and the force to move the ring from the liquid phase to the air phase was determined in duplicate (Rivardo, F. et al. 2009).

For this experiment in particular, the hydrophobin samples were extracted in three different ways. In addition to the extraction made as described in section 2.3, we tested two other ways. Production of bubbles for extraction of hydrophobins was also performed using dry ice. To each solution was added small amount of dry ice and on contact with the solution bubbling was formed and the foam collected. Bubbling was also obtained with a perforated tube dipped into the solution under a nitrogen gas flow that allowed the production of foam.

2.5.6. Scanning electron microscopy (SEM)

Spores, hyphae and individual hyphae from *Bjerkandera sp.* BOS55, L7 and C1.1 isolates, were observed with scanning electron microscope (SEM) in order to evaluate hydrophobin production potential. The procedure was adapted from Castillo (2005) and Patricia Reis (2011, personal communication).

A spore suspension from each isolate was prepared by covering the Petri dishes with 4-5 ml of sterile water and 1 drop of 20 µl of Triton-X. The plates were stirred to enable the release of spores and the spore suspension was carefully aspirated with a sterile Pasteur pipette. The suspension was placed on an open eppendorf and water evaporated in a heating plate at 60 ºC inside the fume hood.

Individual hyphae were prepared by placing a 0.45 µm filter (Millipore), with numerous small pores obtained using a sterile needle, on the surface of a petri dish with PDA medium. Each sample was inoculated with mycelia with an inoculating loop and a new similar filter, also perforated, was placed on top of the inoculum. After fungal growth for 7 days at 30 °C, individual hyphae were removed with the help of an inoculating loop and placed in sterile eppendorfs with 1 ml sterile water.

Aggregates of hyphae were prepared from mycelial grown in Potato dextrose broth (PDB, Difco) liquid medium. The mycelium was filtered through a Whatman filter Nº1, washed with sterile water and small samples (20 mm^2) were cut with a sterile scalpel.

For each isolate, three samples were obtained and placed in eppendorfs. One sample was suspended in 2% (v/v) SDS for 10 min, a second sample was placed in 2% (v/v) SDS for 10 min followed by 2 h in cold formic acid with constant stirring. The third sample was used as control. All samples were then washed with sterile water, neutralized with 45% NaOH on ice and suspended in cold TFA for 30 min. The TFA was finally removed with a nitrogen gas flow.

Samples were placed into a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate (pH 7.2) and left overnight at 20 °C. One drop of Triton-X was added to each sample and the sections were washed three times with 0.1 M cacodylate buffer pH 7.2 during 10 min. Dehydration was performed with an ethanol series of 10, 30, 50, 70, 80, 90, and 100%, with 15 min steps. Between fixation and dehydration steps, the samples were centrifuged for 10 to 15 min at 15000 rpm at room temperature.

For enhanced visualization, the sample was placed in metallic a stub with carbon

tape and coated with gold/palladium using a Sputter Coater (Polaron, Bad Schwalbach, Germany) and images were recorded by Scanning Electron Microscopy (SEM) using a JEOL-5600 Lv microscope (Tokyo, Japan). SEM was operated at the high vacuum mode, using a spot size of 36-37 and a potential of 20-22 kV. All analyses were performed at room temperature (20 ˚C).

2.6. Total protein quantification

Standard unpurified mixtures of hydrophobins for use in steps described in sections 2.7, 2.8 and 2.9 were obtained through the concentration of the extracted samples obtained as detailed in section 2.2 with ultrafiltration against 50 mM phosphate buffer pH 7.0 using an Amicon Ultra centrifugal filter device (Millipore) 3kDa and 30kDa cut-off and subsequently diluted to 0.2 $mg.ml^{-1}$ in the same buffer.

Determination of total protein concentration was carried out with BCA® Protein Assay Kit (Pierce) adapted for hydrophobic proteins according to the manufacturer's instructions.

2.7. Determination of antimicrobial activity

Determination of minimum inhibitory concentration (MIC) was performed according to Clinical and Laboratory Standards Institute guidelines (2007). Briefly, an inoculum of 0.5 MacFarland (1.5×10^8 $CFU.ml^{-1}$) of *Candida albicans* was prepared from overnight cultures in Yeast Malt Broth and inoculated in Yeast Malt Broth (Oxoid, Hampshire, England) with hydrophobin concentrations ranging from 0.025 $mg.ml^{-1}$ to 0.2 $mg.ml^{-1}$ previously prepared in 10 mM phosphate buffer (pH 7.0). Two controls were simultaneously performed: one with 0.2 $mg.ml^{-1}$ hydrophobin but without inoculum, and another where the hydrophobin mixture was replaced by sterile water and inoculated. An oral elixir formulation that has been shown to possess good results in reducing antibacterial activity was selected for standard comparison of MIC. This formulation includes 60% water and 40% chitosan mixture. The chitosan mixture was formed with 1% low molecular weight chitosan and 1% high molecular weight chitosan (Costa el al., 2012).

Samples were incubated 24 h at 37 ºC in a microplate reader (FlUOstar, OPTIMA, BGM Labtech) with optical density being recorded at 660 nm. The MIC was determined by observing the lowest concentration of hydrophobin which would inhibit

C. albicans growth. All assays were performed in duplicated (Costa, et al., 2012).

2.8. Microtiter-plate test for biofilm inhibition

Quantification of biofilm production in batch and fed-batch was carried out by adapting the protocol of Stepanovic et al. (2000). In a flat bottom 96 microplate wells were filled with 200 µl of test solutions with hydrophobin added at 0.1 $mg.ml^{-1}$ of half the maximum concentration value tested in the MIC assays. Biofilms from *C. albicans* were formed by incubation of the plate at 37 ºC for 48 h. All assays were performed in triplicate in Yeast Malt Broth (YMB, Difco) with 5% sucrose.

To visualize biofilms, the contents of each well were discarded and then washed 3 times with sterile deionized water in order to remove non-adherent cells. The remaining attached *C. albicans* were fixed with 200 µl of ethanol for 15 min. Ethanol was discarded and the wells were air dried. After that, 200 µl of crystal violet solution was added to the wells for 5 min and the excess stain removed by rising the plate under tap water followed by air drying. Adherence was quantified by measuring the OD at 630 nm using a microplate reader (FLUOstar, OPTIMA, BGM Labtech).

Optical density values from wells with only YMB (Difco) were used as negative controls. A positive control with sterile deionised water for each bacterium was also used.

Results for this test were given as percentage of biofilm formation inhibition applying the following formula (Costa, et al., 2012):

$$\% \text{ biofilm formation inhibition} = 100 - (OD_{assay} / OD_{control}) \times 100$$

2.9. Hydrophobin purification

Purification of hydrophobin proteins was carried out by analytic HPLC with a reversed phase column Vydac 214-TP C4 column (240 mm x 4.6 mm, 5 µm) (Grace Vydac). The injection volume was 30µl and a linear gradient with 80% B and 20% A (flow rate: 1 $ml.min^{-1}$; A: H_2O with 0.1% (v/v) TFA; B: acetonitrile with 0.1% (v/v) TFA) was carried out for 20 min. The HPLC system consists of a Beckman Coulter System Gold 508 Autosample, a Beckman Coulter System Gold 126 Solvent Module

and a Beckman Coulter System Gold 168 Detector. Data were collected and analyzed using the Beckman Coulter, INK 32 Karat Software.

Elution of protein was monitored at 260 and 280 nm. Collection of protein was performed when the protein was detected to be eluted from the system.

3. RESULTS AND DISSCUSSION

3.1. Hydrophobins production and extraction

Following the incubation period determined, enough mycelium was produced and thus hydrophobins were potentially secreted into extracellular medium. Very small volumes (approx. 20 ml in total) of mixtures of hydrophobins were obtained after extraction and collection of the target poteins. However, it has been already previously described the difficulty of producing and extraction large quantities of hydrophobins (Askolin et al., 2001; Scholtmeijer et al., 2002; Hektor & Scholtmeijer).

3.1. Total fungal DNA extraction

Strains were characterized macro and microscopically and were tentatively assigned to genera of fungi. One of the strains was already identified as *Bjerkandera sp.* BOS55 and for a reliable identification of the other strains, molecular techniques were used. Strains L7 and C1.1 were subjected to DNA amplification for identification of the ITS region (Fig. 3.1).

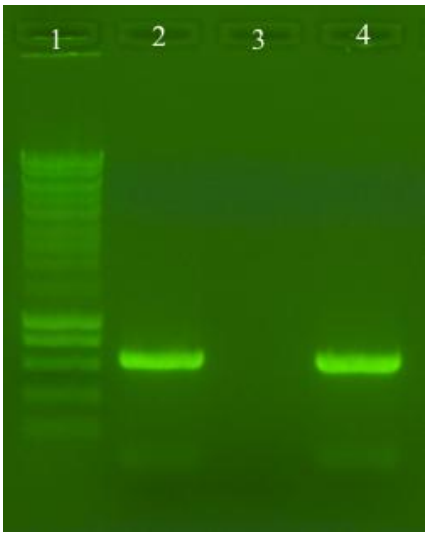

Figure 3.1. DNA amplification with ITS4 and ITS5. **1:** Molecular Weight marker (Smartladder, Eurogentec); **2:** L7 strain; **4:** C1.1 strain

For both strains L7 and C1.1, a very clear, high intensity band at aprox. 650 bp with absence of contamination was obtained.

The samples were purified and sequenced by Macrogen Inc, Korea. Two fungal strains were identified (Table 3.1). The quality of the sequence is good as evaluated in the chromatogram provided by the company (Appendix II, Fig. 1 and 2). The results also presented very low Expected values (E) and the percentage of identities was 99%, giving confidence in the results obtained by the nucleotide blast performed. However L7 was only identified to the genus and C1.1 was not assigned any identification at all.

Table 3.1. BLASTn results for identification of L7 fungal isolate by molecular biology (full sequence).

Strain	Identification	Quality of sequence	E value	Identities		
				Match	**Total**	**PCT (%)**
L7	*Mucor sp.*	Good	0.0	608	614	99
C1.1	Without identification	Good	0.0	599	605	99

Strain L7, as we already suspected due to its morphologic characteristics was identified as a *Mucor sp.* and the results were as expected, since this fungus has been associated with trees and our strain was isolated from a tree from Escola Superior de Biotecnologia gardens.

After analyzing the chromatogram we decided to remove 48 nucleotides from the beginning of the sequence and from nucleotide 630 inclusive to the end (630-655 nucleotides). We also eliminated a G nucleotide due to error and/or external contamination.

The new blast performed with a smaller but better quality sequence enable the species determination (Table 3.2).

Table 3.2. BLASTn first two results for identification of L7 fungal isolate by molecular biology (trimmed sequence).

Strain	Identification	Quality of sequence	E value	Identities		
				Match	**Total**	**PCT (%)**
L7	*Mucor circinelloides*	Good	0.0	569	569	100
L7	*Mucor circinelloides*	Good	0.0	570	571	99

The results indicate that L7 is an isolate of *Mucor circinelloides* first identified in CBS and Centre National de Référence Mycologie et Antifongiques (CNRMA) (Schwarz et al., 2005).

Otherwise strain C1.1 was isolated and sequenced previously in other research works but has not been fully characterized yet.

By analysis of the chromatogram, we decided to re-take the blast of the obtained sequence but without the extremities, that presented much background noise. Nucleotides were removed from the beginning of the sequence up to nucleotide 26 (inclusive) and from nucleotide 606 to the end of the sequence (606-632 nucleotides). The BLASTn performed with the trimmed sequence allowed species level identification for C1.1 isolate (Table 3.3).

Table 3.3. BLASTn first two results for identification of C1.1 fungal isolate by molecular biology (trimmed sequence).

Strain	Identification	Quality of sequence	E value	Identities		
				Match	**Total**	**PCT(%)**
C1.1	*Hypocrea lixii*	Good	0.0	578	579	99
C1.1	*Trichoderma harzianum*	Good	0.0	579	580	99

The results indicate that C1.1 is an isolate of *Hypocrea lixii* for the first time isolated and identified in Facultad de Agronomía, Universidad Nacional de Colombia (Hoyos-Carvajal et al. 2009) or *Trichoderma harzianum* that was identified for the first time in Systematic Botany and Mycology Lab, USA (Chaverri et al. unpublished work). Both are the same fungal species, however *T. harzianum* is asexual (or anamorphic) and the sexual stage (or teleomorph) has been described as *Hypocrea lixii* (Druzhinina I.S. et al., 2010).

3.2. Hydrophobin biochemical characterization

3.2.1. Foam stability

In order to characterize biochemically the hydrophobin mixtures obtained, the stability of the foam was tested in triplicate with the extracellular fluid of each fungal strain grown for 7 days with different treatments. The influence of agitation and heat

treatment (100 º C) were tested and foam formation and degradation was observed, as well as the time it took to total foam fading (Fig. 3.2).

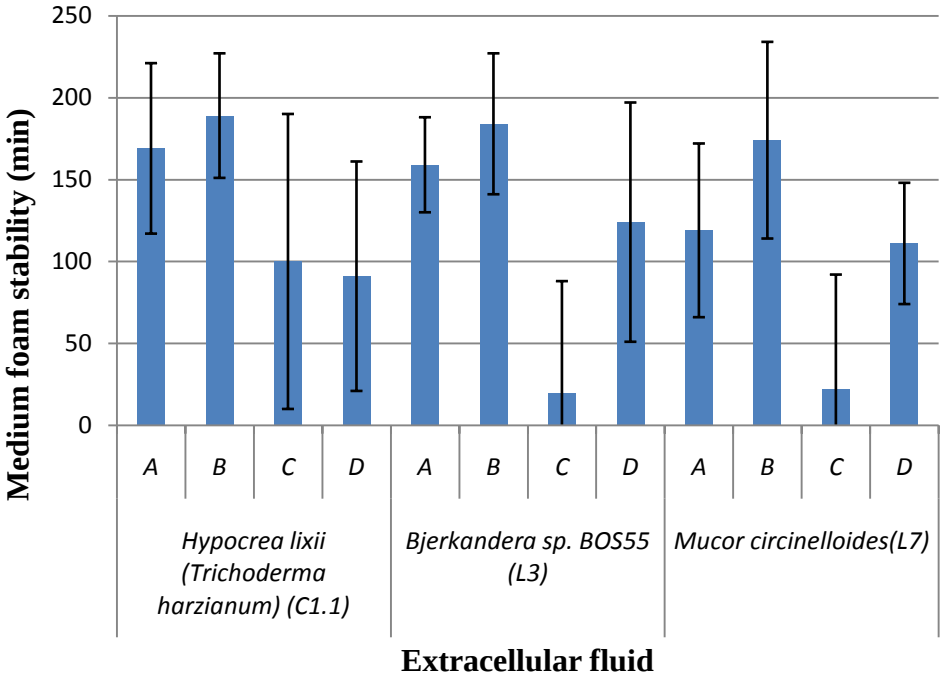

Figure 3.2. Stability of the foam obtained from the extracellular fluid. **A:** Extracellular fluid with agitation treatment; **B:** Extracellular fluid with agitation and heat treatment; **C:** Extracellular fluid without agitation treatment. **D:** Extracellular fluid with heat treatment and without agitation treatment.

Although the results presented high variability as shown by high standard deviations due to low reproducible methodology, they also show a tendency. Higher values of foam stability were obtained when strains were grown with agitation, possibly because of hydrophobins may be protection factor proteins. Similarly after heat treatment, the foam stability is higher, which is not normally expected since there should be denaturation of proteins at 100 °C. This may be explained by the high thermal stability of hydrophobins. Hydrophobins are very hard to denature. Askolin et al. (2006) heated hydrophobins to 90 ºC and did not observed any signs of denaturation. This may also be related to some change in shape when hydrophobins self-assembly (Linder, 2009), or problems in temperature measurement during the assay.

The strain with the best results in foam stability was *Hypocrea lixii (Trichoderma harzianum)* (C1.1) followed by *Bjerkandera sp. BOS55* (L3) and *Mucor circinelloides* (L7).

3.2.2. Oil spreading assay

Oil spreading assay was performed in duplicate in order to evaluate the presence of biosurfactant activity in the extracellular fluid of each strain. *Hypocrea lixii (Trichoderma harzianum)* (C1.1) and *Mucor circinelloides* (L7) strains showed ability for oil displacement associated with production of biosurfactant (Table 3.4), as hydrophobins would be.

Table 3.4. Displacement of oil medium measured for each strain.

Strain	Displacement of oil medium (mm)
Hypocrea lixii (Trichoderma harzianum)(C1.1)	1,5
Bjerkandera sp. BOS55 (L3)	0
Mucor circinelloides (L7)	0,5

The results of the oil displaced area test formed by the activity of surfactants for each isolate are in agreement with the results from the foam stability test. In both experiments, the effects of the presence of hydrophobins in the extracellular fluid were evaluated and *Hypocrea lixii (Trichoderma harzianum)* (C1.1) was the strain with better results, showing the highest-biosurfactant activity.

Although the mechanisms of oil displacement by surfactants have not yet been clarified on the molecular level, this method provided us with a simple sensitive and convenient assay system for surfactants. The method was effective for measuring biosurfactant activity, especially when the activity and the quantity of biosurfactant were not high enough (Morikawa et al., 2000).

3.2.3. Test for mycelium wettability

This test required visual evaluation of strains grown on PDA medium in the presence and absence of light, after drops of water were placed in the surface.

In figure 3.3, the differences between the results of plates A and B were evident. For plate B, grown in the presence of light, none of the drops added to the medium were

absorbed and in plate A, on the other hand grown in the absence of light, all the drops were absorbed.

The strain *Hypocrea lixii (Trichoderma harzianum)* (C1.1) grown in presence of light shows an impermeable surface, hydrophobic, even in the presence of SDS and EDTA. Some loss of hydrophobicity was detected in the surface areas that showed less spores. This may be associated with the mycelia be less hydrophobic than the spores and also the addition of SDS, which may be associated with a change in hydrophobic properties of the layer of hydrophobins in the presence of that detergent.

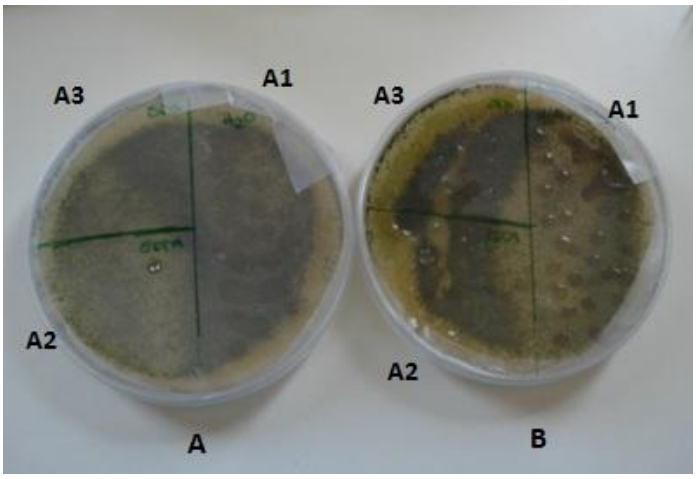

Figure 3.3. Evaluation of wettability of the surface of strain *Hypocrea lixii (Trichoderma harzianum)* (C1.1). **A:** corresponds to growth in the absence of light. **B:** corresponds to growth in the presence of light. Each plate was divided into three areas, respectively being in a clockwise direction. **A1, B1:** water droplets. **A2, B2:** water droplets with EDTA. **A3, B3:** drops of water with SDS.

For *Bjerkandera sp.* BOS55 (L3) strain, it was confirmed that the mycelium grown in these conditions doesn't present surface hydrophobicity, since all droplets applied on the surface were absorbed (Fig. 3.4). No difference was observed on the effect of light.

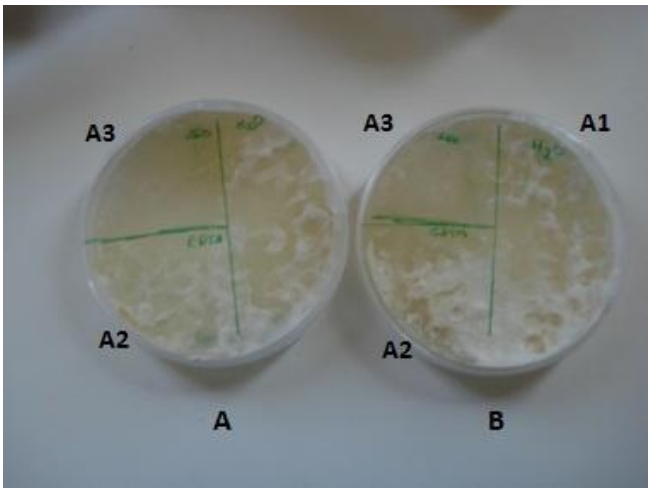

Figure 3.4. Evaluation of wettability of the surface of strain *Bjerkandera sp*. (L3). **A**: corresponds to growth in the absence of light. **B**: corresponds to growth in the presence of light. Each plate was divided into three areas, respectively being in a clockwise direction. **A1, B1:** water droplets. **A2, B2**: water droplets with EDTA. **A3, B3:** drops of water with SDS.

Strain *Mucor circinelloides* (L7) showed the highest hydrophobicity at the growth surface with the total absence of absorption of droplets in all cases (Fig. 3.5). Both **i**n the presence (A) or in the absence of light (B) no drops added to the medium were absorbed, being the surface of the hyphae totally impermeable/hydrophobic.

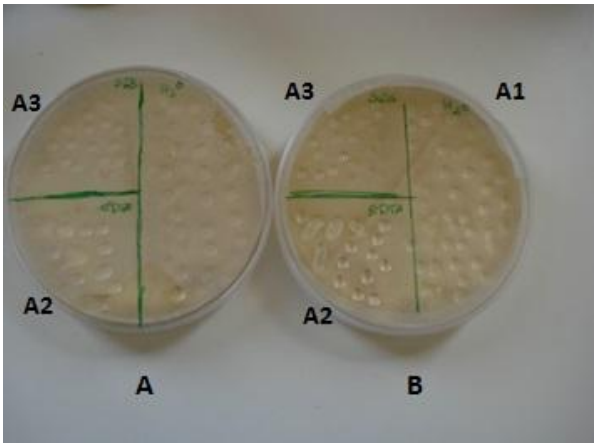

Figure 3.5. Evaluation of wettability of the surface of strain *Mucor circinelloides* (L7). **A:** corresponds to growth in the absence of light. **B**: corresponds to growth in the presence of light. Each plate was divided into three areas, respectively being in a clockwise direction. **A1, B1:** water droplets. **A2, B2:** water droplets with EDTA. **A3, B3:** drops of water with SDS.

The three strains tested present different results for the performed assay. For in *Hypocrea lixii (Trichoderma harzianum)* (C1.1), the spores covered part of the surface of hyphae and as they are hydrophobic, prevented the droplets absorption. The fungal mycelia doesn´t appear so hydrophobic, since partial absorption was detected in regions with less quantity of spores.

Strain *Bjerkandera sp*. (L3) didn't show any hydrophobicity of the mycelia, and also didn´t produce any spores.

For strain *Mucor circinelloides* (L7) there were no differences in the presence and absence of light, and this was the strain with the best results in this test.

3.2.4. Assessment of conidial hydrophobicity

The measurement of the rate of hydrophobicity of conidia was performed with a method adapted from a Shan (2010), using the following equation:

$$Hr = \left(1 - \frac{C}{C0}\right) \times 100$$

Hr is the conidia hydrophobicity rate

C is the residual concentration of spores in the aqueous phase after partitioning,

C0 is the concentration of spores in the aqueous phase before addition of the paraffin.

The hydrophobicity of conidia was determined as a percentage for the two strains which produce spores on solid medium under the conditions tested, strain *Hypocrea lixii (Trichoderma harzianum)* (C1.1) and *Mucor circinelloides* (L7). For these strains the hydrophobicity values obtained were respectively 62% and 76%.

3.2.5. Surface tension

The results of surface tension measurements are tabulated in Table 3.5. The values correspond to the percentages of reduction of surface tension in relation to the control of 50 mM phosphate buffer, pH 7.0. The mean value for surface tension control was 72 $mN.m^{-1}$. The values obtained are within the range of values expected for the effect of hydrophobins according to the bibliography (Wösten, 2001; Linder et al., 2009).

Table 3.5. Surface tension measurements for fungal isolates.

Strain	Method[a]	Mean value of surface tension (mN/m)	Mean value of surface tension decrease (%)
***Bjerkandera sp*. BOS55 (L3)**	DI	53	26
	DI A	61	15
	CA	56	22
	CA A	49	32
	U	50	31
	UA	38	47
***Mucor circinelloides* (L7)**	DI	49	32
	DI A	48	33
	CA	55	24
	CA A	46	36
	U	42	42
	UA	41	43
***Hypocrea lixii (Trichoderma harzianum)* (C1.1)**	U	40,5	44
	UA	39	42

[a]**DI**: dry ice; **CA**: Compressed air; **U**: ultraturrax; **A**: agitation during fermentation

The dry ice foam formation method, produced large amounts of foam, but the bubbles liquefied very rapidly. Another disadvantage of this method is also the high cost. On the other hand, the compressed air method (perforated tube with a nitrogen flow) was time consuming and the bubble collection was difficult. The bubbling using ultra-turrax stirrer not only was a quick and simple method, but also performed better results since by comparison with the others, the surface tension decreased slightly more.

3.2.6. Scanning electron microscopy (SEM)

Spores, agglomerate of hyphae (mycelium) and samples of individual hyphae were observed using SEM for surface characterization.

Results of surface characterization are tabulated in Table 3.6. Original images are in Appendix III.

Table 3.6. Spores, mycelia and individual hyphae surface characterization

Strain	Method	Treatment[a]	Surface appearance[b]
Bjerkandera sp. BOS55 (L3)	Individual hyphae	WT	Irregular
		SDS	
		SDS FA	Smooth
	Agglomerate hyphae	WT	Irregular
		SDS	
		SDS FA	Smooth
	Spores	WT	Not clear
		SDS	
		SDS FA	
Mucor circinelloides (L7)	Individual hyphae	WT	n.d.
		SDS	
		SDS FA	
	Agglomerate hyphae	WT	Irregular
		SDS	
		SDS FA	Smooth
	Spores	WT	Not clear
		SDS	
		SDS FA	
Hypocrea lixii (Trichoderma harzianum) (C1.1)	Individual hyphae	WT	Irregular
		SDS	
		SDS FA	Smooth
	Agglomerate hyphae	WT	Irregular
		SDS	
		SDS FA	Smooth
	Spores	WT	Not clear
		SDS	
		SDS FA	

[a]**WT**: without treatment, **SDS**: treatment with 2% SDS, **SDS FA**: treatment with 2% SDS and formic acid ; [b]**n.d.:** not determined

Technical restriction related to equipment didn't allow the acquisition of high amplification and quality images to compare changes in spore surface subjected to different treatments. Likewise, for strain L7, it was not even possible to obtain percetible images of individual hyphae.

When comparing results for mycelia, it was possible to observe that the untreated hyphal surface has a rough and irregular appearance due to the possible formation of aggregate layers of hydrophobins. When samples were treated with 2 % SDS, the surface hyphae appearance did not change, probably due to the fact that 2 %

SDS won't eliminate hydrophobins. Conversely, all the samples treated with 2 % SDS and formic acid showed a very smooth surface. This result might be consequence of the elimination of the hydrophobin surface layer in the hyphae by the use of a detergent (SDS) followed by a strong acid such as formic acid.

3.3. Total protein quantification

The concentration of hydrophobin mixtures with Amicon Ultra Centrifugal Filter Devices (Millipore) with cut-off 3 KDa and 30 KDa, allowed the incorporation of hydrophobins in a biological pH buffer and removal of the impurities and contaminant salts lower than 3 KDa and higher than 30 KDa.

Determination of protein concentration was carried out with Pierce BCA Protein Assay Kit adapted to hydrophobic proteins. Solutions of each hydrophobin were read on a spectrophotometer at 562 nm.

The standard BSA curve (Appendix III) yield the straight line equation, $Y = 0{,}0013x + 0{,}0414$ used for calculation of the concentration of hydrophobin mixtures (Table 3.7).

Table 3.7. Protein concentrations of hydrophobin mixtures.

Strain	**Concentration (µg.ml^{-1})**
Bjerkandera sp. **BOS55** (L3)	40,5
Mucor circinelloides (L7)	92,8
Hypocrea lixii (Trichoderma harzianum) (C1.1)	51,2

3.4. Hydrophobin purification

In the HPLC chromatograms it is possible to observe the similarities and differences of the location and shape of some peaks between the three samples (Fig. 10, 11, 12). In all cases the measured absorbance is very low.

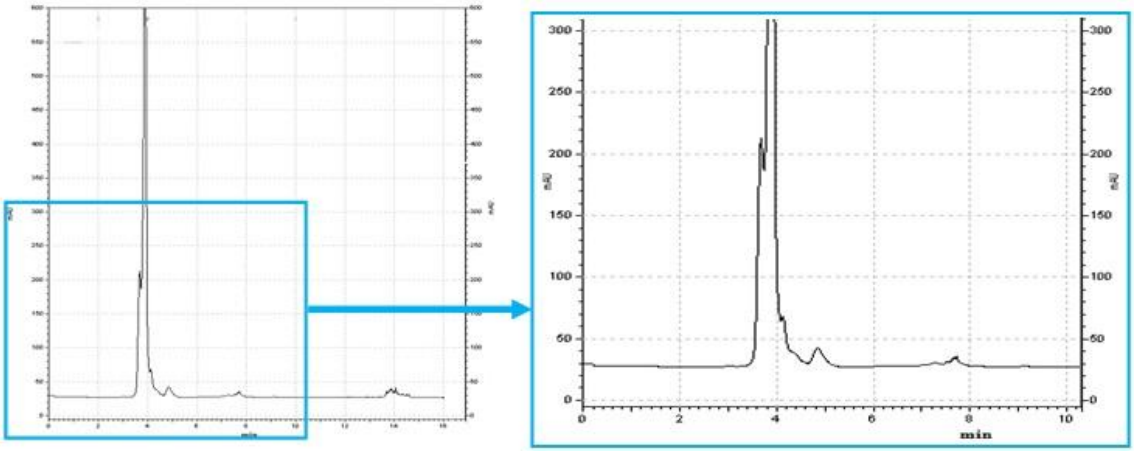

Figure 3.6. Chromatogram of the hydrophobin mixture from *Bjerkandera sp.* BOS55 (L3) measured at 280 nm.

In *Bjerkandera sp.* BOS55 (L3) chromatogram (Fig.3.6) two well-defined peaks are visible, one peak close to 5 min and another at approximately 8 min.

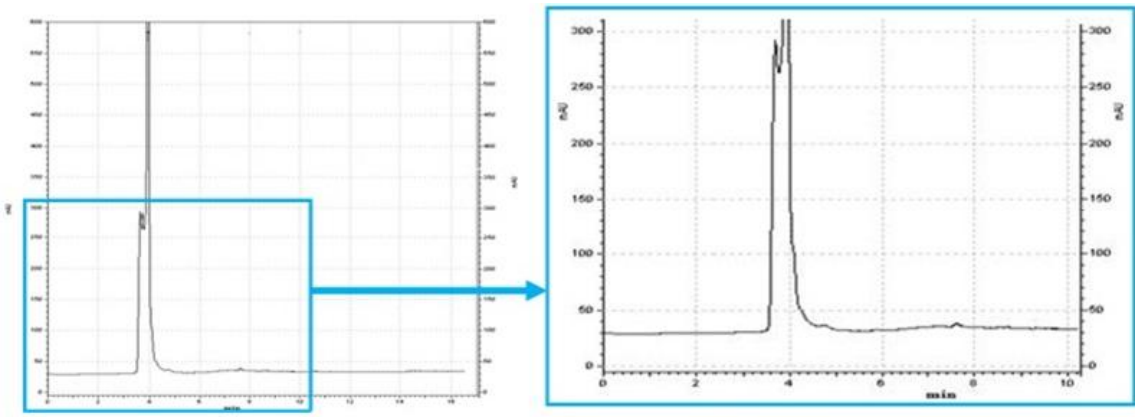

Figure 3.7. Chromatogram of the hydrophobin mixture from *Mucor circinelloides* (L7) measured at 280 nm.

In *Mucor circinelloides* (L7) chromatogram (Fig.3.7) no relevant peaks were visible. This may be explained by a very low protein concentration in the sample, since we combined a pool of extractions obtained from different batches, and possibly final concentration was lower than the limit required to be detected by HPLC. Further studies are required to purify enough quantities to characterize.

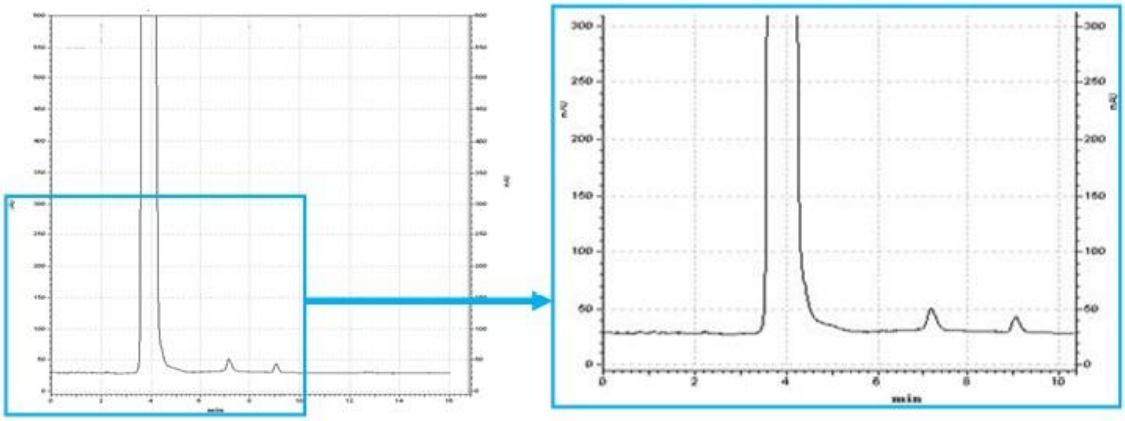

Figure 3.8. Chromatogram of the hydrophobin mixture from *Hypocrea lixii (Trichoderma harzianum)* (C1.1) measured at 280 nm.

For the *Hypocrea lixii (Trichoderma harzianum)* (C1.1) isolate, the chromatogram (Fig.3.8) shows two well-defined peaks, one peak close to 7 min and another at approximately 9 min.

The peaks present in all samples between 3 and 5 min probably correspond the hydrophobic proteins, possibly hydrophobin polymers.

When comparing *Bjerkandera sp.* BOS55 (L3) and *Hypocrea lixii (Trichoderma harzianum)* (C1.1) chromatograms, it's possible to verify that although the peaks that appear latter, between 5 and 9 min have a slightly different retention times, they possibly correspond to the similar proteins with hydrophobic characteristics as expected from hydrophobins.

3.5. Determination of antimicrobial activity

Determination of minimum inhibitory concentration (MIC) for *C. albicans* was performed with hydrophobin concentrations ranging from 0.03 $mg.ml^{-1}$ to 0.20 $mg.ml^{-1}$. Two controls were simultaneously performed: one with 0.2 $mg.ml^{-1}$ hydrophobin but without inoculum, and another where the hydrophobin was replaced by sterile water and inoculated. Another control also used was an elixir that has been shown to possess good antibacterial activity. In Fig. 3.9, 3.10, 3.11, 3.12, 3.13, 3.14 and 3.15 it is possible observe the different antimicrobial activity of the samples tested and elixir on *C. albicans.*

Antimicrobial activity was tested in yeast *C. albicans* as oral marker, because

they cause oral infections. *Candida* is an opportunistic pathogen, causing infections in immunocompromised people and in some cases when natural microbiota is altered.

It should be also tested the *Streptococcus mutans* as bacterial marker in the future to evaluate the actual effect on oral health. This bacterium is common in the human mouth and is the main factor in the development of caries.

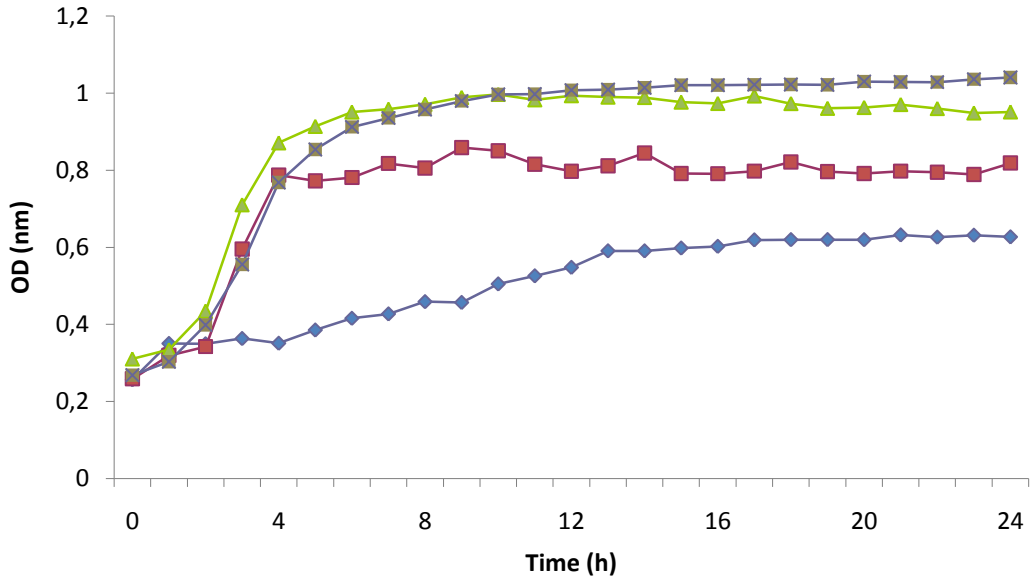

Figure 3.9. Inhibition growth curves obtained for different percentages of hydrophobin mixture from *Bjerkandera sp*. BOS55 (L3) (0.2 mg.ml^{-1}) upon *C. albicans.* C+ Control with medium and inoculum; 12,5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) *Bjerkandera sp.* BOS55 (L3); 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) *Bjerkandera sp.* BOS55 (L3); 100 Medium with inoculum and 98% (0.2 mg.ml^{-1}) *Bjerkandera sp.* BOS55 (L3).

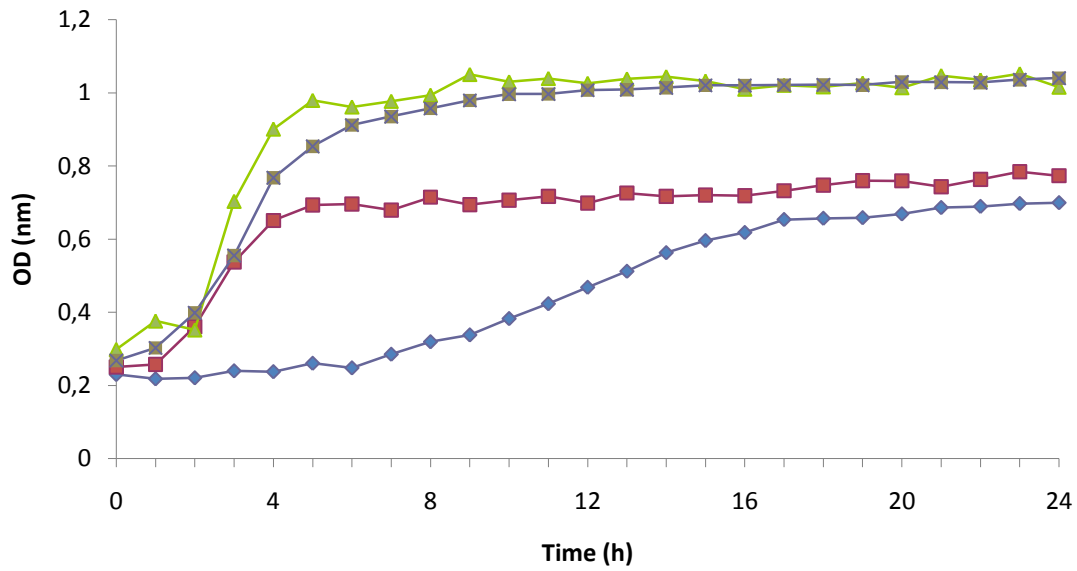

Figure 3.10. Inhibition growth curves obtained for Mucor *circinelloides* (L7) upon *C. albicans*. C+ control with medium and inoculum; 12,5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) *Mucor circinelloides* (L7); 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) *Mucor circinelloides* (L7); 100 inoculum and 98% (0.2 mg.ml^{-1}) *Mucor circinelloides* (L7).

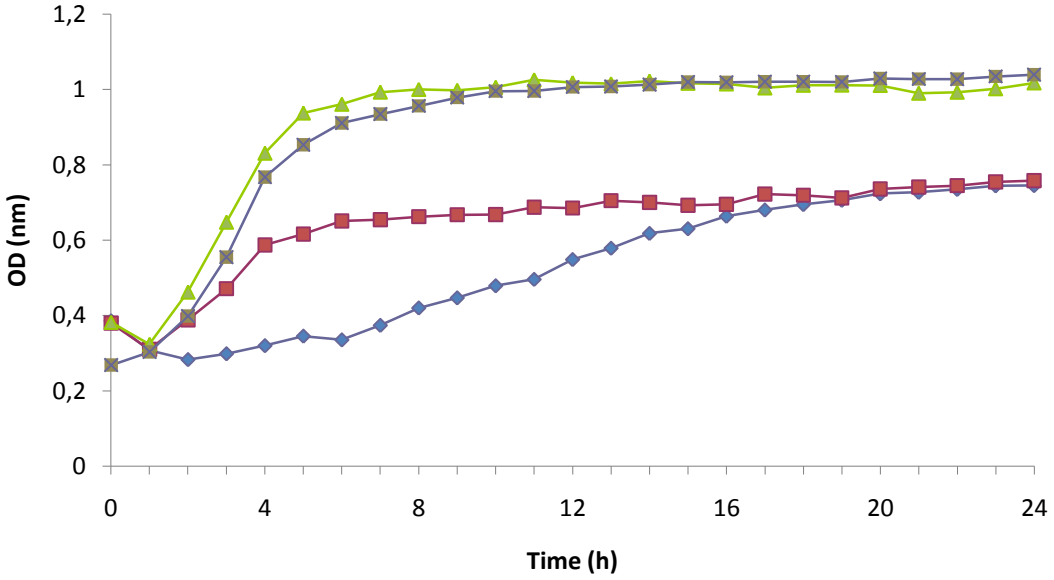

Figure 3.11. Inhibition growth curves obtained for hydrophobin mixture from *Hypocrea lixii (Trichoderma harzianum)* (C1.1) upon *C. albicans*. C+ Control with medium and inoculum; 12,5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) *Hypocrea lixii (Trichoderma harzianum)* (C1.1); 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) *Hypocrea lixii (Trichoderma harzianum)* (C1.1); 100 inoculum and 98% (0.2 mg.ml^{-1}) *Hypocrea lixii (Trichoderma harzianum)* (C1.1).

When testing hydrophobins mixtures from all strains (Fig. 3.9, 3.10 and 3.11) in *C. albicans* it was observed that in general the solution at concentrations higher than 0.1 mg.ml^{-1}possess capacity to reduce microbial growth, which increase with increasing concentrations. Although these concentrations allow partial inhibition, total inhibition was not observed for the highest concentration tested *(*0.2 mg.ml^{-1} of mixed hydrophobins), and for that reason no MIC was determined in this range of concentration.

We've tested a prototype elixir – QUITORAL (Fig. 3.12) proving efficient antimicrobial activity (Costa et al, 2013). In this case different concentrations of elixir were tested upon *C. albicans* and MICs could be achieved, showing efficiency in the range of concentrations tested, to a limit of elixir dilution of 12.5%.

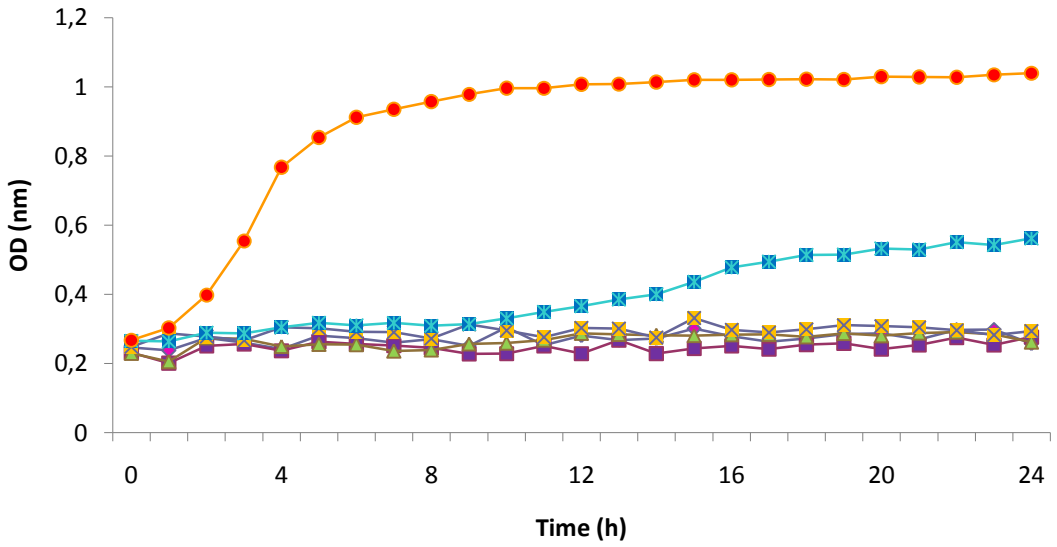

Figure 3.12. Inhibition growth curves obtained for control sample (Quitoral elixir) upon *C. albicans*. C+ control with medium and inoculum; 12,5 Medium with inoculum and 12.5 % elixir (0.03 mg.ml $^{-1}$); 25 Medium with inoculum and 25 % elixir (0.05 mg.ml^{-1}); 50 Medium with inoculum and 50 % elixir (0.1 mg.ml^{-1}); 75 Medium with inoculum and 75 % elixir (0.15 mg.ml^{-1}); 100 inoculum and 98 % elixir (0.2 mg.ml^{-1}).

Since some combination of different antimicrobial solutions may be synergetic or combine activities, we tested the incorporation of hydrophobin mixtures in Quitoral elixir to assess if the interaction with matrix could promote the antimicrobial activity or on other hand could have an antagonist effect. The oral elixir was tested with a mixture of hydrophobins of all strains (Fig. 3.13, 3.14, 3.15) and results showed that there was a slight reduction in final effectiveness of oral elixir with mixture of hydrophobins at 0.03 mg.ml^{-1}, but not significantly for the overall effect. Although the antimicrobial activity is not potentiated, the hydrophobins can be introduced as reinforcement of antibiofilm properties that are relevant to avoid the biofilm formation in teeth, a promoting factor of caries.

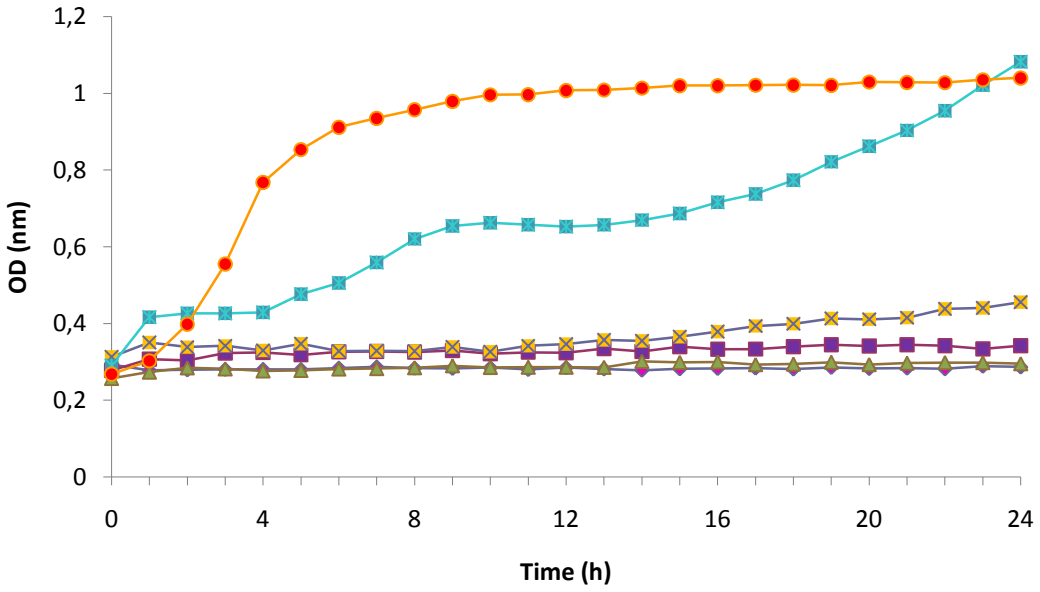

Figure 3.13. Inhibition growth curves for *Bjerkandera sp.* BOS55 (L3) (0.2 mg.ml^{-1}) and Quitoral elixir upon *C. albicans*. C+ control with medium and inoculum; 12.5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) hydrophobin mixture and elixir; 25 Medium with inoculum and 25% (0.05 mg.ml^{-1}) hydrophobin mixture and elixir; 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) hydrophobin mixture and elixir; 75 Medium with inoculum and 75% (0.15 mg.ml^{-1}) hydrophobin mixture and elixir; 100 inoculum and 98% (0.2 mg.ml^{-1}) hydrophobin mixture and elixir.

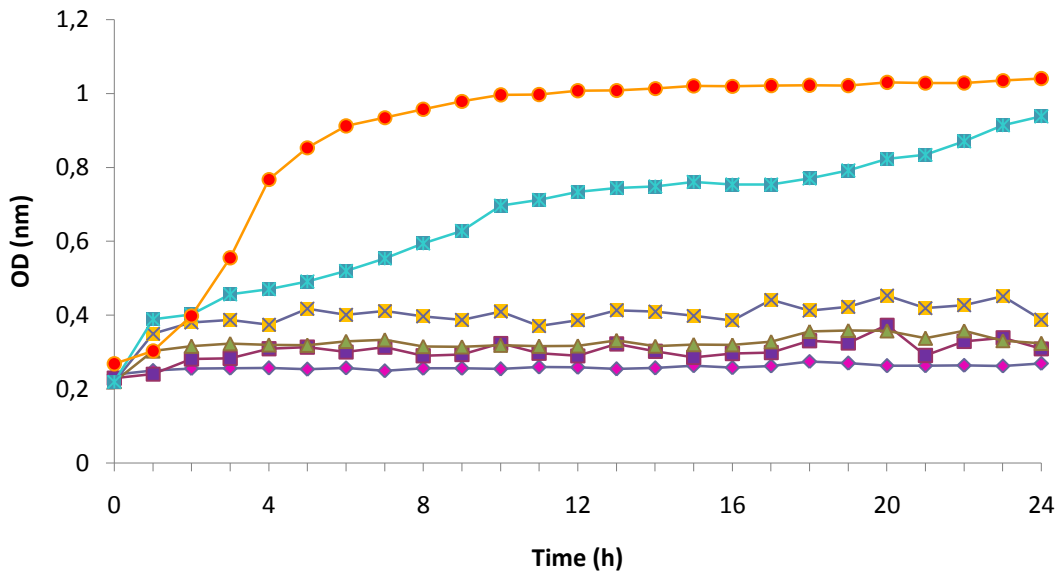

Figure 3.14. Inhibition growth curves obtained for hydrophobin mixture from *Mucor circinelloides* (L7) (0.2mg.ml^{-1}) and Quitoral elixir upon *C. albicans*. C+ control with medium and inoculum; 12.5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) hydrophobin mixture and elixir; 25 Medium with inoculum and 25% (0.05 mg.ml^{-1}) hydrophobin mixture and elixir; 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) hydrophobin mixture and elixir; 75 Medium with inoculum and 75% (0.15 mg.ml^{-1}) hydrophobin mixture and elixir; 100 inoculum and 98% (0.2 mg.ml^{-1}) hydrophobin mixture and elixir.

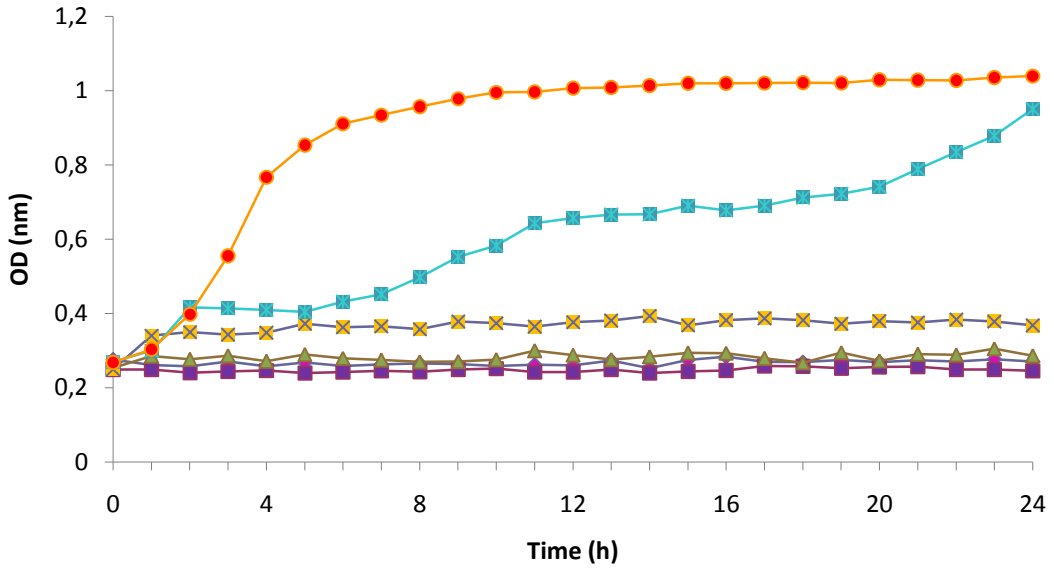

Figure 3.15. Inhibition growth curves obtained for *Hypocrea lixii (Trichoderma harzianum)* (C1.1) (0.2mg.ml^{-1}) and Quitoral elixir upon *C. albicans*. C+ control with medium and inoculum; 12.5 Medium with inoculum and 12.5% (0.03 mg.ml^{-1}) hydrophobin mixture and elixir; 25 Medium with inoculum and 25% (0.05 mg.ml^{-1}) hydrophobin mixture and elixir; 50 Medium with inoculum and 50% (0.1 mg.ml^{-1}) hydrophobin mixture and elixir; 75 Medium with inoculum and 75% (0.15 mg.ml^{-1}) hydrophobin mixture and elixir; 100 inoculum and 98% (0.2 mg.ml^{-1}) hydrophobin mixture and elixir.

Although we were unable to obtain MIC of any mixtures of hydrophobins, it was found that all reduce the concentration of microorganisms in the medium at concentrations greater than 0.1 mg.ml^{-1}. Though antimicrobial activity was not very effective in the range of concentrations tested as it is the oral elixir case, the potential anti-biofilm activity of mixtures of hydrophobins may be conciliated with base antimicrobial activity of natural elixir.

3.6. Microtiter-plate test for biofilm inhibition

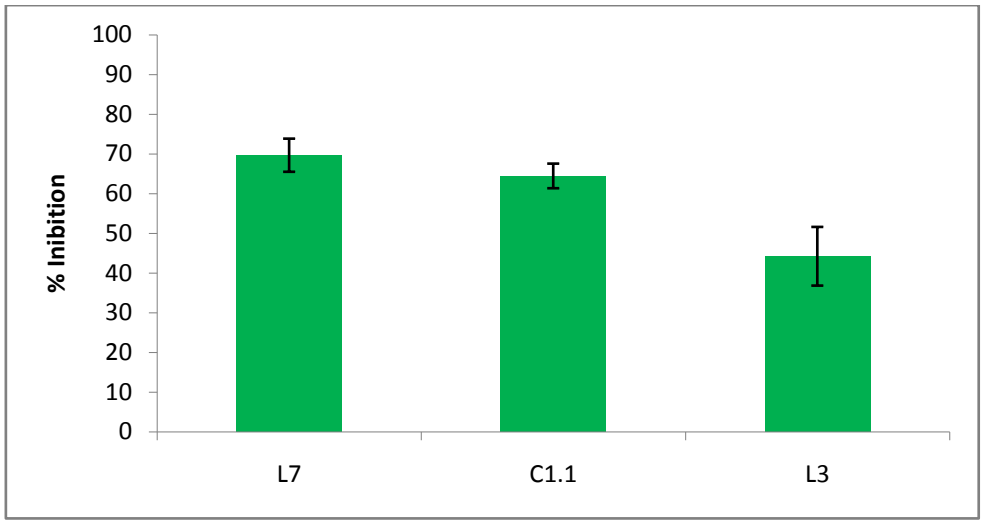

Figure 3.16. Inhibition of biofilm formation for *Candida albicans* with hydrophobin mixtures from the three isolates. **L7:** *Mucor circinelloides;* **C1.1:** *Hypocrea lixii (Trichoderma harzianum);* **L3:** *Bjerkandera sp. BOS55*.

Mixtures of hydrophobins were determined to be active in the inhibition of biofilm formation for *C. albicans* at 0.1 $mg.ml^{-1}$ (of half of the maximum concentration tested for MIC assays), having obtained inhibition values between 40 and 70 %, with the protein solution obtained from strain *Mucor circinelloides* (L7), standing out with the highest percentage compared to the other strains. It is the first time that such activity is reported for hydrophobins.

During the test it was possible to visually the formation of a macroscopic protein film that could be the cause for the inhibition occurred.

4. CONCLUSIONS

The main purpose of this study was to produce and characterize hydrophobins with potential applications in oral medicine. The strategy for selection of the production fungi was based in traditional methods for fungal growth on solid medium, their differentiation and selection criteria based on morphology, growth, production of aerial hyphae and origin of the sample. One strain identified as *Bjerkandera sp.* BOS55 (L3) from the laboratory collection was also used and tested, because no available information existed on a possible production of hydrophobins by this genus. Based on this, it was possible to make a selection of 3 strains (L3, L7 and C1.1).

The use of molecular biology methodologies was crucial to identify strains and to confirm differences detected earlier. The strain L7 isolate from Escola Superior de Biotecnologia garden trees was identified as *Mucor circinelloides* and the strain C1.1 as *Hypocrea lixii (Trichoderma harzianum)*.

For the biochemical characterization of the hydrophobins several parameters were tested. When testing foam stability of the extracellular fluid of each strain, it was found that the results showed large standard deviations, maybe due to the typical very heterogeneous growth in liquid of filamentous fungi and hence didn't allow any conclusions to be drawn. However there was a tendency, for solutions of hydrophobins that were shaken and heated to 100 °C to perform better and the strain with the best results was *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1). The increase in foam stability with agitation may be related to the release of hydrophobins into the extracellular environment as a consequence of action and protection for the mechanical stress induced by agitation in the hyphae and spores. Furthermore there is also an increase in foam stability with heat treatment, probably because hydrophobins are very difficult to denature and thermal stress may induce conformational changes.

In the oil spreading assay, only two strains showed ability for displacement of oil associated with biosurfactant activity production in extracellular fluid. The strain with better results was again *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1). Likewise, for the mycelium wettability test, the mycelium with more impermeable / hydrophobic was the same strain.

On the other hand, in the assessment of conidial hydrophobicity test, strain *Mucor circinelloides* (L7) was evaluated as having more hydrophobic conidia than strain *Hypocrea lixii (Trichoderma harzianum)* (C1.1).

By measuring the change in surface tension for hydrophobin solutions, it was found that the strain *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1) hydrophobin solutions was the most effective. The values obtained are within the range of values expected for the effect of hydrophobins according to the bibliography. Both strains *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1) and *Mucor circinelloides* (L7) significantly decreased surface tension values. For the first time strain *Bjerkandera sp. BOS55* (L3) decrease of the surface tension was reported. There is a trend relationship between the decline of the surface tension and the protein concentrations expected, and its presence in higher concentration results in a greater decrease in surface tension.

In SEM visualization, in different types of samples, the results indicate that in the surface of the sample could be a layer of hydrofobins only extracted / removed in the presence of a strong acid, formic acid. Hydrophobins present may belong to the class I because class I form self-assemble rodlets soluble in trifluoroaceticacid (TFA) and formic acid (FA) (Linder, 2009; Rocha-Pino et al., 2011), whereas class II are easily dissolved in ethanol or sodium dodecyl sulfate (SDS) (Rocha-Pino et al., 2011).

The amount of protein in samples as determined by Pierce BCA protein kit was in the order of $\mu g.ml^{-1}$ that illustrates the difficulty of extracting these proteins as well as the large-scale production of them. Such small amount of protein didn't allow performance of all idealized tests.

The HPLC chromatograms showed that *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1) and *Bjerkandera* sp. BOS55 possessed well-defined peaks with similar retention times which seem to belong to similar proteins with hydrophobic characteristics, as expected from hydrophobins. This result is not directly related to the concentration of hydrophobins determined with Pierce BCA protein kit. The concentration of samples was performed with the intention of obtaining more concentrated solutions but the amount of protein present was not measured.

In determining of minimal inhibitory concentration, there was some inhibition of the antimicrobial effect of Quitoral elixir in the presence of hydrophobins mixture. This elixir has been used because it has been shown to possess good results in reducing antibacterial activity. When the elixir and the hydrophobin mixture were together, a reduction of anti-microbial activity was observed. However, it was also possible to verify the antimicrobial effect of the solutions of the hydrophobin proteins by themselves. Again, the strain *Hypocrea lixii* (*Trichoderma harzianum*) (C1.1) has

demonstrated better results which was verified by a greater reduction in bacterial growth, since all protein mixtures were used with the same concentration

Solutions of hydrophobins also inhibited biofilm formation in *C. albicans* at 0.1 $mg.ml^{-1}$. This activity in hydrophobins was described for the first time with o this research results.

Although the measured absorbance is very low due to the low concentration of the sample used, for the first time hydrophobins were described for the *Bjerkandera* genus. The amount is too small to make further characterization, but attempts for partial sequencing with MALDI-TOF are underway.

5. FURTHER WORK

Hydrophobins exhibit great potential due to their capability for coating surfaces of hydrophobic / hydrophilic characteristics, changing their nature, making this subject of particular interest to the dental industry. To further advance on this research subject it is suggested to produce larger quantities of protein to be able to:

i. Repeat HPLC runs with more concentrated samples in order to be able to collect and purify larger quantities of protein;
ii. Perform SDS-PAGE and Maldi-Tof for the identification and full characterization of the proteins;
iii. Perform tests for inhibition of biofilms with representative of normal microflora in the mouth (*Streptococcus, Lactobacillus, Actinomyces, Bacteroides, Veillonella, Neisseria, Haemophilus, Fusobacterium, Treponema, Staphylococcus, Corynebacterium and Candida);*
iv. Apply and test hydrophobins in real samples of teeth.

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Appendix I

Table 1: List of hydrophobin patents

Patent	Date of publication	Applicant	Title
US2399161	30 April 1946	Claude R Wickard	Process for producing glues and adhesives from keratin protein materials
US3751280	7 August 1973	Ici Ltd	Method of producing a photographic film base having a subbing layer
US4129706	12 December 1978	Basf Aktiengesellschaft	Manufacture of styrene suspension polymers
US4241191	23 December 1980	Basf Aktiengesellschaft	Manufacture of styrene suspension polymers
US5015677	14 May 1991	Bio-Polymers, Inc.	Adhesives derived from bioadhesive polyphenolic proteins
US5049504	17 September 1991	Genex Corporation	Bioadhesive coding sequences
US5110835	5 May 1992	Basf Aktiengesellschaft	Antistatic expandable styrene polymers in bead form
US5290819	1 March 1994	Basf Aktiengesellschaft	Preparation of bead-form expandable styrene polymers
US5859198	12 January 1999	Haber; Meir	Plant proteins
US6977239	20 December 2005	Cognis Deutschland Gmbh & Co. Kg	Detergent tablets
US20030049726	13 Mach 2003	Holloway James L.	Human phermone polypeptide
US20030113454	19 June 2003	De Vocht Marcel Leo	Method of stabilizing a hydrophobin-containing solution and a method of coating a surface with a hydrophobin
US20030134042	17 July 2003	De Vocht Marcel Leo	Method of treating a surface of an object with a hydrophobin-containing solution
US20030217419	27 November2003	L'oreal	Cosmetic use of at least one hydrophobin for treating keratin materials, and compositions used
US20040238170	2 December 2004	Bj Services Company	Aqueous storable cement spacer system and method of making and using the same
US20060040349	23 February 2006	Barry Stieglitz	Thermophilic hydrophobin proteins and applications for surface modification
US20070077619	5 April 2007	Basf Aktiengesellscaft	Secretion of proteins from yeasts
DE2609104A1	15 September 1977	Basf Ag	Method for production of styrene suspension polymers
DE2638839A1	2 March 1978	Basf Ag	Method for production of styrene suspension polymers
DE4220225A1	23 December 1993	Basf Ag	Method for production of styrene suspension polymers
DE19942539A1	8 March 2001	Cognis Deutschland Gmbh	Detergents

DE102004025805A1	29 December 2005	Basf Ag	Cosmetic compound for incorporation in pharmaceutical products for treatment of e.g. human skin, hair and nails
DE102005007480A1	21 September 2006	Basf Ag	New hydrophobin polypeptide not connected in normal manner with an external hydrophobin useful for coating of a glass surfaces
EP0252561A2	13 January 1988	SCLAVO S.p.A.	Expression and secretion vector in yeasts, useful for preparing heterologous proteins
EP0470455A2	12 February 1992	BASF Aktiengesellschaft	Antistatic expansible polystyrene pearls
EP0611824A1	24 August 1994	Institut Francais Du Petrole	Composition comprising a xanthan broth and proteins and use thereof in a well drilling fluid
EP0662515A1	12 Jul 1995	Korea Institute Of Science And Technology	Signal sequences for secretion of heterologous proteins from yeast
EP0773296A1	14 May 1997	Asahi Glass Company Ltd.	Secretory signal gene and expression vector having the same
EP1010748A1	21 June 2000	Ck Witco Corporation	Diesel fuel antifoam composition
EP1223219A2	17 July 2002	Asahi Glass Company Ltd.	Inducible promoter and secretion signal for use in schizosaccharomyces pombe, expression vector containing them and their use
EP1252516B1	14 April 2004	Applied NanoSystems B.V.	Method of treating a surface of an object with a hydrophobin-containing solution
WO1994009094A1	8 Abril 1994	Won Jae Yim	A process for preparing emulsified fuel oil
WO1996041882A1	27 December 1996	Griensven Leonardus Johannes L	Hydrophobins from edible fungi, genes, nucleotide sequences and dna-fragments encoding for said hydrophobins, and expression thereof
WO2000023039A2	27 April 2000	Burt D Ensley	Recombinant hair treatment compositions
WO2000058342A1	5 October 2000	Fagerstroem Richard	Process for partitioning of proteins
WO2001038476A1	31 May 2001	Cognis, Deutschland Gmbh	Detergent tablets
WO2001057066A2	9 August 2001	Applied Nanosystems Bv	Method of stabilizing a hydrophobin-containing solution and a method of coating a surface with a hydrophobin
WO2001057528A1	9 August 2001	Applied Nanosystems Bv	Method of treating a surface of an object with a hydrophobin-containing solution
WO2001060916A1	23 August 2001	Chalen Papier Europ Service	Use of compositions based on proteins, polysaccharides and ethylene oxide

			derivatives as surfactants
WO2001074864A1	11 October 2001	Applied Nanosystems Bv	Protein capable of self-assembly at a hydrophobic-hydrophilic interface and uses thereof
WO2002020651A2	14 March 2002	Zymogenetics Inc	Human phermone polypeptide
WO2002046342A2	13 June 2002	Wolfgang Metzger	Interface-active combination, which is effective in cleaning, which is comprised of renewable raw materials, and which has a high grease solubilizing power
WO2002046369A2	13 June 2002	Davey John	Yeast-based assays involving gpcrs
WO2003010331A2	6 February 2003	Applied Nanosystems Bv	Immobilisation of proteins and electroactive compounds on a sensor surface by means of a hydrophobin coating
WO2003018673A1	6 March 2003	Warren Glen Bryson	The production of biopolymer film, fibre, foam and adhesive materials from soluble s-sulfonated keratin derivatives
WO2003031500A1	17 April 2003	Heinz Axmann	Spray-dried dispersions, method for the production thereof and use of the same
WO2003053383A2	3 July 2003	L'Oreal	Cosmetic use of at least a hydrophobin for treating keratinous materials
WO2003080137A1	2 October 2003 .	Magnus Qvist	Method for attaching two surfaces to each other using a bioadhesive polyphenolic protein and periodate ions
WO2004000880A1	31 December 2003	Applied Nanosystems Bv	Method of binding a compound to a surface
WO2005033316A2	14 April 2005	Basf Ag	Secretion of proteins from yeasts
WO2005068087A2	28 July 2005	Applied Nanosystems Bv	Method for coating an object with hydrophobin at low temperatures
WO2005115306A2	8 December 2005	Barg Heiko	Keratin-binding polypeptides
WO2006082251A2	10 August 2006	Heiko Barg	Novel hydrophobin fusion products, production and use thereof
WO2006082253A2	10 August 2006	Basf Ag	Method for coating surfaces with hydrophobins
WO2006103215A1	5 October 2006	Basf Ag	Use of hydrophobin for hard surface soil-repellent treatment
WO2006103225A1	5 October 2006	Basf Ag	Use of polypeptides in the form of adhesive agents
WO2006103230A1	5 October 2006	Basf Ag	Use of hydrophobins for the surface treatment of hardened mineral building materials, natural stone, artificial stone and ceramics
WO2006103251A1	5 October 2006	Basf Ag	Use of proteins as demulsifying agents
WO2006103252A2	5 October 2006	Basf Ag	Use of hydrophobin as a phase stabiliser

WO2006103253A2	5 October 2006	Basf Ag	Drilling fluid containing hydrophobin
WO2006131555A1	14 December 2006	Basf Ag	Hydrophobin as a coating agent for expandable or expanded thermoplastic polymer particles
WO2006131564A2	14 December 2006	Basf Ag	Novel cysteine-depleted hydrophobin fusion proteins, their production and use thereof
WO2006136607A2	28 December 2006	Basf Ag	Use of hydrophobin-polypeptides and conjugates from hydrophobin-polypeptides having active and effect agents and the production thereof and use thereof in the cosmetic industry
WO2007006765A1	18 January 2007	Basf Ag	Aqueous monomer emulsions containing hydrophobin
WO2007014897A1	8 February 2007	Basf Ag	Use of surface-active non-enzymatic proteins for washing textiles
WO2007042487A2	19 April 2007	Basf Ag	Use of proteins as an antifoaming constituent in fuels

Appendix II

Figure 1: Chromatogram for ITS amplification for L7 strain

Figure 2: Chromatogram for ITS amplifications for C1.1 strain

Appendix III

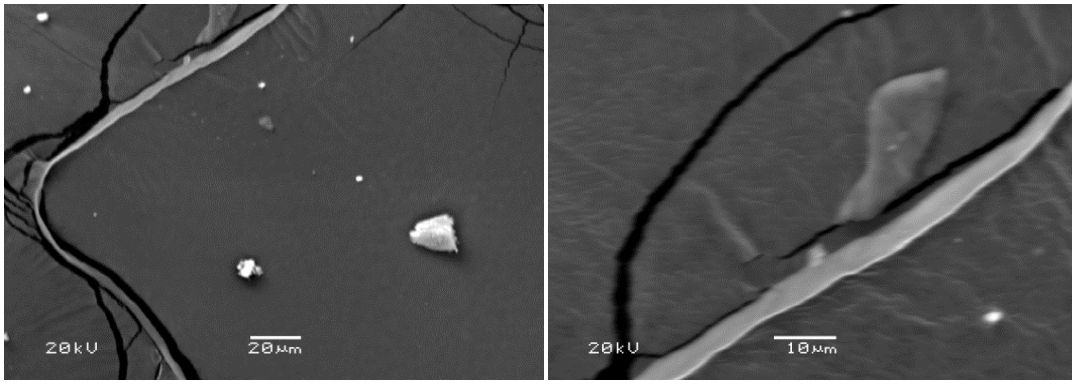

Figure 1: SEM views of the hyphae surface of C1.1 strain without treatment with (x600 and x1500 ampliation)

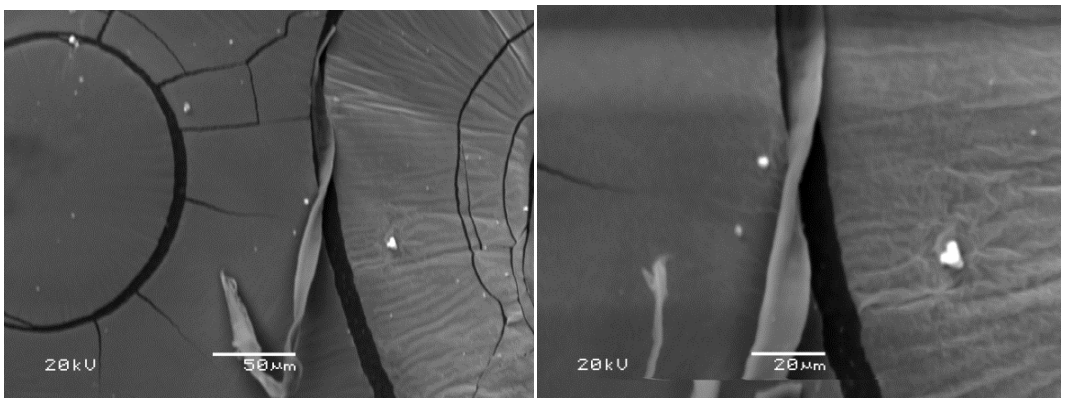

Figure 2: SEM views of hyphae surface of c1.1 strain with 2 % SDS treatment with (x400 and x900 ampliation)

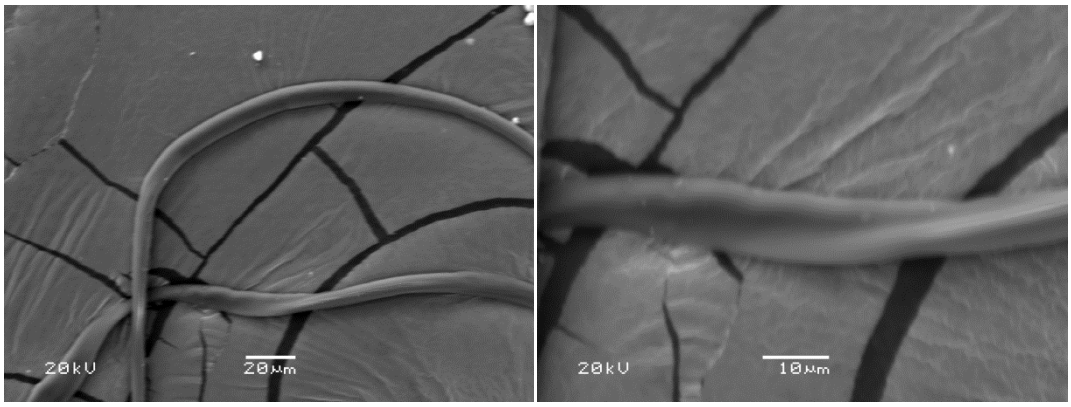

Figure 3: SEM views of hyphae surface of C1.1 strain with 2 % SDS and formic ácid treatment with (x600 and x1600 ampliation)

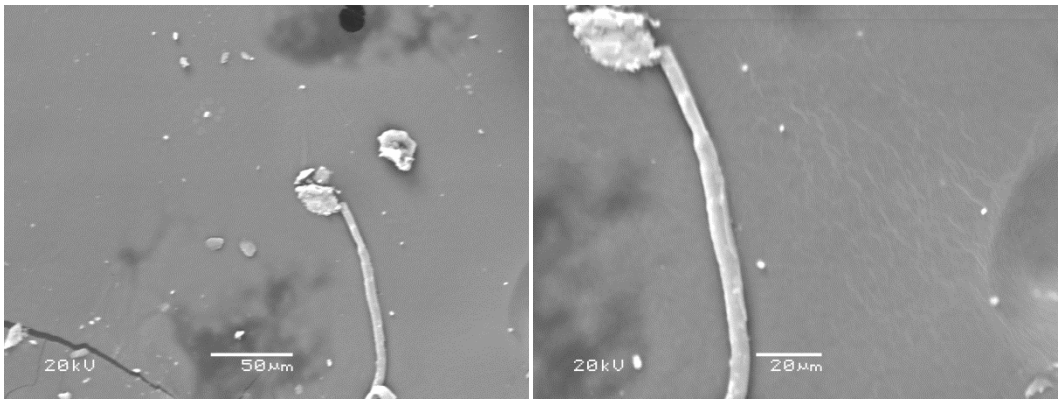

Figure 4 : SEM views of hyphae surface of L3 strain withou treatment (x400 and x800 ampliation)

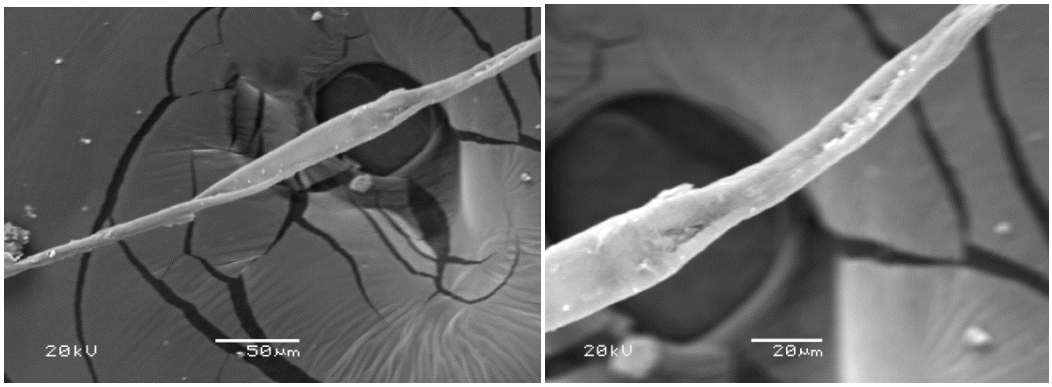

Figure 5 : SEM views of hyphae surface of L3 strain with 2 % SDS treatment with x400 and x 900 ampliation

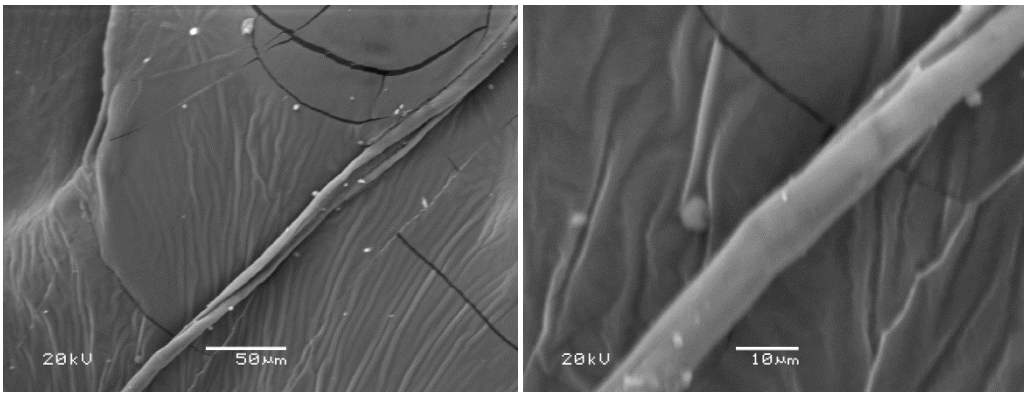

Figure 6: SEM views of hyphae surface of L3 strain with 2 % SDS and formic ácid treatment with (x 400 and x1600 ampliation)

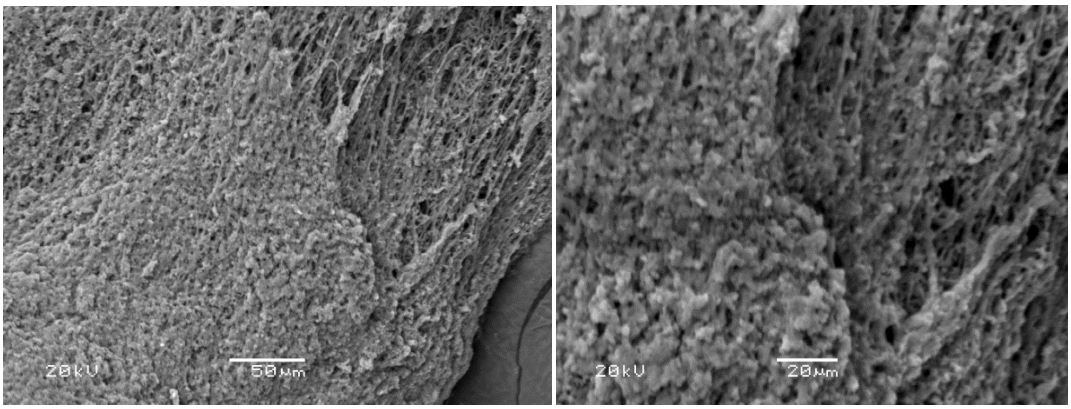

Figure 7: SEM views of surface of aglomerate hyphaes of C1.1 strain without treatment (x350 and x 750 amplification)

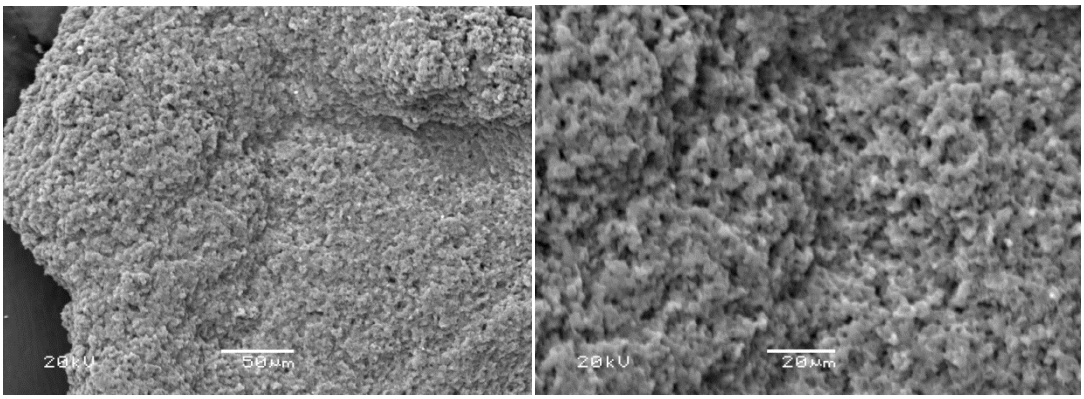

Figure 8 : SEM views of surface of aglomerate hyphaes of C1.1 strain with 2 % SDS treatment (x350 and x 800 amplification)

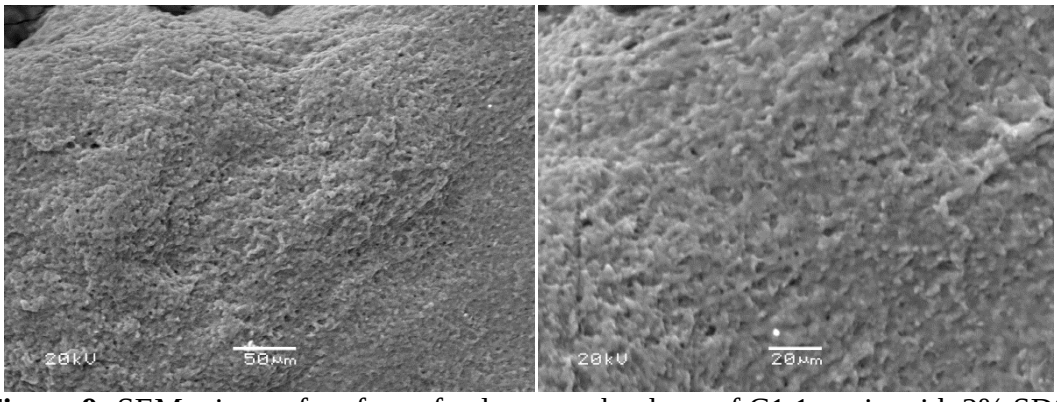

Figure 9: SEM views of surface of aglomerate hyphaes of C1.1 strain with 2% SDS and formic ácid treatment (x 300 and x 650 amplification)

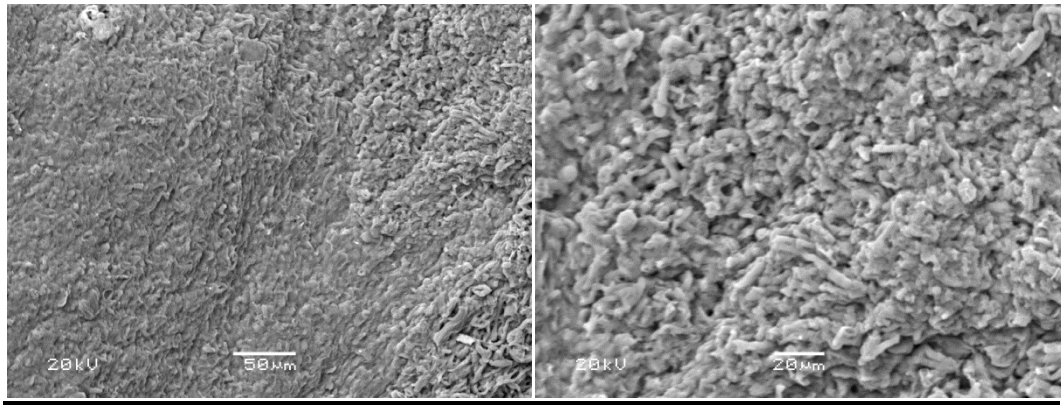

Figure 10: SEM views of surface of aglomerate hyphaes of L7 strain withou treatment (x300 and x600 amplification)

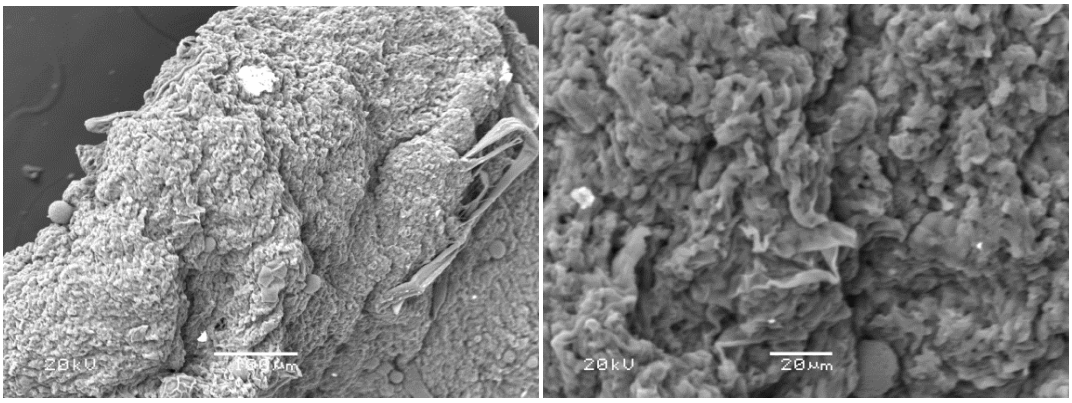

Figure 11: SEM views of surface of aglomerate hyphaes of L7 strain with 2 % SDS treatment (x200 and x 750 amplification)

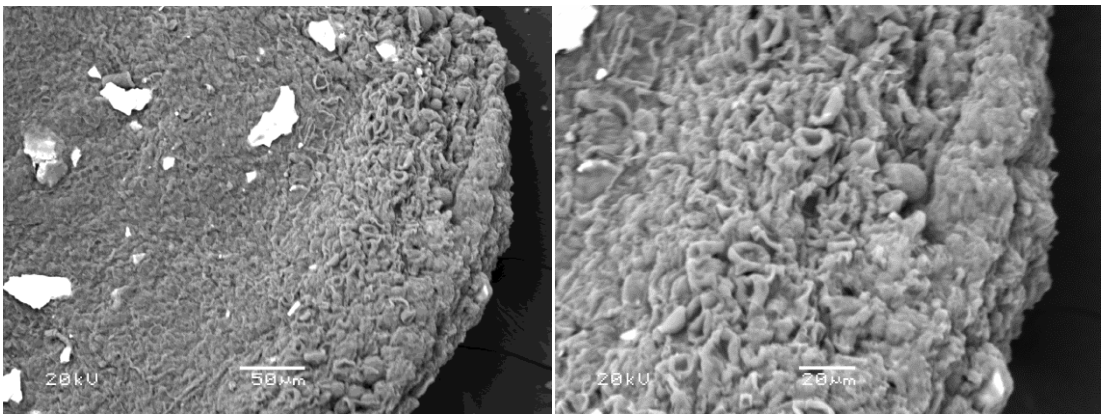

Figure 12: SEM views of surface of aglomerate hyphaes of L7 strain with 2 % SDS and formic ácid treatments (x300 and x 650 amplification)

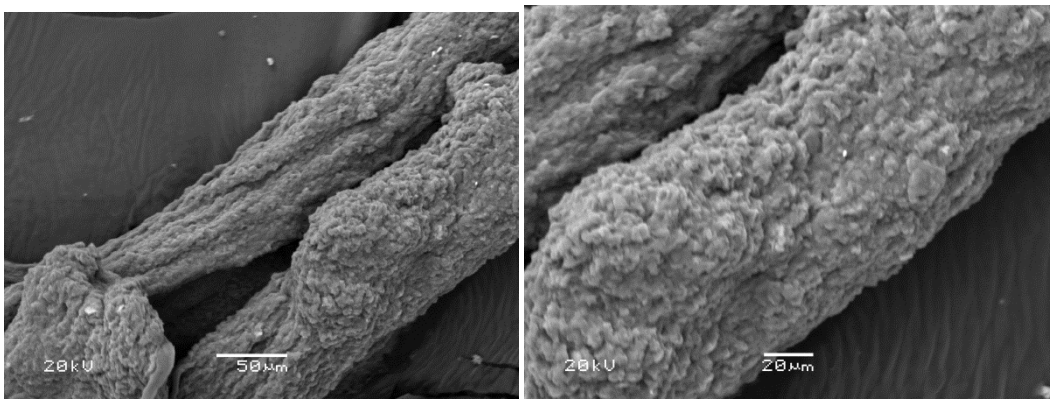

Figure 13: SEM views of surface of aglomerate hyphaes of L3 strain withou treatment (x 350 and x600 amplification)

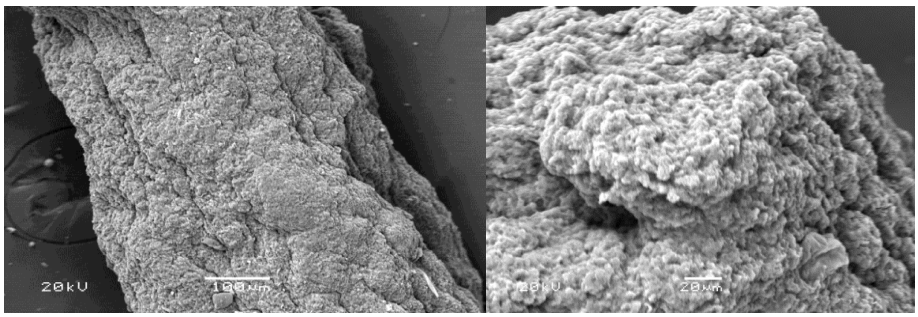

Figure 14 : SEM views of surface of aglomerate hyphaes of L3 strain with 2 % SDS and formic ácid treatment (x 170 and x550 amplification)

Appendix III

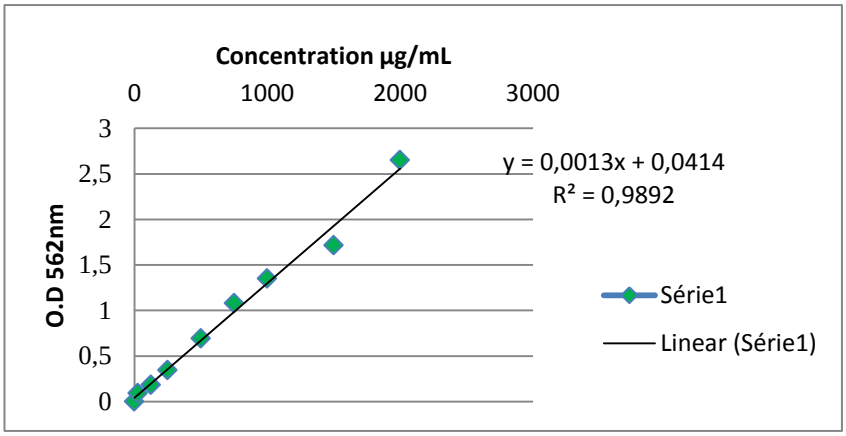

Figure 1: Standard BSA curve

Table 1: Optical absorbance of hydrophobins solutions at 562nm

Strain	O.D. 562nm	Mean values O.D. 562nm
Bjerkandera sp. BOS55 (L3)	0.091	0.094
	0.097	
Mucor circinelloides (L7)	0.173	0.162
	0.150	
Hypocrea lixii (Trichoderma harzianum) (C1.1)	0.122	0.180
	0.094	