Biofilm formation by zygomycetes: quantification, structure and matrix composition

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INTRODUCTION

Biofilms are dense, highly hydrated cell clusters that are irreversibly attached to a substratum, to an interface or to each other, and are embedded in a self-produced gelatinous matrix composed of extracellular polymeric substances (EPS) (Harding et al., 2009). The micro-organisms in these biofilms exhibit an altered phenotype with respect to growth rate, gene transcription, and resistance to physical, chemical and biological stresses (Chandrasekar & Manavathu, 2008; Harding et al., 2009; Mowat et al., 2009). Biofilm formation has been widely implicated in the pathogenesis of implant-associated and chronic infections (Hall-Stoodley et al., 2004).

Most of the work describing biofilm genesis, architecture, chemical composition, genetic regulation and antimicrobial drug resistance has focused on bacteria and yeasts (Chandrasekar & Manavathu, 2008; Harding et al., 2009; Mowat et al., 2009). Occasionally, filamentous fungi have also been reported to form biofilms (Chandrasekar & Manavathu, 2008; Harding et al., 2009; Mowat et al., 2009). Although filamentous fungi often penetrate the substrates that they grow on, and this invasive growth may not accurately represent the term biofilm (Harding et al., 2009), the yeasts Candida albicans and Trichosporon asahii have been shown to require differentiation to hyphal forms during biofilm formation (Harding et al., 2009; Ramage et al., 2009). Filamentation in fungi may therefore be a prerequisite for robust biofilm development and virulence, and fungal biofilms perhaps represent much more than a mere biological coating (Harding et al., 2009). Biofilm formation is claimed to be involved in the pathogenesis of localized as well as invasive diseases caused by Aspergillus fumigatus (Beauvais et al., 2007; Chandrasekar & Manavathu, 2008; Harding et al., 2009; Loussert et al., 2010; Mowat et al., 2007, 2008, 2009). Aspergilloma, a localized infection, has been shown to consist of highly agglutinated hyphae encased in an extracellular matrix (Loussert et al., 2010; Mowat et al., 2009). A similar exopolysaccharide matrix is also produced at the surface of fungal hyphae during invasive pulmonary aspergillosis (Loussert et al., 2010).

Fungi belonging to the class Zygomycetes and the order Mucorales, including Rhizopus, Rhizomucor, Mucor, Lichtheimia, Apophysomyces, Cunninghamella and Saksenaea, often cause opportunistic infections which are similar to those caused by Aspergillus and are characterized by angioinvasion...
and fungal-ball formation (Chakrabarti et al., 2001, 2006, 2009; Goodnight et al., 1993; Kirkpatrick et al., 1979; Lahiri et al., 2001; Robey et al., 2009). Mucormycosis is categorized into rhino-orbito-cerebral (ROC), pulmonary, gastro-intestinal, cutaneous and disseminated types depending upon the clinical presentation and the anatomical sites involved (Chakrabarti et al., 2001, 2006, 2009). While the ROC type is often associated with individuals having uncontrolled diabetes and diabetic ketoacidosis, the pulmonary, gastrointestinal and cutaneous types occur in patients with haematological malignancies or neutropenia, severe malnutrition, and trauma or burns, respectively (Chakrabarti et al., 2001, 2006, 2009). The incidence of mucormycosis has increased globally over the last two decades, with a phenomenal rise in the number of cases reported from India (Chakrabarti et al., 2001, 2006, 2009). Three case series on mucormycosis have been reported from our tertiary-care centre: 129 cases over 10 years (1990–1999), 178 cases during the subsequent five years (2000–2004) and then 75 cases in an 18 month period during 2006–2007 (Chakrabarti et al., 2001, 2006, 2009). Rhizopus oryzae and Apophysomyces elegans (sensu lato) were the predominant isolates in these series (Chakrabarti et al., 2001, 2006, 2009).

Since more than 99 % of microbes have been reported to grow as biofilms, and these surface-attached communities have also been implicated in the pathogenesis of both localized as well as invasive diseases caused by Aspergillus, the present study was planned to elucidate the biofilm-forming potential of four common pathogenic zygomycetes: Rhizopus oryzae, Lichtheinia corymbifera, Rhizomucor pusillus and Apophysomyces elegans.

### METHODS

#### Fungal strains

*Rhp. oryzae* NCCPF 700004, *L. corymbifera* NCCPF 700002, *Rhm. pusillus* NCCPF 720004 and *A. elegans* NCCPF 102033 were used in this study. The strains were obtained from the National Culture Collection of Pathogenic Fungi (NCCPF), Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, and were preserved in 20 % glycerol at −70 °C until use.

#### Isolation of sporangiospores

*Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* were grown on Saboraud dextrose agar (SDA) at 37 °C for 4–5 days. *A. elegans* was cultured on water agar at 37 °C for 7 days. The plates were flooded with 10 ml PBS (0.1 M, pH 7.2) and sporangiospores were harvested by repeated washing of the mycelia with the added buffer. The resulting suspensions were centrifuged at 6000 r.p.m. at 4 °C for 10 min; the pellets were washed twice with PBS and finally suspended in 1 ml PBS. Spores were counted using a Neubauer haemocytometer and counts expressed as c.f.u. ml⁻¹.

#### Standardization of biofilm formation

Biofilm formation was determined according to the method of Pierce et al. (2008). The spores were adjusted to the required density (*Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* – 1 x 10⁶, 1 x 10⁶, 1 x 10⁵ and 1 x 10⁴ c.f.u. ml⁻¹; *A. elegans* – 1 x 10⁶, 1 x 10⁵, 1 x 10⁴ c.f.u. ml⁻¹) in RPMI 1640 (pH 7.2) buffered with MOPS (165 mM). Two hundred microlitres of this suspension was inoculated per well in 96-well, flat-bottomed polystyrene microtitre plates. Media-only blanks were also set up. The plates were incubated at 37 °C for 24 h (up to 48 h for *A. elegans*) and the resulting biofilms were washed twice with PBS, fixed with 200 μl 95 % ethanol at 37 °C for 15 min and stained with 200 μl 0.1 % safranin for 5 min. Biofilm formation was observed under an inverted microscope (Olympus CKX 41) with a x 40 objective lens and was quantified by measuring the absorbance of the bound safranin, eluted with 200 μl 30 % glacial acetic acid, at 490 nm. Experiments were performed in quadruplicate. The cut-off absorbance was calculated as the mean absorbance of the media-only blanks plus three times their SD (A₄₉₀ = 0.075).

#### Adhesion kinetics

Two hundred microlitres of the standardized spore suspension was inoculated per well in 96-well, flat-bottomed polystyrene microtitre plates and incubated at 37 °C for 4, 8, 12 and 24 h. The experiments were performed in quadruplicate and media-only blanks were set up in parallel. At each time point, the biofilms were washed twice with PBS and were quantified as described previously.

#### Confocal laser scanning microscopy (CLSM) of biofilms

Three millilitres of the standardized spore suspension was inoculated in 35 mm Petri dishes and incubated at 37 °C for 24 h. The biofilms formed were washed twice with PBS, and then stained with wheatgerm agglutinin conjugated to Oregon green (WGA-OG, 0.1 mg ml⁻¹; Invitrogen BioServices India) for 15 min (Singh et al., 2010) or with concanavalin A conjugated to Alexa Fluor 488 (Con-A, 50 μg ml⁻¹) and 10 μM FUN-1 (Chandra et al., 2008) (Invitrogen BioServices India) at 37 °C for 30 min in the dark. The biofilms were then washed twice with PBS. CLSM was performed with an LSM 510 Meta (Carl Zeiss Microimaging) attached to an Axioplan II microscope using a ×10/0.3 objective lens.

During CLSM using WGA-OG, HFT 488 was selected as the excitation laser and WGA-OG was detected by fluorescence in the green spectrum using BP505–530. Fungal hyphae were detected by refraction of light in the red spectrum using LP560. NFT 545 was used as the beam splitter and the images were acquired in multitrack mode. For CLSM using dual staining with Con-A/FUN-1, the excitation wavelengths were set to 488 nm and 543 nm for Con-A and FUN-1, respectively. NFT 490 (Con-A) and NFT 545 (FUN-1) were used as the beam splitters, and LP 505 (Con-A) and LP 560 (FUN-1) were used as the emission filters. Image analysis was done using z-series image stacks from four randomly chosen spots of each biofilm and the biofilm architecture and mean thickness were elucidated using LSM image browser version 4.2.0.121 and ZEN 2009.

#### Extraction of biofilm matrix

Ten millilitres of the standardized spore suspension was inoculated per flask in 50 ml polystyrene tissue-culture flasks and incubated at 37 °C for 24 h. The biofilms formed were washed twice with PBS, flooded with 10 ml sterile MilliQ water per flask and vortexed mildly for 30 s. The suspensions were then pooled and centrifuged at 6000 r.p.m. at 4 °C for 20 min. The supernatant was collected, filter-sterilized and treated with 2 volumes of chilled 95 % ethanol overnight at 4 °C. The resulting precipitates were collected by centrifugation at 6000 r.p.m. at 4 °C for 1 h, washed with chilled 95 % ethanol and dried. The EPS matrix was dissolved in water and centrifuged at 6000 r.p.m. at 4 °C for 10 min. The water-soluble fraction (supernatant) was collected and the water-insoluble material (pellet) was then dissolved in trichloroacetic acid (TCA). The experiment was performed three times and the matrix was characterized biochemically each time.

#### Characterization of biofilm matrix

The intracellular enzyme glucose-6-phosphate dehydrogenase (G6PDH) was used as a marker for detecting cell lysis and thereby a contamination of the harvested matrix with cellular components (Flemming & Wingender, 2010). Ten microlitres of the water-soluble fraction was added to 290 μl of
the assay buffer containing 250 mM glycine buffer (pH 7.4), 60 mM glucose 6-phosphate, 20 mM NADP and 300 mM MgCl₂, and incubated at 25 °C for 5 min. The production of NADPH was elucidated by measuring the change in absorbance at 340 nm. A molar absorption coefficient of 6.22 mM⁻¹ cm⁻¹ was used and 1 U GlcPDH was defined as the enzyme activity catalysing the formation of 1 mmol NADPH min⁻¹. Protein concentration was determined in the water-soluble fraction by the bicinchoninic acid method (Smith et al., 1985). Total carbohydrates in the water-soluble fraction were determined by the phenol/sulphuric acid method (Dubois et al., 1951). Glucose was quantified in the water-soluble fraction after acid hydrolysis by using a glucose oxidase kit (Sigma-Aldrich) according to the manufacturer’s instructions. The presence of amino sugars in the water-soluble fraction was detected by UV spectroscopy (Kumirska et al., 2010). Briefly, the UV absorption of the fractions was scanned over 190–350 nm in 1 nm step increments. The first-derivative spectra of the samples were elucidated and compared with those of standard solutions containing N-acetylglucosamine (GlcNAc) and glucosamine (GlcN); GlcNAc only, GlcN only, and GlcNAc and GlcN mixed in different ratios (4:1, 3:2, 1:1, 2:3 and 1:4). The percentage of acetylated and non-acetylated hexosamine in the samples was determined from standard curves of λmin vs percentage GlcNAc as well as calibrated peak height (H) vs percentage GlcNAc. GlcNAc was quantified in the water-soluble and TCA-soluble fractions by enzyme-linked lectin sorbent assay (ELLA) according to the method of Singh et al. (2010), using a standard curve of GlcNAc (μg) vs A₄₀₀. GlcN in the water-soluble fraction was determined by its N-acetylation using acetic anhydride in methanol (Levvy & McAllan, 1959), followed by quantification of the GlcNAc formed by ELLA. All experiments were performed in quadruplicate.

**Statistical analysis.** The two-tailed, unpaired t-test was used for comparing the adhesion capacity of test strains versus the media-only blanks.

**RESULTS AND DISCUSSION**

Mucorales usually cause acute angioinvasive ROC, pulmonary, gastrointestinal or cutaneous infections in immunocompromised patients (Chakrabarti et al., 2001, 2006, 2009). In addition, these fungi have been implicated in central-venous-catheter-associated fungaemia (Chan-Tack et al., 2005), peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (Branton et al., 1990; Fergie et al., 1992; Nakamura et al., 1989; Nannini et al., 2003; Nayak et al., 2007; Polo et al., 1989; Serna et al., 2003), pleuritis following implantation of drainage catheters (Kimura et al., 2009), endocarditis on native (Mehta et al., 2004; Mitchell et al., 2010) and artificial heart valves (Gubarev et al., 2007; Sanchez-Recalde et al., 1999), osteomyelitis (Chaudhuri et al., 1992; Eaton et al., 1994; Lopes et al., 1995; Meis et al., 1994; Wilkins et al., 2009), and paranasal fungal balls (Goodnight et al., 1993; Kirkpatrick et al., 1979; Lahiri et al., 2001; Robey et al., 2009). Many of these infections are associated with biofilm formation (Costerton et al., 1999; Hall-Stoodley et al., 2004; Louisert et al., 2010) and were reported to exhibit a chronic course (Eaton et al., 1994; Lahiri et al., 2001; Lopes et al., 1995). Furthermore, surface-attached growth of zygomycetes has often been used for industrial purposes (Cao et al., 1997). However, biofilm formation by zygomycetes has not yet been demonstrated. This is believed to be the first study to elucidate the biofilm-forming capacity of Rhp. oryzae, L. coryniformis, Rhm. pusillus and A. elegans, and study their adhesion kinetics, biofilm morphology and matrix composition.

**Standardization of biofilm formation**

The spore density of Rhp. oryzae, L. coryniformis and Rhm. pusillus in the initial inoculum was found to be important for germination of the adhered spores as well as structural integrity of the biofilms formed (Fig. 1). Whilst very high seeding density (Rhp. oryzae, 1 × 10⁶ and 1 × 10⁶ c.f.u. ml⁻¹; L. coryniformis and Rhm. pusillus, 1 × 10⁷, 1 × 10⁶ and 1 × 10⁵ c.f.u. ml⁻¹) resulted in poor germination of the adhered spores, low inoculum concentration (Rhp. oryzae, 1 × 10⁴ and 1 × 10⁶ c.f.u. ml⁻¹; L. coryniformis and Rhm. pusillus, 1 × 10⁴ c.f.u. ml⁻¹) reduced the hyphal density. Similar results have been previously reported for Aspergillus fumigatus (Mowat et al., 2007), Trichosporon asahii (Di Bonaventura et al., 2006) and various Candida species including C. albicans (Ramage et al., 2009), and may be associated with the production of quorum-sensing molecules (Ramage et al., 2009). Initial seeding densities of 1 × 10⁵ c.f.u. ml⁻¹ (Rhp. oryzae) and 1 × 10³ c.f.u. ml⁻¹ (L. coryniformis and Rhm. pusillus) produced robust, filamentous adherent structures and were selected for further experiments. At these inoculum concentrations, the mean adherence of Rhp. oryzae, L. coryniformis and Rhm. pusillus on polystyrene plates after 24 h of incubation was 2.06 ± 0.02, 2.74 ± 0.29 and 1.44 ± 0.05 (A₄₀₀, mean ± SD), respectively.

**Apophysomyces elegans** spores germinated at all the three seeding densities tested but adherence and biofilm formation were not detected. No significant difference (P>0.05) was observed between the A₄₀₀ of wells inoculated with A. elegans compared to the cut-off A₄₀₀ for biofilm detection [1 × 10⁷ c.f.u. ml⁻¹, 0.092 ± 0.0007 (24 h) and 0.091 ± 0.0 (48 h); 1 × 10⁶ c.f.u. ml⁻¹, 0.078 ± 0.0 (24 h) and 0.083 ± 0.0007 (48 h); 1 × 10⁵ c.f.u. ml⁻¹, 0.073 ± 0.018 (24 h) and 0.097 ± 0.013 (48 h)]. A. elegans is usually associated with superficial infections in immunocompetent hosts, although infections in deep tissue have also been described (Chakrabarti et al., 2003, 2010). We reported an upsurge of such A. elegans infections over the past decade in India (Chakrabarti et al., 2003, 2010). These findings indicate that biofilm formation may not play an important role in the pathogenesis of A. elegans infections. Species-specific differences in biofilm-forming capacity have also been reported in Candida, and have been correlated with the pathogenesis and the associated risk factors (Hawser & Douglas, 1994; Kuhn et al., 2002; Li et al., 2003; Ramage et al., 2009).

**Adhesion kinetics**

A direct correlation was observed between biomass, quantified by measuring the absorbance of the bound
safranin, and hyphal development (Figs 2 and 3). Following initial seeding, the spores adhered to the polystyrene surface and began to swell and germinate in 4–6 h. Hyphae were observed within 8–10 h of incubation. These hyphae formed monolayers by 10–12 h, and then further intertwined and increased in density over the next 12 h. This pattern resembles the kinetics of biofilm formation in *Aspergillus fumigatus* (Mowat et al., 2007).

The intertwining of the mycelial mass provides stability and integrity to the biofilms and is achieved by extensive branching, followed by elongation of the hyphal branches by apical growth (Chandrasekar & Manavathu, 2008; Mowat et al., 2009).

CLSM of biofilms

Microbial biofilms are characterized by the presence of surface-attached cells enmeshed in a self-produced gelatinous matrix made primarily of polysaccharides (Costerton et al., 1999; Hall-Stoodley et al., 2004). This matrix, also termed slime or EPS, forms a scaffold for the three-dimensional architecture of the biofilm (Flemming & Wingender, 2010; Hall-Stoodley et al., 2004). It provides stability to the biofilm structure, helps in adherence as well as in trapping the nutrients, and also protects the biofilm cells from desiccation, UV radiation, protozoan grazers, antimicrobials and host immune defences (Flemming & Wingender, 2010; Hall-Stoodley et al., 2004).

To detect the presence of polysaccharide matrix and elucidate the biofilm architecture, confocal microscopy was performed using the plant-based lectins WGA and concanavalin A. These markers bind to GlcNAc (WGA) (Singh et al., 2010), glucose and mannose (concanavalin A) (Chandra et al., 2008), and therefore detect these specific residues in the hyphae and the matrix, if any. Individual fungal hyphae were also detected via the red light refracted by the cells (WGA-OG staining) or FUN-1 (Con-A/FUN-1 staining), and the images obtained were compared with WGA-OG or Con-A fluorescence for differentiation between the binding of WGA or Con-A to the hyphae and the matrix.

Phase-contrast images of the confocal micrographs revealed a haze-like film, suggestive of a polysaccharide matrix, covering the fungal hyphae in the biofilms (Fig. 4). CLSM images also indicated the presence of highly intertwined mycelia mass, with WGA-OG staining many inter-hyphal regions as well (Fig. 4). These results demonstrate the existence of an exopolysaccharide matrix in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms and further satisfy the criteria proposed for detecting biofilm formation in filamentous fungi (Harding et al., 2009). Also, the staining of many inter-hyphal regions with WGA-OG suggests the presence of GlcNAc in the matrix. In contrast, Con-A detected the fungal hyphae but inter-hyphal regions were not significantly stained with this marker, indicating that glucans and mannans were not a major component of the matrix. This was further confirmed by biochemical characterization of EPS extracted from the biofilms. The thickness of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms was 109.67 ± 10.02 μm, 242 ± 23.07 μm and 197 ± 9.0 μm (mean ± SD), respectively.
Characterization of the biofilm matrix

EPS has often been called the ‘dark matter’ of biofilms, owing to the large range of biopolymers it may contain, and the difficulty in analysing them (Flemming & Wingender, 2010). It accounts for a major component of the biofilm biomass and consists primarily of polysaccharides, although proteins (including many enzymes), lipids and extracellular DNA may also be present (Flemming & Wingender, 2010).

G6PDH activity was not detected in the matrices extracted from Rhp. oryzae, L. corymbifera and Rhm. pusillus biofilms, confirming an absence of contaminating cellular components. Hexosamine and N-acetylhexosamine constituted 93.4–95.6% and 4.4–6.6%, respectively, of the amino sugars in the water-soluble EPS fraction. GlcN was found to be the primary component of Rhp. oryzae, L. corymbifera and Rhm. pusillus biofilm matrix, constituting $745.38 \pm 115.25$, $822.20 \pm 178.99$ and $803.94 \pm 135.43$ mg (mg EPS)$^{-1}$ (mean $\pm$ SD), respectively. In contrast, GlcNAc constituted $43.49 \pm 6.72$, $47.97 \pm 10.44$ and $46.91 \pm 7.90$ mg (mg EPS)$^{-1}$ (mean $\pm$ SD) in Rhp. oryzae, L. corymbifera and Rhm. pusillus biofilms, respectively. These results suggest the presence of the chitin-derivative chitosan, a partially acetylated $\beta$-1,4-linked GlcN polymer, which is usually soluble in water at a high degree of deacetylation (Kumirska et al., 2010). In contrast to other fungi, chitin and chitosan constitute a major component of the cell wall in zygomycetes (Bartnicki-Garcia, 1968). Chitosan cements the cell wall, protects chitin from enzymic attack and also adsorbs ionic material by salt or complex formation (Bartnicki-Garcia, 1968). The content of this aminopolysaccharide in zygomycetes’ cell wall may be up to three times the amount of chitin (Bartnicki-Garcia, 1968). A similar partially deacetylated $\beta$-1,6-linked GlcNAc polymer termed polysaccharide intercellular adhesin (PIA) is the primary component of Staphylococcus aureus and Staphylococcus epidermidis biofilm matrix and it mediates cell-to-cell interaction in these bacteria (Singh et al., 2010). Hexosamine has also been reported as an important component of EPS in Candida tropicalis biofilms (Al-Fattani & Douglas, 2006).

The high concentration of amino sugars interfered with the determination of carbohydrates by the phenol/sulphuric acid method. Upon reaction with this reagent, the samples exhibited a peak absorbance at 405 nm, compared to the usual 490 nm observed in sugars. Glucose content was therefore determined in the water-soluble fraction using a
glucose oxidase kit. Although glucose constitutes 32–74% of the biofilm matrix in *Aspergillus fumigatus* (Beauvais et al., 2007) and *Candida* species (Al-Fattani & Douglas, 2006; Silva et al., 2009), it accounted for only a small proportion of the biofilm-matrix weight in the Mucorales that we studied. *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilm EPS contained 7.42 ± 0.92, 7.92 ± 1.20 and 7.17 ± 0.71 mg glucose (mg EPS)^−1^ (mean ± SD), respectively. Proteins were also detected in the matrix preparations, constituting 2.23 ± 0.10, 7.70 ± 0.31 and 3.17 ± 0.61 mg (mg EPS)^−1^ (mean ± SD) in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus*, respectively. However, these results probably underestimate the protein content of the slime. The solubilized matrix harvested from the biofilms was concentrated using ethanol, an organic solvent which preferentially precipitates polysaccharides (Flemming & Wingender, 2010). The protein content of the suspensions before ethanol precipitation was about sevenfold higher than that of the precipitated EPS.

The TCA-soluble EPS fraction was processed for the detection of chitin, a β-1,4-linked GlcNAC polymer insoluble in water and many acids (Kumirska et al., 2010), and its monomer GlcNAc was quantified by ELLA. The concentration of GlcNAc in the TCA-soluble fraction of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilm matrix was 59.43 ± 0.80, 74.28 ± 8.09 and 56.89 ± 4.40 μg (mg EPS)^−1^ (mean ± SD), respectively.

Thus, the extracellular matrix in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms is primarily composed of amino sugars (GlcN and GlcNAc), with glucose and proteins also...
being present in small amounts. By contrast, glucose is the major component of Candida and Aspergillus fumigatus biofilms (Al-Fattani & Douglas, 2006; Baillie & Douglas, 2000; Beauvais et al., 2007; Lal et al., 2010; Silva et al., 2009). In A. fumigatus biofilms, the EPS consists of glucose (74 %), mannitol (18 %), trehalose (3 %), glycerol (5 %), and melanin and proteins (2 %) (Beauvais et al., 2007). Immunolabelling studies have further revealed the presence of galactomannan, galactosaminogalactan and α-1,3 galactans (Beauvais et al., 2007; Loussert et al., 2010). C. albicans biofilms usually contain glucose (16–32.2 %), hexosamine (3.3 %), proteins (5 %) and uronic acid (0.1 %) as well as mannose, rhamnose and galactose in the matrix (Al-Fattani & Douglas, 2006; Baillie & Douglas, 2000; Lal et al., 2010). However, C. tropicalis biofilm matrix comprises mainly hexosamine (27.4 %), with smaller amounts of other carbohydrates (3.3 %, including 0.5 % glucose), protein (3.3 %) and phosphorus (0.2 %) (Al-Fattani & Douglas, 2006).

In conclusion, our results reveal the biofilm-forming potential of Rkh. oryzae, L. corymbifera and Rhm. pusillus, but not A. elegans. At appropriate seeding densities, these fungi produced robust, highly intertwined, filamentous, adherent structures that were encased in an extracellular matrix composed primarily of GlcN and GlcNAc. Although Mucorales are usually implicated in angio-invasive infections, biofilm formation may be involved in the pathogenesis of paranasal fungal balls, endocarditis, osteomyelitis and catheter-based infections caused by these pathogens. It may also be important for the survival of these saprophytic fungi in the environment (Ramage et al., 2009).

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