Insights into the lignocellulosic physiology of the yeast pathogen *Cryptococcus neoformans* var. *grubii*

Angela Botes

Dissertation presented in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Faculty of Natural Sciences at Stellenbosch University

Promoter: Prof A. Botha

April 2014

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification

Signature: _____ Date: April 2014

ABSTRACT

ABSTRACT

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic pathogen that causes cryptococcal meningitis, predominantly in immuno-compromised individuals, particularly in those suffering from human immuno virus (HIV) or acquired immuno-deficiency syndrome (AIDS). This basidiomycetous yeast species is sub-divided into two main varieties, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), as well as a rare hybrid variety, *C. neoformans* (serotype AD). The global incidence of cryptococcosis among AIDS sufferers is approximately 30 % with 90 % of these cases being attributed to serotype A.

Cryptococcus neoformans has been isolated from numerous environmental sources including guano, soil, and particularly decaying wood and tree hollows. Infection occurs when particles originating from these environments are inhaled. The ecological niche of *C. neoformans* was thought to be avian guano; however, recent findings indicate that the true ecological niche may rather be woody material. Representatives of this species, particularly *C. neoformans* var. *grubii*, were found to grow on agar plates containing carboxymethyl cellulose as carbon source; however, little is known about its ability to degrade hemi-cellulose.

As such, the overall aim of this project was to study the interactions of *C. neoformans* var. *grubii* ATCC H99 with cellulosic and hemi-cellulosic materials.

Growth studies revealed that *C. neoformans* var. *grubii* ATCC H99 was capable of utilizing carboxymethyl cellulose, glucomannan and galactomannan as sole carbon sources. This yeast also assimilated simple degradation products of lignocellulose such as L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose. D-Mannose and D-glucose resulted in the highest maximum specific growth rates.

Screening the genome of *C. neoformans* var. *grubii* ATCC H99 resulted in the identification of three putative cellulases, specifically an endo-glucanase (EC7) and two cellulases (CC1 and CC6). Evaluation of the deduced amino acid sequences indicated that all three enzymes belong to glycoside hydrolase family 5 (GHF5). Phylogenetic analyses revealed that the three enzymes grouped in distinct clades with other GHF5 members.

Automated homology modeling of the three-dimensional structure revealed that CC1 and CC6 displayed the classical $(\alpha/\beta)_8$ TIM barrel fold associated with

GHF5. Modeling of EC7 did not produce the classic GHF5 structure, suggesting that this enzyme may be classed in a separate GHF. Green fluorescent protein (GFP) tagging confirmed that CC6 forms part of the cryptococcal secretome.

Real-time quantitative PCR (qPCR) analyses indicated that the three proteins responded differently on a transcriptional level in the presence of various carbohydrates. Cellulase CC6 displayed the most dynamic expression profile, indicating up-regulation in the presence of mannose, galactose and cellobiose. The use of *Acacia mearnsii* debris and the aqueous extract thereof also resulted in a significant up-regulation of all three enzymes. This confirms previous findings that the woody phyloplane is a natural habitat of *C. neoformans* var. *grubii*.

In the presence of mucin, the transcription of CC6 was up-regulated. Similar to laccase and urease, CC6 may aid the survival of *C. neoformans* within the human respiratory system. Understanding the carbohydrate metabolic regulatory system and its impact on virulence would increase our overall knowledge of this pathogen's survival capabilities and infection strategies.

OPSOMMING

Cryptococcus neoformans (Sanfelice) Vuillemin is ‘n opportunistiese patogeen wat kriptokokkale meningitis veroorsaak, hoofsaaklik in immuun-gekompromiteerde individue en veral diegene wat die menslike immunovirus (MIV) of verworwe immuniteitsgebreksindroom (VIGS) onderlede het. Hierdie basidiomisete gisspesie word in twee hoof variëteite verdeel, naamlik *C. neoformans* var. *neoformans* (serotipe D) en *C. neoformans* var. *grubii* (serotipe A), asook ‘n skaars hibried-variëteit, *C. neoformans* (serotipe AD). Die wêreldwye voorkoms van kriptokokkose onder VIGS-lyers is ongeveer 30 %, met 90 % van hierdie gevalle wat aan serotipe A toegeskryf kan word.

Cryptococcus neoformans is uit talle omgewingsbronne geïsoleer, insluitende guano, grond en veral verrottende hout en boomholtes. Infeksie vind plaas wanneer partikels uit hierdie omgewings ingeasem word. Voëlguano is voorheen as die ekologiese nis van *C. neoformans* beskou, maar onlangse bevindinge dui aan dat die ware ekologiese nis eerder houtagtige materiaal mag wees. Verteenwoordigers van hierdie spesie, veral *C. neoformans* var. *grubii*, kon op agar plate te groei wat karboksimetielsellulose as koolstofbron bevat; min is egter bekend oor sy vermoë om hemisellulose af te breek.

Die hoofdoel van hierdie projek was dus om die interaksies van *C. neoformans* var. *grubii* ATCC H99 met sellulosiese en hemi-sellulosiese materiale te bestudeer.

Groeistudies het getoon dat *C. neoformans* var. *grubii* ATCC H99 in staat was om karboksimetielsellulose, glukomannaan en galaktomannaan as enigste koolstofbronne te benut. Hierdie gis het ook eenvoudige afbraakprodukte van lignosellulose, L-arabinose, D-galaktose, D-glukose, D-mannose, L-rhamnose en D-xylose geassimileer. D-Mannose en D-glukose het die hoogste maksimum spesifieke groeitempo’s tot gevolg gehad.

Die sifting van *C. neoformans* var. *grubii* ATCC H99 se genoom het gelei tot die identifisering van drie moontlike sellulases: ‘n endo-glukanase (EC7) en twee sellulases (CC1 en CC6). Evaluering van afgeleide die aminosuur-volgordes het aangedui dat al drie ensieme aan die glikosied hidrolase familie 5 (GHF5) behoort. Filogenetiese analises het getoon dat die drie ensieme in duidelike taksonomiese groepe saam met ander lede van die GHF5 voorkom.

Outomatiese homologie-modellering van die drie-dimensionele struktuur het getoon dat CC1 en CC6 die klassieke $(\alpha/\beta)_8$ TIM vaatjie-vou, wat met GHF5 geassosieer word, vertoon. Modellering van EC7 het nie die klassieke GHF5 struktuur geproduseer nie, wat daarop mag dui dat hierdie ensiem moontlik in 'n afsonderlike GHF geklassifiseer kan word. Groen fluoresserende protein (GFP) merking het bevestig dat CC6 deel van die kriptokokkale sekretoom vorm.

Intydse kwantitatiewe PCR (qPCR) analises het getoon dat die drie proteïene verskillend op 'n transkripsievlak in die teenwoordigheid van verskillende koolhidrate reageer. Sellulase CC6 het die mees dinamiese uitdrukkingsprofiel getoon, wat dui op op-regulering in die teenwoordigheid van mannose, galaktose en sellobiose. Die gebruik van *Acacia mearnsii* molm en die waterige ekstrak daarvan het ook 'n beduidende op-regulering van hierdie drie ensieme tot gevolg gehad. Dit bevestig vorige bevindings dat die houtagtige plantoppervlak 'n natuurlike habitat vir *C. neoformans* var. *grubii* is.

Die transkripsie van CC6 is ook in die teenwoordigheid van musien op-gereguleer. Net soos lakkase en urease, mag CC6 tot die oorlewing *C. neoformans* in die menslike asemhalingstelsel bydra. 'n Begrip van die koolhidraat metaboliese reguleringsisteem en die impak daarvan op virulensie sal ons algehele kennis van hierdie patogeen se vermoë en strategie om te oorleef, verbeter.

“Somewhere, something incredible is waiting to be known”

-Carl Sagan

“If I have seen further than others, it is by standing upon the shoulder of giants”

-Isaac Newton

Dedicated to my family

“To live in the hearts we leave behind is not to die”

-Thomas Campbell

ACKNOWLEDGMENTS

My heartfelt thanks, love and sincere appreciation to everyone involved with this research over the years:

* My parents, brother, grandparents, aunts and cousins for all their love, support, encouragement, understanding and immense patience
* Alf Botha for his creativity, enthusiasm, input, passion, paranoia, patience, sense of humour and amazing ability to "get it done" under so much pressure
* The Botha Lab for all the coffee, input, coffee, insight, coffee, humour, coffee, understanding, coffee, patience, coffee, sanity, coffee, outings and of course the coffee
* Dr Shelly Deane for the coffee, trouble-shooting and stock taking sessions
* Friends closer to home and further afield for all the love, support, encouragement, late night growth curves, trouble-shooting, coffee drinking, park running, squash playing and wine touring
* Lonnie van Zyl for spending countless hours trouble-shooting and encouraging me to complete this research
* The Harry-Crossley Foundation, National Research Foundation, Stellenbosch University and the Department of Microbiology for financial support during the study

"Call it a clan, call it a network, call it a tribe, call it a family: Whatever you call it, whoever you are, you need one." - Jane Howard

MOTIVATION

MOTIVATION

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for cryptococcosis among immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1998; Mitchell *et al.*, 1995), particularly those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). Global incidence of this infection among these individuals was estimated at approximately 30 % (Park *et al*., 2009; Vilchez *et al*., 2003; Husain *et al*., 2001; Chuck *et al.*, 1989) with a mortality rate between 30 – 60 %. In Sub-Saharan Africa, cryptococcosis is the fourth highest cause of mortality amongst infectious diseases (Park *et al.*, 2009). A survey conducted within the Gauteng Province of South Africa by the Gauteng Cryptococcal Surveillance Group during 2002 and 2004, identified 2753 cryptococcosis cases (McCarthy *et al*., 2006). A more recent survey conducted in 2012 by the National Health Laboratory Service of Johannesburg, revealed an overall incidence of 119 cryptococcosis cases per 100 000 HIV-infected persons in South Africa (GERMS-SA Annual Report 2012). All these cryptococcosis cases were caused by basidiomycetous pathogenic cryptococci however; only 4% were ascribed to *Cryptococcus gattii*, while *C. neoformans* was implicated in the rest of the cases. Cryptococcal meningitis accounted for 89 % of cryptococcosis cases, while a previous survey indicated an in-hospital mortality rate of 27 % for patients suffering from cryptococcosis (McCarthy *et al*., 2006). These relatively high mortality rates, despite antifungal treatments, emphasize the need to further examine the biology of this ubiquitous yeast pathogen.

Two main varieties of *C. neoformans* are known, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), with a hybrid variety, *C. neoformans* (serotype AD) also identified (Boekhout *et al*., 2001). *Cryptococcus neoformans* var. *grubii* is regarded as being the more virulent of the two varieties with approximately 90 % of all clinical cases being attributed to this serotype (Mitchell *et al.*, 1995). It is widely believed that infection occurs when propagules such as desiccated yeast cells or viable basidiospores are inhaled (Feldmesser *et al*., 2000). These propagules may remain localized within the lungs, where they are thought to be relatively dormant prior to dissemination.

The yeast has been isolated from a number of environmental sources including soil, avian guano contaminated soil, avian guano, fir trees, almond trees, eucalyptus

trees, woody debris and decaying wood (Trilles *et al*., 2003; Halliday *et al*., 1999; Sorrell *et al*., 1997; Lazéra *et al*., 1996). The identification of a putative cellulase within the cryptococcal genome (Loftus *et al*., 2005), the repeated isolation of this pathogen from woody habitats (Chowdhary *et al*., 2012; Randhawa *et al*., 2011; 2008; 2006; 2000; Trilles *et al*., 2003; Sorrell *et al*., 1997; Lazéra *et al*., 1996), as well as the finding that this yeast is able to grow and complete its life cycle on woody debris in the absence of additional nutrients (Botes *et al*., 2009), strongly suggest that the true ecological niche of *C. neoformans* is woody material.

By identifying and understanding the functioning and regulation of the cryptococcal metabolism within its woody ecological niche, we may gain further insights into this pathogen's survival in a clinical setting that may possibly aid in the future treatment of this disease. Therefore, the overall goal of this project was to study the physiology of *C. neoformans* with regards to a woody habitat. Given the high prevalence of *C. neoformans* var. *grubii* among both environmental and clinical isolates (Litvintseva *et al*., 2009; McCarthy *et al*., 2006; Mitchell *et al*., 1995) the type strain, *C. neoformans* var. *grubii* ATCC H99, was selected as the model strain in this study.

The first objective was to determine if *C. neoformans* var. *grubii* ATCC H99 was capable of assimilating the various components of lignocellulose, namely cellulose and hemi-cellulose, as well as their associated simple sugars (Chapter 2). The second objective was to phylogenetically identify any putative cellulases, cellobiohydrolases and/or mannanases within the genome of *C. neoformans* var. *grubii* ATCC H99 (Chapter 3). Thirdly, we aimed to determine the physical and chemical properties of any identified enzymes, as well as their tertiary structure and cellular localization (Chapter 4). The fourth goal was to isolate and express the identified enzymes to evaluate their specific activity (Chapter 5). Fifthly, we aimed to gain an insight into the regulation of these genes in *C. neoformans* var. *grubii* ATCC H99 when the yeast is introduced into carbohydrate differing environments that range from media containing simple degradation products of lignocellulose, to media containing complex wood polymers (Chapter 6). The final objective was to determine if the presence of carbohydrate-containing mucin, comparable to the coating of respiratory epithelium, would result in an increase in the transcription of the identified proteins (Chapter 7).

REFERENCES

Boekhout T., van Belkum A., Leenders A. C. A. P., Verbrugh H., Mukamurangwa P., Swinne D., Scheffers W. A. (1997). Molecular typing of *Cryptococcus neoformans*: Taxonomic and epidemiological aspects. *International Journal of Systematic Bacteriology* **47(2):** 432-442.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* **147:** 891-907.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment – the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chowdhary A., Randhawa H.S., Prakash, A., Meis, J.F. (2012) Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* in India: An update. *Critical Reviews in Microbiology* **38:** 1–16.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **321:** 794–799.

Feldmesser M., Kress Y., Novikoff P., Casadevall A. (2000). *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infection and Immunity* **68:** 4225-4237.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *Journal of Clinical Microbiol*ogy **36(8):** 2200-2204.

GERMS-SA Annual Report. (2012). Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa. Available from: http://www.nicd.ac.za/units/germs/germs.htm.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and a mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gattii* strains from Australia. *Journal of Clinical Microbiol*ogy **37:** 2920-2926.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerging Infectious Disease* **7:** 375-381.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

Litvintseva A. P., Mitchell T. G. (2009). Most environmental isolates of *Cryptococcus neoformans* var. *grubii* (Serotype A) are not lethal for mice. *Infection and Immunity* **77(8):** 3188-3195.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T. R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M., Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science* **307:** 1321-1324.

McCarthy K. M., Morgan J., Wannemuehler K. A., Mirza S. A., Gould S. M., Mhlongo N., Moeng P., Maloba B. R., Crewe-Brown H. H., Brandt M. E., Hajjeh R. A. (2006). Population-based surveillance for cryptococcosis in an antiretroviral-naive South African province with a high HIV seroprevalence. *AIDS* **20:** 2199–2206.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* **8(4):** 515-548.

Park B. J., Wannemuehler K. A., Marston B. J., Govender N., Pappas P. G., Chiller T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Powderly W. G. (1993). Cryptococcal meningitis and AIDS. *Clinical Infectious Disease* **17:** 837–842.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans. Revista Iberoamericana de Micologia* **14:** 42-43.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Medical Mycology* **41(5):** 383-390.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transplant International* **16:** 336-340.

TABLE OF CONTENTS

CHAPTER 1

Literature Review

CHAPTER 2

The assimilation of complex lignocellulosic substrates and their associated monosaccharides by the yeast pathogen *Cryptococcus neoformans* var. *grubii*

CHAPTER 3

Identification and phylogenetic analyses of three putative cellulase genes within the genome of *Cryptococcus neoformans* var. *grubii*

CHAPTER 4

Predicting the three-dimensional structure and cellular localization of three putative cellulases identified within the genome of the yeast pathogen *Cryptococcus neoformans* var. *grubii*

CHAPTER 5

Heterologous expression of glycoside hydrolases from *Cryptococcus neoformans* var. *grubii* in recombinant hosts.

CHAPTER 6

Carbon regulation of two putative cellulases and an endo-glucanase identified in the genome of *Cryptococcus neoformans* var. *grubii*

CHAPTER 7

Growth of *Cryptococcus neoformans* var. *grubii* ATCC H99 on mucosal associated glycoproteins

CHAPTER 8

General Conclusions and Future Research

APPENDICES

CHAPTER 1

Literature Review

1. *CRYPTOCOCCUS NEOFORMANS*

Cryptococcus neoformans (Sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis in immuno-suppressed individuals, such as those suffering from acquired immuno-deficiency syndrome (AIDS), cancer, and those receiving immuno-suppressive therapy (Casadevall *et al*., 2003; Harrison, 2000). It belongs to the order Tremellales, also known as the jelly fungi, which is predominantly comprised of yeasts (Boekhout *et al*., 2001; Fell *et al*., 2000) and is usually characterized by the production of melanin, resulting in typical brown colony pigmentation when cultured on differential media, particularly Niger seed (*Guizotia abyssinica*) agar (Yarrow, 1998; Staib, 1962).

The genus *Filobasidiella* is currently comprised of five species namely; *F. neoformans*, *F. bacillispora*, *F. depauperata*, *F. lutea* and *F. amylolenta* (Kwon-Chung, 2010). Interestingly, *Filobasidiella* is the only genus of the basidiomycota that produces long chains of basidiospores on the apex of the holobasidia (Kwon-Chung, 1975), and includes pathogens causing systemic infections in both immuno-compromised and immune-competent hosts (Kwon-Chung, 2010). Members of *Filobasidiella* are regarded as being either pathogenic or parasitic. Both *F. neoformans* and *F. bacillispora* are capable of infecting species of protozoa as well as a wide range of vertebrates and invertebrates (London *et al.*, 2006; Steenbergen *et al.*, 2003; 2001; Miller *et al.*, 2002; Kwon-Chung *et al.*, 1992). *Filobasidiella depauperata* and *F. lutea* are known myco-parasites infecting the ascomycete *Verticullium lecanii* (Ginns *et al.*, 2003) and basidiomycete *Hypochnicium vellereum* (Roberts, 1997), respectively, while *F. amylolenta* is associated with insect frass (Van der Walt *et al.*, 1972).

Currently, *F. neoformans* and *F. bacillispora* are the only two members of *Filobasidiella* known to possess a yeast stage, namely; *C. neoformans* and *C. gattii*. The yeast, *C. neoformans* is encapsulated and can appear either round or oval shaped, is able to utilize a wide variety of carbon compounds as growth substrate and is prototrophic for the majority of sugars, amino acids and lipids (Casadevall *et al*., 2003; Steenbergen *et al*., 2003). The ability to grow at physiological temperatures (37 °C) is characteristic of most pathogens; however, *C. neoformans* does not require a host cell to replicate and is able to tolerate a range of environmental temperatures.

Cryptococcus neoformans has a defined life cycle that involves vegetative growth as budding yeast capable of undergoing filamentous dimorphic transitions due to the conjugation of yeast cells of the opposite mating types, namely mating type *a* (MAT*a*) and mating type alpha (MATα) (Kwon-Chung, 1980). The result is a dikaryotic mycelium that gives rise to basidia followed by meiosis and the production of haploid basidiospores. More recently, the phenomena of monokaryotic fruiting and same sex mating have also been observed in strains of both *C. neoformans* and *C. gattii*.

Until 1949, *C. neoformans* was regarded as a homogeneous species, although; antigenic properties of the polysaccharide capsule revealed the existence of four different serotypes, namely *C. neoformans* var. *neoformans* (serotype D); *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *gattii* (Sanfelice) Vuillemin (serotypes B and C). Nonetheless, heterogeneity remained unclear until the identification of two distinct teleomorphs in the 1970s. Serotypes A and D gave rise to *F. neoformans*, while serotypes B and C yielded *F. bacillispora* (Kwon-Chung *et al.*, 1976; 1975). Since then, research has identified a number of differences between the two anamorphic species in terms of their biochemistry, environmental distribution, DNA composition, chromosome numbers, as well their host preference. Despite morphological, molecular and evolutionary research confirming that *C. neoformans* and *C. gattii* are indeed two separate species, the development of new genotyping techniques has shown that interspecific diversity does occur between to these species.

Similarly, the advancement in molecular science has led to the development of numerous molecular typing methods to determine the genotype of both clinical and environmental isolates of *C. neoformans*. These typing techniques include Southern blot hybridization with DNA probes (Varma *et al.*, 1995), karyotyping (Perfect *et al.*, 1993), polymerase chain reaction (PCR) fingerprinting (Meyer *et al.*, 1999; 1993), DNA fingerprinting (Varma *et al.*, 1992), random amplified polymorphic DNA (RAPDs) (Meyer *et al.*, 1999, Chen *et al.*, 1997; Sorrell *et al.*, 1996), restriction fragment length polymorphism (RFLP) of the phospholipase B1 (PLB1) and uracil 5 (URA5) gene (Jain *et al.*, 2005; Latouche *et al.*, 2003); multilocus enzyme electrophoresis (MLEE) (Brandt *et al.*, 1996); amplified fragment length polymorphism (AFLP) (Boekhout *et al.*, 2001); multilocus sequencing type (MLST) (Hagen *et al.*, 2012; Illnait-Zaragozi *et al.*, 2010); matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method (MALDI-TOF) analysis

(Firacative *et al.*, 2012; Posteraro *et al.*, 2012) and sequence analysis (Franzot *et al.*, 1997). At present, *C. neoformans* is comprised of five major genotypes, namely VNI, VNII, VNB, VNIII and VNIV (Cogliati, 2013). Excluding Australia and Papua New Guinea, VNI is the predominant genotype across the globe (Cogliati, 2013). Genotype VNII is considered as rare, but has been isolated from all continents except Europe. Interestingly, the genotype VNB was originally thought to be endemic to sub-Saharan Africa (Litvintseva *et al.*, 2003), but this genotype has recently been isolated in Brazil, Colombia (Ngamskulrungroj *et al.*, 2009) and Italy (Cogliati *et al.*, 2011).

2. CRYPTOCOCCOSIS

Cryptococcus neoformans var. *grubii* is the leading cause of fungal meningitis in immune impaired individuals world-wide (Park *et al.*, 2009) and results in an inflammation of the meninges, the membranes that cover and protect the brain and spinal cord (Saag *et al.*, 2000). The yeast was recognized as a human pathogen in 1894 before the development of antimicrobial drugs, venous catheters, immuno-suppressive drugs and the increasing prevalence of the human immuno virus (HIV) and AIDS. Until then, systemic fungal infections, such as cryptococcosis, were considered to be extremely rare (Casadevall, 2005; Steenbergen *et al.*, 2003).

Globally, *C. neoformans* is responsible for approximately 13 - 44 % of all AIDS-related infections and 5 % of infections seen in organ transplant recipients (Park *et al.*, 2009; Vilchez *et al.*, 2003; Husain *et al.*, 2001; Liu *et al.*, 1999; Williamson, 1997; Wang *et al.*, 1996; Chuck *et al.*, 1989). Mortality rates are generally high, up to 33 - 42 % in organ transplant patients and an estimated 55 % amongst HIV/AIDS patients, despite the use of anti-fungal therapies to combat the yeast. In resource limited areas such as sub-Saharan Africa and South-South East Asia the yearly incidence of cryptococcosis has remained high, approximately 3.2 and 3.0 % respectively (Chowdhary *et al.*, 2012; Park *et al.*, 2009; Mirza *et al.*, 2003; Kumarasamy *et al.*, 2003; Vajpayee *et al.*, 2003). In 2009, the estimated mortality rate of cryptococcosis was as high as 70 %, making it the fourth highest cause of mortality in Sub-Saharan Africa after malaria, diarrheal disease and childhood-cluster diseases (Park *et al.*, 2009).

Interestingly, while *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are routinely isolated from immune impaired individuals, *C. gattii* has been reported in an ever-increasing number of cases involving immune-competent

individuals, particularly in British Columbia's Vancouver Island in Canada (Fraser *et al*., 2003; Taylor *et al*., 2002).

3. PATHOGENESIS OF *CRYPTOCOCCUS NEOFORMANS*

The exact mechanism of infection for *C. neoformans* is not yet clearly understood. It is suspected that infectious propagules, such as desiccated yeast cells and aerosolized basidiospores, are inhaled (Fig 1) to establish a pulmonary infection (Feldmesser *et al*., 2001). Pulmonary cryptococcosis is still regarded as being asymptomatic and is often misdiagnosed as viral infections (Goldman *et al*., 2001; 2000) or pulmonary tuberculosis (Jarvis *et al.*, 2010). Subsequently, the immune system may either eradicate the yeast from the lungs, sequester the yeast cells within the alveolar macrophages where they lie dormant to be reactivated at a later stage, or the yeast may actively disseminate throughout the body displaying a strong preference for the central nervous system (CNS) (Feldmesser *et al*., 2001; Saag *et al*., 2000).

Studies have revealed that exposure to *C. neoformans* occurs at an early age and that the majority of children two years and older acquire life-long antibodies targeted towards the pathogen (Goldman *et al*., 2001). Given that paediatric cryptococcosis cases are extremely rare (Othman *et al.*, 2004; Sweeney *et al.*, 2003; Abadi *et al.*, 1999), it would suggest that childhood infections may remain relatively asymptomatic and that the yeast cells may lie dormant only to be "re-activated" at a later stage once the immune system is compromised. Indeed, studies have shown that *C. neoformans* infection may be latent for up to 18 years or more. Based on molecular profiling, random amplified polymorphic DNA (RAPD) and *C. neoformans* repetitive element 1 (CNRE-1) restriction fragment length polymorphism (RFLP), Garcia-Hermoso and co-workers (1999) were able to show that *C. neoformans* isolates from African patients differed from those isolated from French patients despite their having left the African continent 13 years previously.

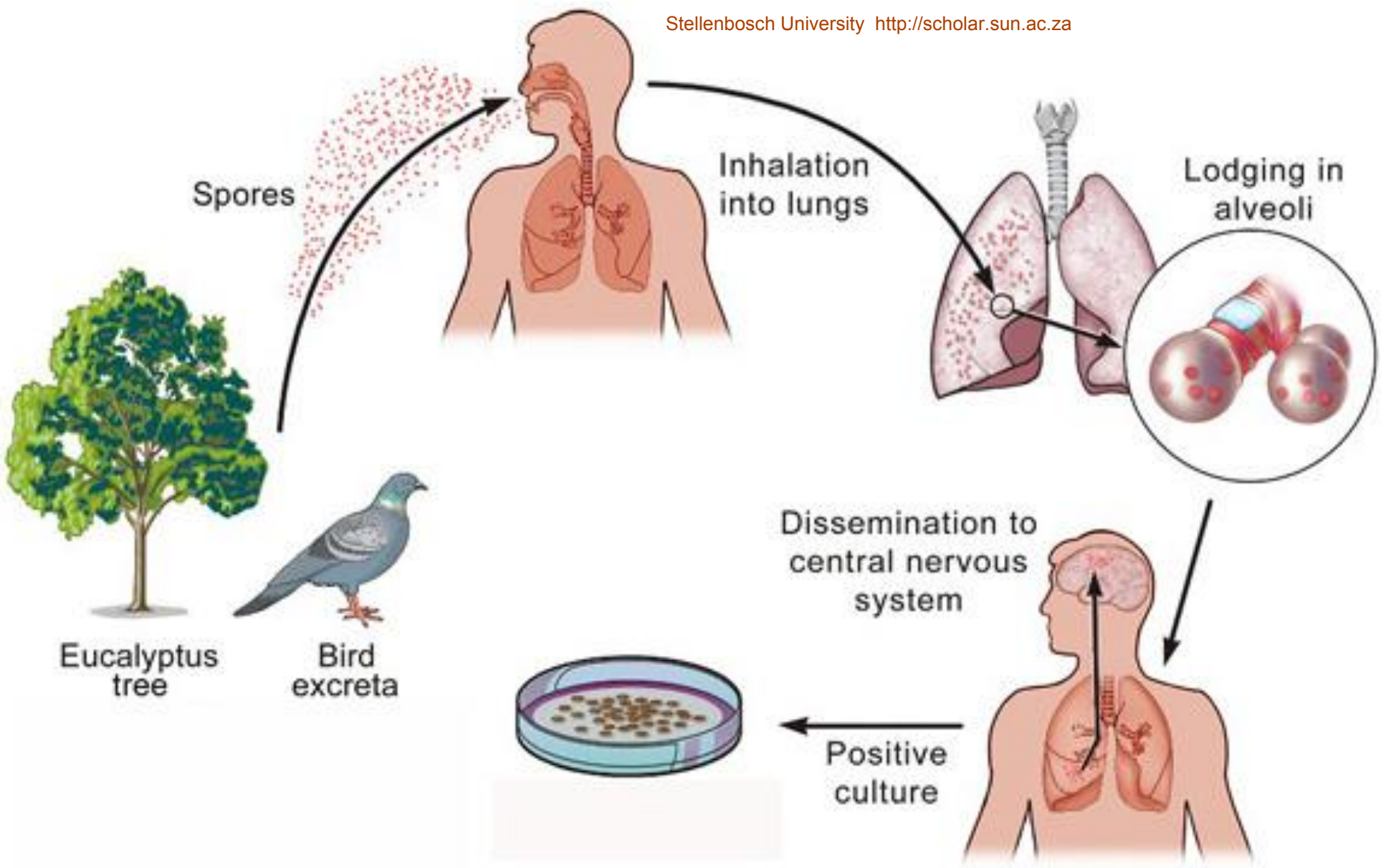

Figure 1. The proposed infectious pathway of the pathogen *C. neoformans* (Hull *et al.*, 2002).

The survival of *Cryptococcus* within the alveolar macrophages, monocytes and endothelial cells differs greatly from other intracellular pathogens such as *Histoplasma capsulatum, Listeria monocytogenes, L. pneumophila, Mycobacterium avium* and *Shigella flexneri*. The yeast's ability to reside and proliferate within the phagosome and lysosome has been attributed to a number of virulence factors expressed by *C. neoformans*, including the polysaccharide capsule, antiphagocytic protein 1 (App 1), mannoproteins, superoxide dismutases (SODs), melanin production, phospholipase B1 (Plb1), phytoceramide and glucosylceramide (Akhter *et al.*, 2003; Steen *et al*., 2002; Cox *et al.*, 2001; Feldmesser *et al*., 2001; 2000; Zhu *et al*., 2001; Liu *et al*., 1999; Wang *et al.*, 1995; 1994). Indeed this intracellular location has been shown to provide multiple benefits for *C. neoformans* as it reduces the yeast's overall exposure to anti-fungal therapies and allows it to avoid extracellular immune mechanisms (Feldmesser *et al*., 2001).

Re-activation of latent cryptococcal infections results in the dissemination of the yeast throughout the human body (Fig 1). *Cryptococcus neoformans* is able to escape the phagosome using multiple strategies such as host cell lysis, expulsion and lateral transfer. The lack of pore-forming proteins within the cryptococcal genome suggest that Plb1 is solely responsible for host cell lysis (Noverr *et al.*, 2003), however, no research has shown a direct link between Plb1 and cryptococcal cells escaping from the phagosome. As such, lysis due to the mechanical burden placed on the cytoplasmic membrane as yeast cells accumulate within the host cell, cannot be ignored (Alvarez *et al.*, 2006; Tucker *et al.*, 2002). Interestingly, expulsion of *C. neoformans* from macrophages appears to resemble exocytosis and is initiated by the yeast itself (Alvarez *et al.*, 2006; Ma *et al.*, 2006).

Two major mechanisms by which *C. neoformans* is able to traverse the blood-brain barrier (BBB) have been suggested and include the "Trojan-horse" mechanism and trans-cellular penetration. Chrétien and co-workers (2002) observed that *C. neoformans* cells circulate throughout the body within blood monocytes that are capable of entering endothelial cells of leptomeningeal capillaries, highlighting a possible means of traversing the BBB. However, it was confirmed in 2009 that the blood monocytes infected with *C. neoformans* were capable of freely entering the brain and would therefore carry the pathogen over the BBB (Charlier *et al.*, 2009). Alternatively, *C. neoformans* has been shown to bind to brain micro-vascular endothelial cells (Chang *et al.*, 2004; Chen *et al.*, 2003). These bound cells are

subsequently internalized and trans-cellular penetration occurs. The use of para-cellular penetration, the passage of microbes between barrier cells, by *C. neoformans* has been proposed (Charlier *et al.*, 2005; Kanoh *et al.*, 2008), but the research is regarded as largely circumstantial as yeast cells have yet to be "caught in the act".

The central nervous system however, is not the only bodily organ that may be affected. *Cryptococcus neoformans* is also known to infect the bones and visceral organs, as well the skin, causing cutaneous cryptococcosis that manifests in the form of lesions and ulcers. Once again, dissemination is believed to occur by means of infected macrophages and blood monocytes (Feldmesser *et al.*, 2001; Saag *et al.*, 2000).

4. TREATMENT OF CRYPTOCOCCOSIS

Until the onset of the 1950s, disseminated cryptococcosis was uniformly fatal (Saag *et al.*, 2000). With the introduction of a number of anti-fungal agents, including amphotericin B (AMB), flucytosine, fluconazole and itraconazole, the successful treatment of cryptococcosis has improved. The introduction of AMB in the 1960s resulted in the successful treatment of up to 70 % of cases. AMB was replaced with flucytosine, an orally bio-available agent that demonstrated a potent activity against *C. neoformans*. However, the over-use of flucytosine led to the rapid development of flucytosine resistance in *C. neoformans*. Later research would also implicate the extended use of flucytosine in toxicity, particularly in patients with a compromised immune system (Viviani, 1996). Towards the early 1980s, two new orally bio-available azole anti-fungal agents, namely fluconazole and itraconazole, were introduced. Both displayed *in vivo* activity against *C. neoformans*.

Treatment is primarily determined by the site of infection, such as pulmonary or meningitis, as well as the status of the patient's immune system (Saag *et al.*, 2000). The preferred treatment in HIV negative patients is determined largely by the site of infection, namely the respiratory or central nervous system. Patients presenting mild to moderate pulmonary infections are usually treated with 200-400 mg/d fluconazole or itraconazole for six to 12 months (Saag *et al.*, 2000; Pappas *et al.*, 1998; Dromer *et al.*, 1996; Denning *et al.*, 1989). In severe pulmonary infection or those presenting with a CNS infection, a combination of AMB and flucytosine is prescribed for two weeks followed by 400 mg/d fluconazole for a minimum of 10 weeks (Saag *et al.*, 2000; van der Horst *et al.*, 1997).

Patients suffering with a compromised immune system, such as HIV and AIDS, are prescribed a primary therapy followed by extended therapy, termed maintenance therapy. Maintenance therapy involves the lifelong supplementation of the immune system with one or more anti-fungal agents. Patients presenting pulmonary infections are generally prescribed 200-400 mg/d fluconazole or itraconazole lifelong, or alternatively, 400 mg/d fluconazole supplemented with flucytosine for a minimum of 10 weeks (Saag *et al*., 2000; Jones *et al*, 1991; Denning *et al*., 1989). Those patients presenting central nervous system infections are generally treated with a combination of AMB and flucytosine for two weeks, followed by 400 mg/d fluconazole for a minimum of 10 weeks (van der Horst *et al*., 1997; Powderly, 1993). Fluconazole (200-400 mg/d) is generally prescribed as a prophylaxis as this azole is considered to be more effective, although itraconazole (200 mg/d) and AMB have also proven to be successful (Saag *et al*., 2000; Mondon *et al*., 1999; Saag *et al*., 1999; Powderly *et al*., 1992).

In the era of anti-retrovirals (ARVs) and highly active anti-retroviral therapies (HAARTs), the use of prophylaxis has become more common. In some patients, the initiation of HAART subsequent to being diagnosed with a cryptococcal infection results in an exaggerated immune response termed cryptococcal immune reconstitution inflammatory syndrome (IRIS) (Jenny-Avital *et al.,* 2002). Cryptococcal IRIS is associated with a clinical worsening of cryptococcosis, paradoxical cryptococcal IRIS, or with the new presentation of cryptococcal disease or ART-associated cryptococcosis (Haddow *et al.,* 2010; Breton *et al.,* 2002; Shelburne *et al.,* 2002; Trevenzoli *et al.,* 2002; Blanche *et al.,* 1998; Woods *et al.,* 1998). Similar cases have been reported in association with other pathogens such as *M. avium, M. tuberculosis*, cytomegalo-virus and the hepatitis viruses (Hirsch *et al.,* 2004; Sungkanuparph *et al.,* 2003; Jenny-Avital *et al.,* 2002; Cheng *et al.,* 2000; French *et al.,* 2000; Race *et al.,* 1998). Interestingly, cryptococcal IRIS has recently been identified in organ transplant patients who have been treated with adalimumab and alemntuzmad (Cadena *et al.,* 2009; Ingram *et al.,* 2007; Singh *et al.,* 2005).

Numerous studies reported the development of cryptococcal IRIS in 8 – 50 % of AIDS patients after the initiation of HAART (Antinori *et al.,* 2009; Lawn *et al.,* 2005a; Lortholary *et al.,* 2005; Jenny-Avital *et al.,* 2002; Shelburne *et al.,* 2002), but, delaying the initiation of HAART therapy to avoid IRIS is still a controversial issue. The onset of cryptococcal IRIS subsequent to the initiation of HAART ranges from

between three and 27 months (Skiest *et al.*, 2005), but, patients that presented with non-CNS cryptococcal IRIS displayed longer incubation periods and lower mortality rates than patients with CNS cryptococcal IRIS. A study conducted in Zimbabwe indicated a significantly greater mortality rate (82 %) in those cases that received immediate anti-retroviral treatment compared to those that delayed anti-retroviral treatment (34 %) (Makadzang *et al.*, 2010). Similar results have been reported from small studies conducted in France (Lortholary *et al.*, 2005) and South Africa (Lawn *et al.*, 2005a) where 30 and 66 % of patients displayed early mortality, respectively. It should be noted, however, that the mortality rate among HIV and AIDS patients who do not initiate anti-retroviral treatment remains high in Sub-Saharan Africa. Two studies conducted in South Africa reported mortality rates of 67 and 87 % in the three months leading up to anti-retroviral treatment (Fairall *et al.*, 2008; Lawn *et al.*, 2005b). Given these high mortality rates, the early initiation of HAART often out-weighs the associated risks of developing cryptococcal IRIS.

The prophylactic use of fluconazole by AIDS patients is of concern with regards to the emergence of more resistant strains within the population. Numerous reports have highlighted the decreased susceptibility of *C. neoformans* to amphotericin B, fluconazole, flucytosine and itraconazole (Cuenca-Estrella *et al.*, 2001; Armengou *et al.*, 1996; Birley *et al.*, 1995; Currie *et al.*, 1995). In contrast, a recent study conducted by Chowdhary and co-workers (2011) determined that the majority of isolates remain susceptible to antifungals. Similarly, both Pfaller (2005) and Brandt (2001) concluded that the *in vitro* susceptibility of *C. neoformans* isolates has remained relatively constant over a 15 and 4 year period, respectively. Despite the relative uncommon resistance seen in *C. neoformans*, continued surveillance of resistance needs to be undertaken to limit the emergence of less susceptible strains of this yeast pathogen.

5. SEXUAL REPRODUCTION OF *CRYPTOCOCCUS NEOFORMANS*

In 1975 and 1976 the perfect state of the pathogenic yeasts *C. neoformans* and *C. gattii*, respectively *Filobasidiella neoformans* and *Filobasidiella bacillispora*, were identified by Kwon-Chung. The co-culturing of clinical strains in differing combinations on various sporulation agar led Kwon-Chung to observe the development of hyphae with fused clamp connections, characteristic of basidiomycetes. Further observation revealed the development of hyphae into basidia

and the production of basidiospores arranged in chain-like structures on the apex of the holobasidia (Kwon-Chung, 1980).

5.1. Sexual Mating in *Cryptococcus neoformans*

During the sexual reproduction of heterothallic yeasts, one partner generally initiates a signal that results in the development of conjugation tubes by both partners. During the mating of *C. neoformans*, however, the mating pheromone produced by MAT*a* (*MFa*) is expressed in response to certain environmental signals such as nitrogen limitation, copper ions, plant material and myo-inositol (Kent *et al.*, 2008; Xue *et al.*, 2007; Hull *et al.*, 2002; Shen *et al.*, 2002; Kwon-Chung *et al.*, 1992) which induces the development of a conjugation tube in MATα cells. Cells of opposite mating types then fuse and form a heterokaryon that develops into dikaryotic hyphae possessing un-fused nuclei and fused clamp connections (Fig 2) (Kwon-Chung, 1980). Unlike other model yeasts, karyogamy and meiosis will only occur once the hyphae have developed into basidia. The subsequent process of sporogenesis can be erratic; nevertheless, it results in basidiospores of each mating type forming chain-like structures on the basidial head. Dispersion of each mating type is done in a random fashion and the basidiospores can be easily dislodged.

Interestingly, research has shown that the migration of mitochondrial DNA (mtDNA) is uni-parental, originating from the MAT*a* cell (Xu *et al.*, 2000), a phenomenon observed in filamentous fungi, but not among yeasts. The migration of the nucleolar genetic material appears to be unidirectional, moving along the conjugation tube of the MATα cell to the MAT*a* cell (Kwon-Chung *et al.*, personal communication).

5.2. Unisexual Mating / Monokaryotic Fruiting in *Cryptococcus neoformans*

Monokaryotic fruiting occurs when haploid cells form true hyphae that give rise to basidia and basidiospores in the absence of the opposite mating type (Esser *et al.*, 1977). Occurring in many higher basidiomycetes such as the mushrooms, *C. neoformans* is the only known lower basidiomycete to undergo monokaryotic fruiting. During the process, the nuclear content is doubled, either via the fusion of two haploid nuclei of the same mating type or endo-duplication (Lin *et al.*, 2006; Tscharke *et al.*, 2003; Wickes *et al.*, 1996; Shadomy *et al.*, 1966). Diploidization can occur at various stages such as before filamentation, during filamentation or within

the basidia, but nuclear reduction is achieved though meiosis in all cases. Although hyphae can appear similar, monokaryotic hyphae can be distinguished firstly by the appearance of un-fused clamp connections and the development of long bead-like structures termed blastospores (Fig 3).

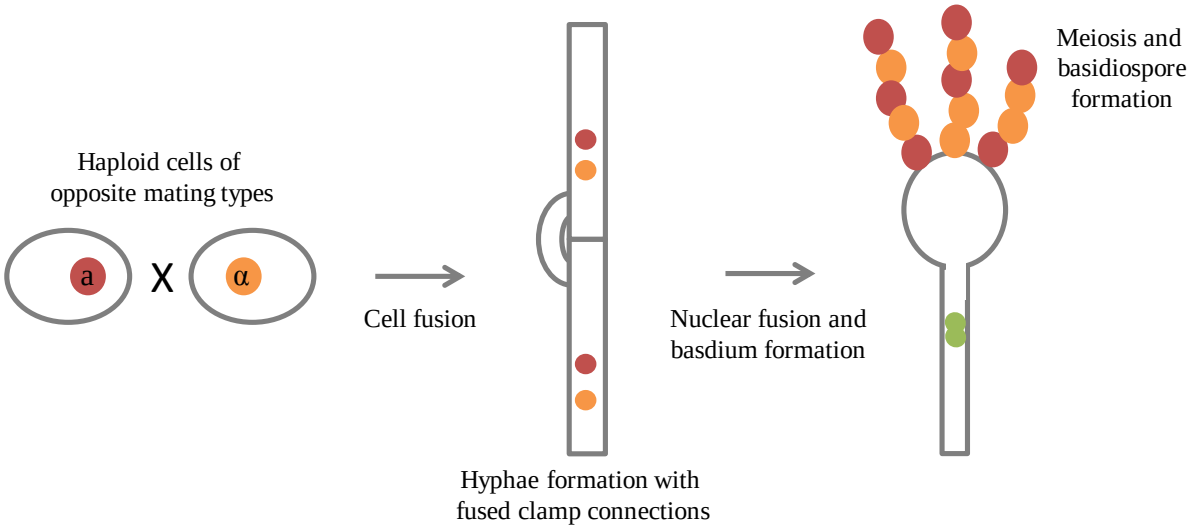

Figure 2. Sexual mating of *C. neoformans* occurs when cells of opposite mating types fuse and form hyphae, with fused clamp connections that ultimately give rise to basidia and basidiospores, the suspected infectious propagules.

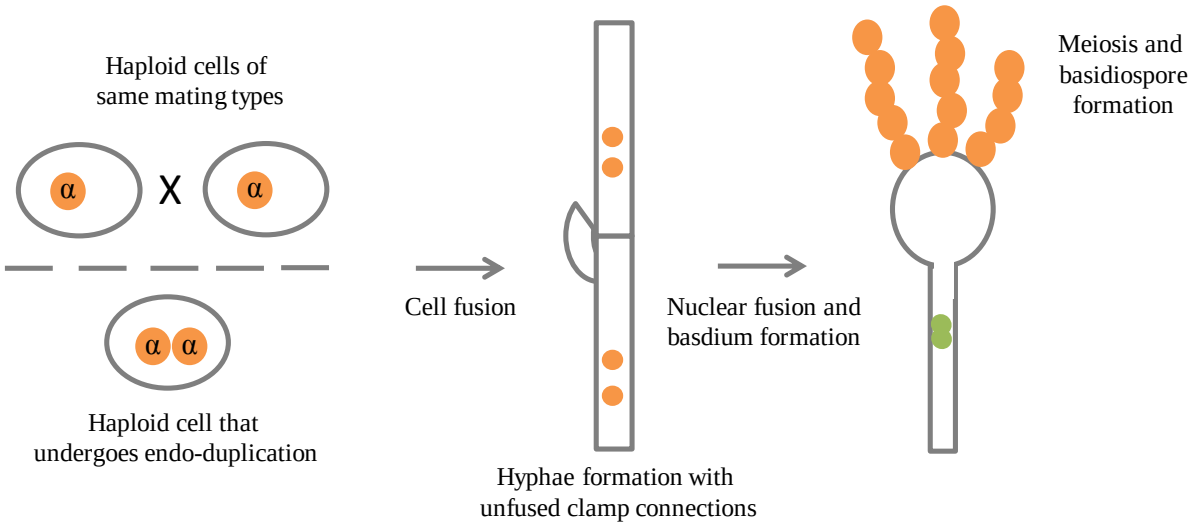

Figure 3. Monokaryotic and same-sex fruiting of *C. neoformans* generally occurs when two MATα cells fuse or when a MATα undergoes endo-duplication. Hyphae with un-fused clamp connections are formed that ultimately give rise to basidia and basidiospores. It should be noted nuclear fusion can occur at any point during the formation of hyphae and basidiospores.

Despite rare reports of hyphal development by *C. neoformans* during infection (Bemis *et al*., 2000; Williamson *et al*., 1996; Neilson *et al*., 1978; Freed *et al*., 1971; Shadomy *et al*., 1971; Shadomy *et al.*, 1966), this pathogen is not regarded as being dimorphic as hyphal production was only observed under mating conditions or as a result of self-fertile diploid stains. In 1996, Wickes and co-workers were able to induce monokaryotic fruiting of MATα strains of *C. neoformans.* The phenomenon occurred under nitrogen starvation conditions at room temperature and resulted in the formation of basidia as well large numbers of viable basidiospores.

The inability of MAT*a* strains to produce monokaryotic hyphae led the authors to conclude that the mating type bias of MATα cells observed in both environmental and clinical isolations may be as a result of monokaryotic fruiting. However, in 2003, Tscharke and co-workers identified two new strains of *C. neoformans* var. *neoformans* MAT*a* that were able to undergo monokaryotic fruiting. They also noted that *C. neoformans* var. *neoformans* (serotype D) strains were the most vigorous monokaryotic fruiters, while *C. neoformans* var. *grubii* (serotype A) strains were poor monokaryotic fruiters or did not fruit at all. These findings would contradict the hypothesis that monokaryotic fruiting is responsible for the mating type bias as the majority of strains isolated are *C. neoformans* var. *grubii*, serotype A, MATα.

A major outbreak of cryptococcosis in 2005 on Vancouver Island resulted in the isolation of *C. gattii* MATα strains that appeared to be more virulent than those seen elsewhere (Fraser *et al.*, 2005). Genotypic analysis of these *C. gattii* strains revealed evidence of sexual recombination but, the offspring appeared to have descended from two MATα parents. Shortly thereafter, the same phenomenon was observed in 31 *C. neoformans* var. grubii MATα isolates from infected cats and dogs (Bui *et al.*, 2008). The isolation of αADα diploid strains confirmed that similar to *C. neoformans* var. *grubii*, same-sex mating also occurs within populations of *C. neoformans* var. *neoformans* (serotype D) (Lin., *et al.*, 2007). These studies indicate that same-sex mating does occur in natural populations of *C. neoformans* and *C. gattii* and may account for the mating type bias observed in populations of both these pathogenic yeast species.

Interestingly, the closely related species *F. depauperata* is known to be homothallic and grows exclusively as a filamentous fungus (Kwon-Chung *et al.*, 1995; Guéhol *et al.*, 1993). Although these hyphae are monokaryotic, the

basidiospores are produced as a result of meiosis and could occur in a similar manner seen during the same-sex mating of *C. neoformans*.

5.3. Genetic Composition of the MAT locus of *Cryptococcus neoformans*

In contrast to a number of basidiomycetes, *C. neoformans* has a bipolar mating system with two opposite mating types, MAT*a* or MATα (McClelland *et al*., 2002; Kwon-Chung *et al.*, 1992; 1976). This first mating-linked gene to be characterized from *C. neoformans* MATα was the mating pheromone (*MF*α) gene containing part of the MAT locus (Moore *et al.*, 1993). In 1997, Wickes and co-workers were able to identify *STE12*α that showed homology to the *Saccharomyces cerevisiae STE12* gene; while Chang and co-workers identified the corresponding *STE12a* in 2001. Subsequent mapping of the MATα locus revealed the presence of several MATα-specific homologs, such as a pheromone response mitogen activated protein kinase (MAPK) cascade genes; *STE11*, *STE20* and *STE12*, two additional pheromone genes; *MF*α*2* and *MF*α*3*, the pheromone receptor CPRα, a myosin gene as well as the translation initiation factor (Karos *et al*., 2000). The presence of the latter two genes is unusual as genes unrelated to mating type are not located in the MAT locus of other fungi. A second pheromone receptor, Cpr2, has also been identified and appears to be unlinked to the MAT locus (Chang *et al.*, 2001). The gene has been found to be modestly expressed during pheromone exposure however; transcription is increased after cell fusion. Deletion of this gene did not abolish fruiting, but hyphae displayed an abnormal morphology suggesting that Cpr2 is involved in the stabilization of the dikaryon (Hsueh *et al.*, 2009; Kwon-Chung *et al.*, 1998).

Final sequence data of the MAT loci from both mating types has shown that both loci contain more than 20 genes; while the MAT*a* locus is approximately 120 kb while the MATα is only 100 kb (Lengeler *et al*., 2002). The entire locus is inherited as single unit, thereby limiting recombination and preventing the generation of sterile progeny. To date, no other heterothallic fungus has revealed the same genetic organization of the MAT locus as seen in *C. neoformans*.

5.4. Pheromone Response Pathway of *Cryptococcus neoformans*

Opposite cell types of *C. neoformans* are able to signal and respond during mating through a conserved-pheromone receptor system (Wickes, 2002). The pheromones of each mating type, designated *MFa* for MAT*a* pheromone and *MF*α for MATα

pheromone, are small multi-copy hydrophobic peptides (McClelland *et al*., 2002). Three genes have been identified that encode for the *MFα* pheromone, termed *MFα1*, *2* and *3* (Shen *et al*., 2002; Davidson *et al*., 2000; Karos *et al*., 2000; Moore *et al.*, 1993), and all are induced under nutrient limiting conditions and co-culture with *MATa* cells. Similarly, three related genes, sharing little amino acid sequence homology with *MFα* (McClelland *et al*., 2002) encoding the *MFa* pheromone, have also been identified. However, unlike *S. cerevisiae*, the deletion of the pheromone genes does not inhibit the mating or spore production of *C. neoformans*.

Mating is initiated when a pheromone binds to a cognate receptor, subsequently leading to the activation of a MAP kinase cascade of which some components appear to be mating type specific (Lengeler *et al*., 2000; Wang *et al.*, 1999). In MATα cells, the *MFa* pheromone activates the MAP kinase cascade by binding to a G-protein coupled receptor and a heterotrimeric G-protein (Fig 4). The G-β-subunit transduces the signal to Ste20α, a PAK-kinase that is known to be mating specific (Wang *et al*., 2002; Wang *et al*., 2000). The PAK kinase relays the signal to the MAP kinase cassette comprised of Ste11α, Ste7, and Cpk1 (Hull *et al.*, 2005; Clarke *et al*., 2001). The MAP kinase cascade activates transcription factor Mat2, which initiates the transcription of *STE12α* and *Sxi1α* that control fruiting and virulence (Kruzel *et al.*, 2012). Once again, unlike *S. cerevisiae*, the deletion of the transcriptional activator genes (*STE12a* and *STE12α*), does not affect the fertility of *C. neoformans*. However, efficiency of monokaryotic fruiting, capsule and melanin production are reduced, indicating that *STE12* may be necessary for other virulence factors (Chang *et al*., 2000; Yue *et al*., 1999).

5.5. The Link between Mating Type and Virulence

The speculation that mating type is linked to virulence is as a result of high isolation frequencies (> 99.9 % with regards to serotype A) of MATα strains from cryptococcosis cases (Halliday *et al*., 1999; Kwon-Chung *et al.*, 1981; 1978). In 1992, Kwon-Chung and co-workers constructed a pair of congenic MAT*a* and MATα strains for *C. neoformans* var. *neoformans* (serotype D), B-4476 (MAT*a*) and B-4500 (MATα). The virulence of these congenic strains was tested using the murine tail vein model. Mice infected with B-4500 displayed a higher mortality rate that generally occurred within in shorter time period, thus indicating that MATα is indeed more virulent than MAT*a* (Nielsen *et al.*, 2005a; Kwon-Chung *et al.*, 1992). It should be

noted, however, that the killing of mice by MAT*a* cells did also occur, albeit at lower levels, indicating that although this mating type is less virulent, it is still lethal.

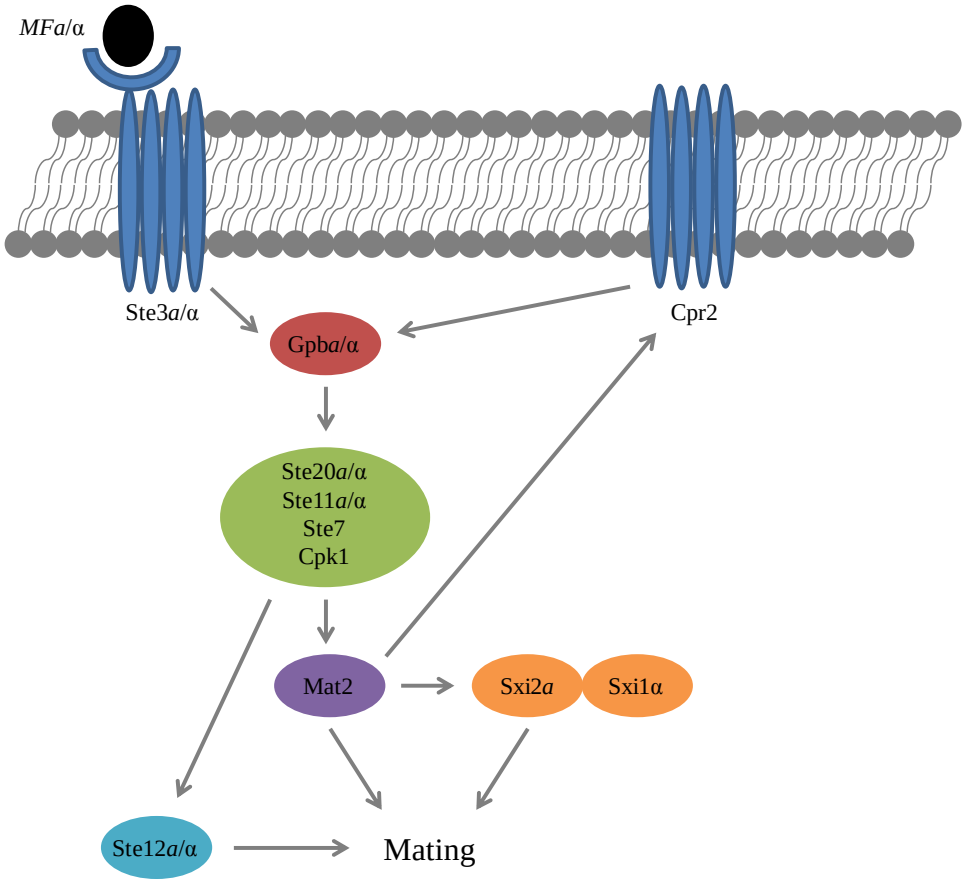

Figure 4. The pheromone signalling pathway of *C. neoformans* is conducted via a MAP kinase cascade

In contrast to the results obtained with *C. neoformans* var. *neoformans*, virulence testing with *C. neoformans* var. *grubii* (serotype A) using the congenic MAT*a* and MATα strains, KN99-*a* and KN99-α, revealed no difference in virulence between the two mating types (Nielsen *et al.*, 2005b; 2003). Interestingly, when mice were infected with KN99-*a* and KN99-α, MATα cells appeared to enter the CNS and cross the BBB more readily.

Virulence testing, as mentioned above, revealed that the genetic diversity between the differing strains utilized can impact on the virulence of *C. neoformans* and would suggest that the MAT locus is capable of interacting with other genes within the genome (Nielsen *et al.*, 2005a; 2005b).

An increase in the number of environmental isolations of *C. neoformans* has revealed the same mating type bias exists within naturally occurring populations of

this pathogen. Ratios as high as 40 : 1 (MATα : MAT*a*) have been reported for environmental isolations (Cogliati, 2013; Chowdhary *et al.*, 2012; Kuchak *et al.*, 2012; Romeo *et al.*, 2012; Mora *et al.*, 2010; Randhawa *et al.*, 2008; Jain *et al.*, 2005; Yan *et al.*, 2002; Halliday *et al.*, 1999), but rare cases of even distribution between mating types have been reported. Litvintseva and co-workers (2003) identified a cryptococcal population in Botswana where MAT*a* cells comprised roughly 10 % of the population. The closely related species *C. gattii* demonstrates the same dominance with regards to uneven mating type distribution, although cases of even ratios have also been reported. In 1999, Halliday and co-workers reported the isolation of *C. gattii* with ratios of approximately 1:1 with regards to mating type.

As such, researchers suggest that a higher frequency of exposure to naturally occurring populations of MATα strains could explain the mating type bias observed within clinical isolations. However, the environmental MATα bias raises questions as to whether or not MATα is better suited for survival than its counterpart. Researchers have suggested that sex-linked lethal mutations associated with MAT*a* could be one possibility to explain the mating type bias. Alternatively, the occurrence of monokaryotic and same-sex fruiting may indicate that sexual reproduction is not essential for survival or overall fitness in *C. neoformans* (Franzot *et al.*, 1997; Brandt *et al.*, 1996; Chen *et al.*, 1995). Indeed, monokaryotic fruiting would allow *C. neoformans* to produce easily dispersed basidiospores during unfavourable conditions while still retaining certain polygenic traits, such as those necessary for virulence (Nielsen *et al.*, 2007; Fraser *et al.*, 2005; Kidd *et al.*, 2005; 2004).

Interestingly, a number of other fungal pathogens, such as *Candida albicans* and *Aspergillus fumigatus*, have retained genes essential for sexual reproduction, but similar to *C. neoformans* their populations are predominantly clonal displaying limited recombination (Nielsen *et al.*, 2007; Heitman, 2006). This limited sexual reproduction appears to act as a virulence strategy enabling fungal pathogens to rapidly adapt to both a host and an environmental niche (Nielsen *et al.*, 2007). However, alternative theories to explain the mating type bias seen within *C. neoformans* populations need to be explored further.

6. VIRULENCE FACTORS OF *CRYPTOCOCCUS NEOFORMANS*

Virulence can be defined as a microbial characteristic that is expressed only in susceptible hosts and involves the microbe's capacity to cause damage to the host

itself (Casadevall *et al.*, 2003; Steenbergen *et al.*, 2003). Three basic criteria must be considered to define a specific microbial characteristic as a virulence factor. Firstly, the characteristic must be associated with the pathogen; secondly, the inactivation of the associated gene must decrease the overall virulence; and finally, the complementation or restoration of the gene product must restore virulence (Steenbergen *et al.*, 2003). Although some mechanisms of virulence remain unresolved, it is clear is that *C. neoformans* is able to avoid the humoral response. Like most pathogens, this phenomenon is not the result of a single virulence factor, but rather the cumulative effect of a number of virulence factors.

As such, there are a number of virulence factors associated with the pathogenesis of *C. neoformans*, but perhaps the best understood include laccase and melanin synthesis, the ability to withstand physiological temperatures, as well the production of a polysaccharide capsule (Akhter *et al.*, 2003; Steen *et al.*, 2002; Cox *et al.*, 2001; Feldmesser *et al.*, 2001; 2000; Zhu *et al.*, 2001; Liu *et al.*, 1999; Wang *et al.*, 1995; 1994). Other virulence factors that need to be considered include phospholipase, urease and proteinase production and as previously mentioned the mating type of the yeast itself.

6.1. The Polysaccharide Capsule

While serving as a major diagnostic feature (Bose *et al.*, 2003), the polysaccharide capsule of *C. neoformans* plays a vital role in the virulence of this pathogen by inhibiting effective phagocytosis and clearance of the yeast by macrophages. As a result, *C. neoformans* is able to persist and multiply within human macrophages however; infections appear to only become life-threatening when the human immune system is compromised (Goldman *et al.*, 2001).

6.1.1. Polysaccharide Capsule Structure. The capsule is regarded as the main virulence factor of *C. neoformans* (Janbon 2004). Early analysis of the capsule identified the sugars galactose, mannose, xylose and also glucuronic acid (Evans *et al.*, 1950). Later research revealed that the capsule is composed of a minimum of three components, namely mannoprotein; galactoxylomannan (GalXM) and glucuronoxylomannan (GXM) (Janbon, 2004; Bose *et al.*, 2003; Steenbergen *et al.*, 2003; Cheriniak *et al.*, 1982; Bhattacharjee *et al.*, 1981; 1980; 1979a; 1979b; Rebers *et al.*, 1958). The latter component (GXM) composes approximately 90% of the

capsule structure and consists of mannose residues that are α-1,3 linked with xylosyl and glucuronyl side groups (Janbon, 2004; Bose *et al.*, 2003; Bhattacharjee *et al.*, 1984; Cheriniak *et al.*, 1982). Certain mannose residues are 6-O-acetylated, usually with an un-branched mannose, but substitution with glucuronic acid can occur. It is the variation in the xylose : mannose : glucuronic acid molar ratio that allows the classification into the four different serotypes, namely serotypes A and D for *C. neoformans* var. *grubii* and var. *neoformans*, respectively, and serotypes B and C for *C. gattii* (Bhattacharjee *et al.*, 1984). However, this classification does not take into account the variation within GXM structure that appears to correlate directly with the virulence of the strain (Janbon, 2004; Bose *et al.*, 2003; Steenbergen *et al.*, 2003). It has therefore been suggested that the yeast be classified on the basis of minimum GXM repeating units in order to determine the virulence of the isolated strain.

The second structural component, GalXM, comprises only 7 % of the polysaccharide capsule (Janbon, 2004; Bose *et al.*, 2003; Vaishnav *et al.*, 1998; Turner *et al.*, 1984). It consists of a α-1,6-linked galactose polymer with a number of side chains of varying lengths. These side chains can consist of a number of structures including galactosyl, mannosyl and xylosyl residues. Once again, the structures vary between the four different serotypes.

The final component, mannoprotein, is perhaps the most vital with regards to the host's immune response (Janbon, 2004; Bose *et al.*, 2003; Levitz *et al.*, 2001; Murphy, 1998). These proteins are responsible for the induction of cell-mediated immunity and cytokine production, both of which are critical to the host during the initial stages of infection.

6.1.2. Regulation of Capsule Biosynthesis. Both *in vivo* and *in vitro* studies have demonstrated that the immediate environment affects capsule synthesis (Busse, 1984). Nutrient availability appears to play a major role in capsule size and is dependent on the available carbon source, amino acids and vitamins (Littman, 1958). High glucose concentrations inhibit capsule synthesis, while low concentrations of glucose, iron, mannose, xylose and sucrose in combination with the amino acids thiamine, L-proline and asparagine enhance capsule production (Vartivarian *et al.*, 1993; Jacobson *et al.*, 1989; Dykstra *et al.*, 1977; Farhi *et al.*, 1970).

During infection, capsule size is also dependent upon the organ infected (Rivera *et al.*, 1998). Isolates originating from lung tissue were shown to have on

average thicker capsules than those originating from brain tissue. Rivera and co-workers suggest that this difference is as a result of the higher iron concentration found within brain tissue. As mentioned previously, low concentrations of this metal enhance capsule synthesis (Vartivarian *et al.*, 1993) and would therefore suggest that the opposite effect would be observed with higher iron concentrations, however the exact mechanisms of inhibition is unknown.

Four *CAP* genes, *CAP10, CAP59, CAP60*, and *CAP64*, have been implicated in the biosynthesis of the polysaccharide capsule, but sequence homology has been unable to determine their precise function (Chang *et al.*, 1998; 1997; 1996; 1994). Signal transduction pathways involved in capsule synthesis are also not yet fully understood (Janbon, 2004). Transcription and translation of capsule genes appears to be regulated by the target of rapamycin (TOR) pathway that responds to changes in the availability of nitrogen and amino acids. Secondly, response to changes in osmotic pressure could potentially be controlled by the high osmolarity glycerol (HOG) pathway as is seen in *S. cerevisiae*. And finally, the cyclic adenosine monophosphate (cAMP) pathway is involved in the regulation of capsule biosynthesis although the surface receptors and a number of lower protein kinase targets are yet to be identified.

6.1.3. Role of the Polysaccharide Capsule during Pathogenesis of *Cryptococcus neoformans*. The capsule enhances the virulence of *C. neoformans* by interfering with and protecting yeast cells from the immune system. Traditionally, the capsule was thought to be primarily anti-phagocytic, but a number of additional factors have been resolved and include induction of macrophage apoptosis, alteration of antigen presentation, inhibition of antibody and cytokine production, reduction of leukocyte migration to inflamed sites, depletion of complement components, *in vitro* invasive growth and macrophage dysfunction (Monari *et al.*, 2008; Zaragoza *et al.*, 2008a; Janbon, 2004; Bose *et al*., 2003; Steenbergen *et al*., 2003; Dong *et al.*, 1995; Collins *et al.*, 1991). Once engulfed, the yeast sheds polysaccharides from its capsule into vesicles around the phagosome that gradually accumulate in the cytosome, resulting in dysfunction of the macrophage and possibly apoptosis (Monari *et al.*, 2008; Steenbergen *et al*., 2003; Dong *et al.*, 1995; Collins *et al.*, 1991). More recently, however, it is thought that the capsule enables the yeast to replicate within the macrophages allowing infections to remain dormant, to be "re-activated" at a later stage (Zaragoza *et al.*, 2008a; Janbon, 2004; Goldman *et al*., 2000). Ingested yeast

cells appear to replicate as rapidly as extra-cellular cells with a single macrophage containing on average 30 to 40 yeast cells (Tucker *et al.*, 2002). Although the exact mechanism of yeast replication within macrophage cells remains unclear, Tucker and co-workers (2002) suggested that the larger capsule present in lung tissue dilutes lysosomal contents and provides physical protection by creating a barrier between the surface of the yeast cell and microbiocidal compounds, such as free radicals released from the phagosomal membrane (Zaragoza *et al.*, 2008b).

6.2. Laccase

Laccases form a large group of enzymes termed the multi-copper or blue oxidase enzymes (Mayer *et al*., 2002; Liu *et al*., 1999; Thurston; 1994). The enzyme is regarded as being ubiquitous in nature and has so far been identified in all domains of life, although the majority have fungal origins (Claus, 2004). Laccases are non-specific regarding their substrate; however enzyme activity varies from laccase to laccase (Thurston, 1994). Generally, any molecule similar to *p*-diphenol will be utilized as substrate by this group of enzymes (Mayer *et al*., 2002).

6.2.1. The Cryptococcal Laccase Enzyme. The laccase produced by *C. neoformans* was originally thought to be a phenol or di-phenol oxidase due to its ability to produce coloured pigments (Liu *et al*., 1999; Thurston; 1994). Atomic absorption revealed that the enzyme contained 4 mol/mol of copper and had absorbance peaks at 610 and 320 nm, both of which are characteristic of type I and III copper laccases, respectively (Zhu *et al.*, 2004; Ikeda *et al.*, 1993). This evidence, combined with the presence of several copper binding sites within the amino acid sequence derived from the cryptococcal laccase (*CNLAC1)* gene, indicated that the enzyme was indeed a fungal laccase. Recent genome projects revealed that *C. neoformans* possesses a second laccase gene, *CNLAC2*. Present in the form of a tandem repeat and in the same orientation as *CNLAC1*, the two genes share 65 % nucleotide homology (Zhu *et al.*, 2004).

Evolutionary studies conducted by Valderrama and co-workers (2003) suggested that the laccase of *C. neoformans* is in fact not a true fungal laccase. While the majority of laccases are extremely diverse in terms of their protein structure and substrate utilization, their catalytic sites are generally conserved (Mayer *et al*., 2002). Valderrama and co-workers (2003) conducted phylogenetic studies by comparing the

active sites of various laccase enzymes and noted that the laccases expressed by *C. neoformans* and *Aspergillus nidulans* fell within their own clade. It is therefore suggested that the cryptococcal laccase is not a true laccase, but rather a representative of a different family of multi-copper oxidases (Valderrama *et al.*, 2003).

6.2.2. Molecular Regulation of the Cryptococcal Laccase Enzyme. Molecular regulation of the *CNLAC1* gene is thought to have evolved due to environmental pressure as opposed to the physiological pressures of the host (Zhu *et al.*, 2004). For example, glucose limited conditions, such as those present in the brain, stimulate the expression of laccase (Salas *et al.*, 1996). Metal induction of the cryptococcal laccase has been well characterized with regards to iron and copper where quantities of the latter as low as 5 µM resulted in an increase in laccase transcription (Zhu *et al.*, 2004; Zhu *et al.*, 2003; Jacobson *et al.*, 1996; Salas *et al.*, 1996; Polacheck *et al.*, 1982).

The regulation of *CNLAC1* also contains features commonly associated with regulation in higher eukaryotes (Zhang *et al.*, 1999). Transcriptional regulation in higher eukaryotes, both mammalian and plants, is usually characterised by the presence of multiple interacting DNA binding sites found over a large upstream region of genes, as well as the ability to use enhancers, such as Sp1, that are rich in glutamine. In contrast, fungi contain fewer transcriptional elements and are generally located closer to the open reading frame (ORF).

Zhang and co-workers (1999) evaluated the 5´-upstream region of *CNLAC1* under glucose repression in order to identify any enhancement and repression regions. Studies revealed two upstream enhancer regions, one of which contains a consensus specificity protein 1 (Sp1) DNA binding site. Specificity protein 1 is a zinc finger transcription factor that binds to GC rich areas of the promoter (Briggs *et al.*, 1986). Further analysis revealed that Sp1 DNA binding sites are also present in the *CAP64* and *CAP59* genes which are involved in polysaccharide capsule synthesis. This may suggest the co-regulation of these virulence factors by a transacting Sp1 protein.

The second enhancer region contained an E2F consensus site, a gene family that is predominantly associated with the regulation of cell growth (Zhang *et al.*, 1999). It is suggested that the *E2F* gene is needed to synchronize genes during the cell cycle to prevent the uptake of iron during the log phase. The uptake of iron at this

stage would prove lethal to cells due to the ferrioxidase activity of the laccase enzyme.

The large number of repressor and enhancer sites implies that the laccase enzyme is under strict regulation to respond to both environmental and host stimuli varying expression under altering conditions (Zhu *et al.*, 2004).

6.2.3. Cellular Location of the Cryptococcal Laccase Enzyme. Studies have shown that *CNLAC1* is tightly associated with the outer region of the cryptococcal cell wall (Zhu *et al*., 2001), while *CNLAC2* can be found in the cytosol (Garcia-Rivera *et al.*, 2005; Missall *et al.*, 2005). From the cell wall, *CNLAC1* is able to interact directly with the host immune system as well as other extracellular products. A second advantage of such a location is that substrate transporters, such as those required for dopamine and other catecholamines, are made redundant. In the absence of *CNLAC1*, it has been found that *CNLAC2* can be targeted towards the cell wall and cytosol (Missall *et al.*, 2005). Interestingly, evidence was obtained for the release of laccase from the cell wall, which suggests that this enzyme may act as an anti-oxidant or iron scavenger (Garcia-Rivera *et al.*, 2005).

6.2.4. Role of the Laccase Enzyme during Pathogenesis of *Cryptococcus neoformans*. Originally thought to only produce the virulence factor melanin, recent studies indicated that the laccase enzyme itself is a potent virulence factor (Zhu *et al*., 2001; Liu *et al*., 1999). Liu and co-workers (1999) compared macrophage-mediated killing between both laccase-positive and laccase-deficient strains. Their study was able to show that the laccase enzyme is able to confer protection without the presence of melanin.

As mentioned previously, fungal laccases belong to the family of copper or blue oxidase enzymes. The iron transporter Fet3, isolated from *S. cerevisiae*, has been shown to be a member of this family (de Silva *et al*., 1997). The oxidation of iron from Fe(II) to Fe(III) is coupled to the transport of iron across the plasma membrane by the Fet3 protein. The cryptococcal laccase shares this iron oxidase activity with Fet3 (Liu *et al*., 1999; Jacobson *et al.*, 1998). Macrophages require Fe(II) to produce toxic oxygen metabolites (TOM) that are essential for macrophage mediated killing of microbes (Zhu *et al*., 2001; Liu *et al*., 1999; Jacobson *et al.*, 1997). These oxidative bursts would therefore prove to be less efficient against *C. neoformans* as *CNLAC1*

would compete for the substrate Fe(II) due to its iron oxidase activity, ultimately aiding the pathogen.

6.3. Melanin

A defining characteristic of *C. neoformans* and *C. gattii* is their ability to synthesize a dark pigment known as melanin (Staib, 1962). Melanin is a negatively charged, high molecular weight compound that is ubiquitous in nature and is produced by a number of organisms that includes bacteria, fungi, plants and animals (Hill, 1992). Although their structures are poorly understood, melanins are described as macromolecules that are insoluble, resistant to acid degradation and form a stable free radical population (Butler *et al.*, 1998).

Melanin is associated with a variety of biological phenomena including Parkinson's disease (d'Ischia *et al.*, 1997; Enochs *et al.*, 1994), malignant melanoma (Hill, 1991; Riley *et al.*, 1991), traumatic anterior chamber uvetis (Kaya *et al.*, 1992; Broekhuyse *et al.*, 1991) and vitiligo (Wassermann *et al.*, 1973). Melanin has also been found to play a role in the pathogenesis of many pathogens such as *Aspergillus fumigatus*, *C. neoformans*, *C. gattii*, *Mycobacterium leprae*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii* and *Wangiella dermatitidis*; with the two most important types of melanin being dihydroxynaphthalene (DHN) and dihydroxyphenylalanine (DOPA) (Langfelder *et al.*, 2003; Butler *et al.*, 1998).

6.3.1. Biosynthesis of Melanin in *Cryptococcus neoformans.* The synthesis of melanin in *C. neoformans* is regulated by a number of factors such as iron, copper and glucose, and requires an exogenous dihydroxy-phenolic (DOPA) as well as oxygen (Zhu *et al.*, 2004; 2001; Alspaugh *et al.*, 1997; Jacobson *et al.*, 1996; Salas *et al.*, 1996; Polacheck *et al.*, 1982). With the well-known neurotropism of *C. neoformans*, the Mason-Raper scheme for the biosynthesis of eumamalian melanin was adapted using L-DOPA (Ito *et al.*, 1993; Polacheck *et al.*, 1988) (Fig 5). According to this pathway, the laccase enzyme oxidizes L-DOPA to form the intermediate dopachrome. Dopachrome is then non-enzymatically decarboxylated to form 5,6-dihydroxyindole (DHI) and 5,6 dihydroxyindole carboxylic acid (DHCI). Further oxidation is required to form indole-5,6-quinone, followed by polymerization to melachrome and finally melanin. Melanin becomes covalently liked to the cell wall and can account for approximately 15 % dry mass of heavily pigmented cells (Zhu *et al.*, 2001; Wang *et*

al., 1996). It should, however, be noted that the Mason-Raper model of melanin biosynthesis is based on the mammalian enzyme tyrosinase, capable of only oxidizing tyrosine while laccase enzymes display a much broader substrate specificity.

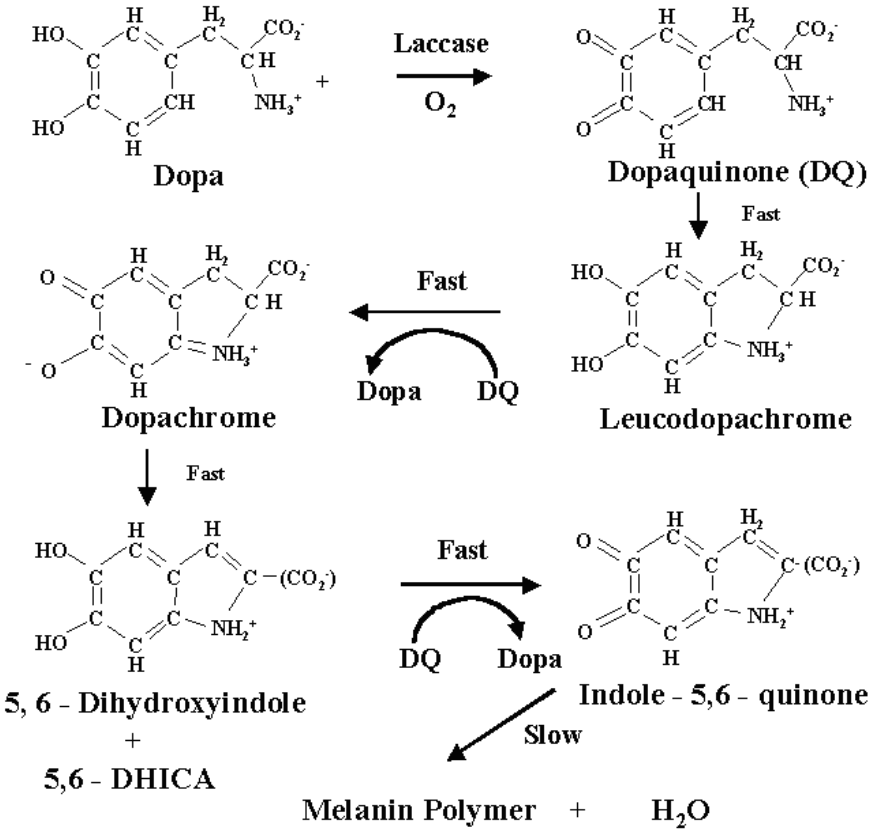

Figure 5 Proposed melanin synthesis scheme in *Cryptococcus neoformans* adapted from the Mason-Raper model (Williamson, 1997).

6.3.3. The Role of Melanin during Pathogenesis of *Cryptococcus neoformans.* Over the years, a number of studies have determined the role of melanisation during the pathogenesis of *C. neoformans*. In 1982, Kwon-Chung and co-workers were able to demonstrate that the infection of mice with wild-type melanin-positive *C. neoformans* strains (Mel^+) proved to be fatal. Mice infected with strains unable to produce melanin (Mel^-) survived and showed clearance of *C. neoformans* cells from the spleen, liver and brain. Similar results were observed by Rhodes and co-workers in 1985 with regards to mortality induced by Mel^+ and Mel^- *C. neoformans* strains. Interestingly, in mice that died after infection with Mel^- strains, approximately 50 %

of cells isolated had reverted to the wild-type phenotype. Microevolution of laboratory strains resulted in isolates displaying lower levels of melanin production and also displayed attenuated virulence (Franzot *et al.*, 1998). Similarly, the disruption of the cryptococcal laccase *LAC1* resulted in isolates with a significant reduction in virulence during the intravenous mouse model (Salas *et al*., 1996).

Melanin contributes to the virulence of *C. neoformans* by acting as an immuno-modulator, thereby protecting the yeast cell from the host immune system. Melanization increases the negative charge of the cell which is thought to inhibit phagocytosis. Similarly, melanin is a powerful anti-oxidant and allows *C. neoformans* to persist in alveolar macrophages and resist macrophage killing by providing resistance to oxidative stress. Melanin has also been shown to counteract microbial peptides (Doering *et al.*, 1999), as well as decreasing the susceptibility of *C. neoformans* towards amphotericin B and caspofungin (Ikeda *et al.*, 2003; Van Duin *et al.*, 2002; Wang *et al.*, 1994).

6.4 Thermo-tolerance

In order for a pathogen to cause disease, it must be able to withstand and proliferate at physiological temperatures (Kraus *et al*., 2004). Indeed, growth at 37 °C often results in a phenotypic switch in many pathogenic yeast species that increase its virulence. It is interesting to note that approximately 270 fungal species are known to cause disease within humans; the majority are dermatophytes that primarily affect the skin and nails. These regions are considered to have a lower temperature than the interior of the body, highlighting the pathogen's struggle to survive at higher temperatures (Perfect, 2006).

Interestingly, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* show great diversity with regards to their thermo-tolerance. Currently, serotype A is regarded as being more thermo-tolerant and is isolated from 90 % of all clinical cases world-wide (Martinez *et al*., 2001; Mitchell *et al.*, 1995). Perhaps the only exception to this trend exists within northern European countries where *C. neoformans* var. *neoformans* (serotype D) displays a greater prevalence (Martinez *et al*., 2001; Dromer *et al*., 1996). This observation has been attributed to the more temperate climate of these northern European countries.

Martinez and co-workers (2001) were also able to demonstrate that at 41 °C serotype D was on average more susceptible to heat killing than serotype A. A second

case study involving the simultaneous infection by *C. neoformans* and malaria, reported that during relapses of malaria, *C. neoformans* cells were cleared from the spinal fluid (Kligman *et al*., 1949). Researchers have speculated that the fever induced by the malaria may have resulted in the elimination of these yeast cells. This thermo-intolerance displayed by *C. neoformans* var. *neoformans* could account for its higher prevalence in European countries, with temperate climates, as well as the high predilection seen for skin tissue that is regarded as having a cooler temperature as the rest of the body (Martinez *et al*., 2001; Dromer *et al*., 1996).

6.5. Additional virulence factors

A variety of additional virulence factors have been observed during pathogenesis of *C. neoformans* and include phospholipase, urease and proteinase expression.

6.5.1. Phospholipase. Phospholipases are grouped in five classes, namely A_1, A_2, B, C and D, depending on the site of phospholipid-ester hydrolysis (van den Bosch *et al.*, 1982). Phospholipases A1 and A2 both cleave the SN-1 chain however; in the case of A2 this hydrolysis results in the release of arachidonic acid. Phospholipase B can cleave both SN-1 and SN-2 chains and are often referred to as lyso-phospholipases. Phospholipase C cleaves before the phosphate, releasing a di-acylglycerol and a phosphate-containing head group. In contrast, phospholipase D cleaves after the phosphate, releasing both a phosphatidic acid and an alcohol.

Two phospholipase classes have been identified in *C. neoformans*, phospholipase B (PLB) and phospholipase C (PLC) (Ghannoum, 2000). The cryptococcal phospholipase B1 (*PLB1*) is expressed by a single gene and is secreted into the environment where it hydrolyzes host phospholipids (Cox *et al*., 2001). This action is suspected of aiding in the spread of the pathogen as *C. neoformans plb1*$^-$ mutant strains were unable to degrade the phagosome membrane, thereby limiting the dispersion of the yeast (Cox *et al*., 2001).

The second phospholipase expressed by *C. neoformans*, P1-PLC1, has been shown to be responsible for regulating several virulence factors, including growth at 37 °C, the production of melanin, the secretion of Plb1 and cell wall integrity (Chayakulkeeree *et al.*, 2008a; 2008b).

6.5.2. Urease. Urease is able to convert urea to ammonia and carbonate, resulting in a localized increase in pH (Casadevall *et al.*, 1998). Urease-expressing cells are generally sequestered within the cerebral micro-capillary beds and as such the enzyme is implicated in aiding the invasion of the CNS by *C. neoformans* (Olszewski *et al.*, 2004; Cox *et al.*, 2000). It is, however, unclear if urease serves any role once *C. neoformans* has entered the brain.

6.5.3 Proteinase. Proteinases are regarded as important virulence factors in both fungi and bacteria (Ruma-Hayens *et al.*, 2000; Brueske *et al.*, 1986). Cryptococcal proteinases are suspected to provide nutrients to the pathogen by degrading proteins such as elastin, collagen, fibrinogen and immunoglobin; although a clear genetic link to virulence has not yet been established (Chen *et al.*, 1996).

7. ORIGINS OF VIRULENCE IN *CRYPTOCOCCUS NEOFORMANS*

The development and subsequent maintenance of virulence factors in *C. neoformans* is largely attributed to selective pressures that the yeast has encountered within its natural habitat (London *et al.*, 2006; Casadevall *et al.*, 2003; Steenbergen *et al.*, 2003; 2001). Consequently, these are commonly termed "dual-use" virulence factors as it has been proposed that factors such as capsule production and laccase expression evolved to ensure the survival of *C. neoformans* within its ecological niche and not for survival within a human host.

7.1. Microbial Interactions

Despite the inability to pin-point the exact ecological niche of *C. neoformans*, it is certain that the pathogen participates in a number of both positive and negative interactions within the microbial community (Allee *et al.*, 1949). Negative reactions will limit the overall size of the population by serving as feedback mechanisms and ultimately preventing over-population and destruction of a favourable niche. Positive interactions combine physical and metabolic capabilities within a microbial community that enhance population. Positive interactions would be regarded as commensalism, synergism and mutualism, while negative interactions include competition and amensalism. An example of a positive interaction was recently demonstrated when growth of *C. neoformans* var. *grubii* was enhanced during co-culture with *Coniochaeta pulveracea* on carboxy-methyl cellulose (CMC) (van

Heerden *et al.*, 2011). Parasitism and predation are considered to be both positive and negative towards a given population, of which the latter plays a vital role in the acquisition and maintenance of the virulence factors of *C. neoformans* (London *et al.*, 2006; Casadevall *et al.*, 2003; Steenbergen *et al.*, 2003; 2001).

7.2. Predation

There is a fine line between what is regarded as parasitism and predation (Atlas *et al.*, 1998). Some would argue that the bacterium *Bdellovibrio* is in fact an ecto-parasite as it does not engulf the susceptible bacteria (Riitenberg, 1983). Others suggest that since *Bdellovibrio* shows no host specificity, except towards Gram-negative bacteria, it is in fact a predator (Slobodkin, 1968). However, co-existence of predator and prey relies on the latter's ability to avoid and survive predation. Many microorganisms produce cell structures, such as spores, that are simply resistant to lyses and digestion by the predator (Kuhlman and Heckman, 1985). Others are able to modify their overall cell structure making engulfment difficult. Environmental structures, such as clay particles, also play a role in physically preventing predation.

Early studies indicated that the amoeba *Acanthamoebae polyphaga* was capable of engulfing large numbers of *C. neoformans* cells that ultimately resulted in the killing of the yeast cell (Neilson *et al.*, 1978). Further research conducted by Steenbergen (2001) and Mylonakis (2002) revealed that the amoeba *A. castenalli* and the nematode *Caenorhabditis elegans* were also capable of ingesting the pathogen *C. neoformans*. However, unlike *A. polyphaga*, ingestion resulted in the death of the amoebae and nematodes and not the yeast *C. neoformans*. Indeed, *C. elegans* was shown to be capable of ingesting other yeasts, namely *C. laurentii*, *C. kuetzingii*, however death only occurred after ingestion of *C. neoformans.*

The interactions of these predators and *C. neoformans* closely resembled the interactions observed between macrophages and this yeast pathogen (London *et al.*, 2006; Casadevall *et al.*, 2003; Mylonakis *et al.*, 2002; Steenbergen *et al.*, 2003; Steenbergen et *al.*, 2001: Neilson *et al.*, 1978). As observed with macrophages, acapsular yeast cells are more readily ingested by the predators, but engulfment of encapsulated *C. neoformans* cells does occur. Once ingested, the yeast cells are internalized and enclosed within a membrane bound vacuole. Here *C. neoformans* is able to replicate and the formation and accumulation of polysaccharide vesicles

occurs. Contact with these vesicles results in cell membrane leaking and ultimately lysis of the amoeboid cell.

Unfortunately, current research does not indicate whether *C. neoformans* shows an increase in virulence after passage through both amoebae and nematodes. Changes in virulence is often associated with phenotypic switching, a characteristic displayed by *C. neoformans* (Guerrero *et al*., 2006). Such phenotypic switching is a result of micro-evolution and is thought to occur during the course of infection, aiding the pathogen to avoid an already compromised immune system. Alternatively, micro-evolution may be as a result of predation by amoebae and nematodes on *C. neoformans* ultimately inducing limited genetic diversity within a predominantly clonal population (Halliday *et al*., 2003; Trilles *et al*., 2003).

8. THE ECOLOGICAL NICHE OF *CRYPTOCOCCUS NEOFORMANS*

Currently, the true ecological niche of *C. neoformans* remains elusive. Despite the wide spectrum of vertebrate hosts, it would appear none are a requisite component for the cryptococcal life cycle. It must therefore be assumed that infection by *C. neoformans* is as a result of exposure to an external reservoir of yeast cells or basidiospores and should be considered an important aspect of cryptococcal research.

As mentioned earlier, the re-classification of *C. gattii* as a new species was supported by the fact that *C. neoformans* and *C. gattii* were isolated from different habitats. *Cryptococcus gattii* occurs predominately in sub-tropical and tropical regions (Sorrell *et al*., 1997) and has been shown to have a strong ecological association with a number of *Eucalyptus* trees, particularly *Eucalyptus camaldulensis*, generally known as the river red gum, and *Eucalyptus tereticornis*, commonly known as the forest red gum (Sorrell *et al*., 1997). Both these species are prevalent in Australia where a high incidence of cryptococcosis is observed amongst native animal and human populations (Halliday *et al*., 1999). By means of random amplified polymorphic DNA (RAPD) and PCR fingerprinting, Sorrell and co-workers (1996) were able to determine that the source of these infections was indeed the *Eucalyptus* species as the genetic composition of those pathogens found on the trees matched those pathogens isolated from infected animal and human populations.

Despite this perceived association, the role that the tree plays in the life cycle of the yeast remains unclear. Halliday and co-workers (2003) suggested that the association between *C. gattii* and the *Eucalyptus* species is not as relevant as

previously thought. They argue that the lack of genetic diversity amongst *C. gattii* species found on *Eucalyptus* species in Australia indicates wide-spread clonality and therefore *C. gattii* is capable of utilizing the favourable environment offered by the decaying wood of these *Eucalyptus* species. Interestingly, different trees harboured genetically distinct populations of *C. gattii*, indicating that there was no dispersal of the pathogen between trees.

As Halliday suggests, these ecological associations are indeed not finite. There are a number of different tree species from which the yeast has been isolated. In Brazil, *C. gattii* has been isolated from the pink shower tree, the fig tree, as well as the pottery tree; isolates of *C. gattii* were found in India on the Ashoka, Spanish cherry and Palu tree; the almond tree has also been colonized in Columbia, while on Vancouver Island; a temperate climate; native Douglas fir, maple and Garry oak trees appear to be the preferred habitat (Randhawa *et al.*, 2008; Kidd *et al.*, 2007; 2004; Fraser *et al*., 2003; Halliday *et al*., 2003). Researchers have also managed to isolate *C. gattii* and *C. neoformans* from a number of different tree species within the Amazon forest and India, suggesting that the two species are rather associated with decaying wood in general as opposed to any particular tree species in this large area (Randhawa *et al.*, 2011; 2008; 2006; 2000; Trilles *et al*., 2003; Fortes *et al*., 2001; Lazéra *et al*., 1996; Bauwens *et al.*, 1986). However, *C. neoformans* does appear to have a wider arboreal distribution when compared to *C. gattii* (Randhawa *et al.*, 2011; 2008; 2006).

The variants *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) have a worldwide distribution, but serotype A is regarded as being more ubiquitous compared to serotype D, which appears to be limited to less tropical regions such as Europe. Avian guano is still considered to be a significant source of both variants (Sorrell *et al*., 1997; Sethi *et al.*, 1966; Emmons, 1955) as *C. neoformans* is able to easily convert the urea within the avian guano to ammonia (Cox *et al*., 2000). Recent research has demonstrated that under laboratory conditions, *C. neoformans* is capable of producing viable basidiospores when mated on pigeon guano (Neilsen *et al.*, 2007), indicating a possible source of infectious basidiospores. Interestingly, research conducted in India determined that despite the infection of birds with a virulent *C. neoformans* strain, the birds did not develop cryptococcosis (Sethi *et al.*, 1968). It is speculated that due to the high internal temperature of the birds (approximately 42 ºC) *C. neoformans* is unable to grow invasively, although

rare cases of subcutaneous and upper respiratory infections have been reported (Malik *et al.,* 2003)

Strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have been isolated from a variety of environmental sources including soil, bat caves, fruits, insects, vegetative debris and particularly decaying wood and the hollows of trees (Randhawa *et al.,* 2011; 2008; 2006; 2000; Baroni *et al.,* 2006; Ergin *et al.,* 2004; Montagna *et al.,* 2003; Trilles *et al.*, 2003; Chakrabarti *et al.,* 1997; Mussa *et al.*, 1997; Lazéra *et al.*, 1996; López *et al.*, 1995; Bauwens *et al.*,1986; Emmons, 1955; 1951). The latter source has shown increasing promise to be the true ecological niche of *C. neoformans.* Completion of the genome in 2005 identified a putative cryptococcal cellulase (Loftus *et al.,* 2005) while Botes and co-workers (2009) demonstrated that strains of *C. neoformans* var. *grubii* were capable of vegetative growth as well as sexual and unisexual mating on two tree species, namely *Acacia mearnsii* and *E. camaldulensis*.

The ability of *C. neoformans* var. *grubii* to utilize woody debris as sole carbon source in the absence of other microbes suggests that this yeast is expressing enzymes responsible for the hydrolysis if ligneous material. The degradation of lignocellulose is achieved through the action of liginanases as well as a combination of various glycoside hydrolases (GHs) such as cellulases, mannanases and xylanases (de Souza, 2013; Lum *et al.,* 2011; Sanchez, 2009).

9. GLYCOSIDE HYDROLASES

Glycoside hydrolases (GHs) are enzymes that catalyze the hydrolysis of the glycosidic bond of glycosides. These enzymes are extremely common and assist in a variety of processes including the degradation of biomass, pathogenic mechanisms, immune defence strategies as well as normal cellular functioning (Henrissat *et al.*, 1996; 1993).

9.1. Classification of Glycoside Hydrolases

Numerous forms of classification have been suggested in order to classify GHs and include the reaction mechanism used, whether the enzyme cleaves internally or externally, the type of chemical reaction they catalyse and finally, their protein sequence and tertiary structure.

9.1.1. Reaction Mechanisms. Enzymatic hydrolysis of glycosidic bonds takes place via general acid catalysis that requires both a proton donor and a nucleophile/base residue (McCarter *et al.*, 1994; Sinnott, 1990; Koshland, 1953). This process is achieved either via a retention or inversion of the anomeric configuration. Hydrolysis by inverting GHs is generally accomplished in a one step, single displacement involving an oxocarbenium ion-like transition state. Retaining enzymes utilize a two-step (glycosylation / de-glycosylation), double-displacement mechanism involving a covalent glycosyl-enzyme intermediate process. During glycosylation, the base residue attacks the anomeric centre, displacing the aglycon and forming a glycosyl enzyme intermediate, while the other residue functions as an acidic catalyst. In step two (de-glycosylation) the glycosyl enzyme intermediate is hydrolyzed by water yielding the hydrolyzed product.

9.1.2. Endo- / Exo-acting. Glycoside hydrolases can also be classified as exo- or endo- acting, dependent upon whether they cleave the substrate at the end (generally the non-reducing end) or in the middle of the chain (Davies, 1995).

9.1.3. Enzyme Commission (EC) numbers. The assignment of EC numbers is based on the chemical reaction that the enzyme catalyzes (Moss, 2006; Webb, 1992). This classification does not specify enzymes and as such, different enzymes catalysing the same chemical reaction will be given the same EC number. Consequently, this system can only be applied to enzymes for which a function has been determined.

9.1.4. Sequence Based Classification. A classification system based on the amino acid sequence of the catalytic domains of glycoside hydrolases was introduced over two decades ago (Henrissat, 1991). Originally comprising of only 35 glycoside hydrolase families (GHFs), this system has been continuously expanded and updated and GHs are currently grouped into 131 families (Cantarel *et al.*, 2009; Henrissat *et al.*, 1996; 1993). The three-dimensional structure, mechanism employed, catalytic machinery, as well as the molecular mechanisms involved are conserved in the majority of GHFs (Davies *et al.*, 1995; Henrissat *et al.*, 1995; Gebler *et al.*, 1992), thereby allowing researchers to accurately predict the function of unknown enzymes. Exceptions include glycoside hydrolases grouped into GHFs 4, 97 and 109. Glycoside hydrolase family 97 is comprised of both inverting and retaining enzymes (Gloster *et*

al., 2008) while GHF 4 and 109 use an NAD-dependent hydrolysis mechanism that allows a single enzyme to hydrolyze both α- and β-glycosides (Yip *et al.*, 2007).

10. STRUCTURE OF LIGNOCELLULOSE

Lignocellulose is the most abundant raw material on the planet (de Souza, 2013; Aro *et al.*, 2005; Pérez *et al.*, 2002). It is composed of two carbohydrate polymers, cellulose and hemi-cellulose, and a more complex aromatic polymer, lignin. Cellulose and hemi-cellulose are the major focus of the biofuels industry as microbes are able to ferment these carbohydrate-rich materials and produce bioethanol (Lynd *et al.*, 2002; Pérez *et al.*, 2002). Cellulose is a crystalline high molecular weight polymer of repeating glucosyl units (Pérez *et al.*, 2002; Klemm *et al.*, 1998; Marchessault *et al.*, 1993). Hemi-cellulose is a heterogeneous polymer that can be linear or branched in structure (de Souza, 2013; Pérez *et al.*, 2002; Klemm *et al.*, 1998). Moreover, the backbone can be comprised of a single repeating sugar unit or a mixture of varying sugars such as D-galactose, D-glucose, D-mannose and D-xylose.

The type of hemi-cellulose is classified according to the main sugar residue within the backbone. Xylan is composed of β-1,4-linked D-xylose units, xyloglucan consists of a β-1,4-linked D-glucose backbone substituted mainly with D-xylose, while mannan may consist of a backbone of β-1,4-linked D-mannose and D-glucose (glucomannans) residues with D-galactose side chains (gluco-galactomannans). The final component, lignin, is comprised of numerous phenylpropane units linked in a complex three-dimensional structure (de Souza, 2013; Pérez *et al.*, 2002; Klemm *et al.*, 1998). Lignin provides rigidity to the plant structure and as such is resistant to both enzymatic and chemical hydrolysis.

The biodegradation of lignaceous material requires a vast mix of glycoside hydrolases such as cellulases, hemi-cellulases and xylanases (Kulkarni *et al.*, 1999; Jeffries *et al.*, 1994; Warren *et al.*, 1996; Reese *et al.*, 1971). Given the wide variety of cellulase and hemi-cellulase producers, it is no surprise that these enzymes are classified into a number of GHFs including GHF2, 5, 6, 7, 8, 9, 10, 11, 12, 16, 18, 19, 20, 26, 27, 42, 43, 44, 45, 48, 51, 53, 67, 74 and 124 (Xie *et al.*, 2012; Tartar *et al.*, 2009).

Interestingly, mucin displays a similar structure to that of lignocellulose. Mucins are heavily O-glycosylated glycoproteins found in mucous secretions and occur as trans-membrane glycoproteins of cell surfaces such as those found within the

human respiratory tract. They aid in the defence against pathogens and are comprised of one to several hundred carbohydrate chains, typically fucose, galactose and small amounts of mannose (Lamblin *et al.*, 2001; 1991). In mucins, the O-glycans are covalently bound to a serine or threonine residue via an *N*-acetylgalactosamine (GalNAc) moiety, the most common of which is Galβ1-3GalNAc (Rose *et al.*, 2006; Hounsell, 1994). This core structure can then be extended by galactose- and *N*-acetylglucosamine transferases yielding Gal-β1,3-GlcNAc or Gal-β1,4-GlcNAc units. Given that cellulase and hemi-cellulases hydrolyze similar β-1,4-glycosidic bonds the *in vivo* expression of the glycoside hydrolases may inadvertently aid the survival of *C. neoformans* in the lung.

10.1. Biodegradation of Cellulose

The successful enzymatic hydrolysis of cellulose requires a consortium of different enzymes termed cellulases (Fig 6) (Warren *et al.*, 1996; Reese *et al.*, 1971). The three major enzymes required are β-1,4-endo-glucanases, β-1,4-exo-glucanases and β-glucosidases (Lyndt *et al.*, 2002; Beguin *et al.*, 1990; Reese *et al.*, 1971). Endo-glucanases attack regions of low crystallinity and cleave internal bonds creating more free chain ends. Exo-glucanases remove cellobiose units from the end of these chains, while β-glucosidases hydrolyse cellobiose forming two glucose units. The latter can be readily assimilated by the cell.

A wide variety of cellulase producing bacteria and fungi have been isolated from numerous habitats, including soil, composting heaps, decaying plant material and debris, fruit, hot springs, both freshwater and marine aquatic environments as well as the gut microbiome of ruminants such as cows and more recently the giant panda (Zhu *et al.*, 2011; Lynd *et al.*, 2002; Li *et al.*, 1997; Van Soest, 1994; Bott *et al.*, 1991; Hoeniger *et al.*, 1985). Cellulose degrading bacteria can be found in various genera such as *Acetivibrio, Butyrivibrio, Caldicellulosiruptor, Cellulomonas, Clostridium, Cytophaga, Erwinia, Fibrobacter, Ruminococcus, Sporocytophaga* and *Thermobifida*. Cellulolytic fungi are distributed throughout the entire fungal kingdom however; the filamentous ascomycete *Trichoderma reesei* is perhaps the best characterized of all (Foreman *et al.*, 2003).

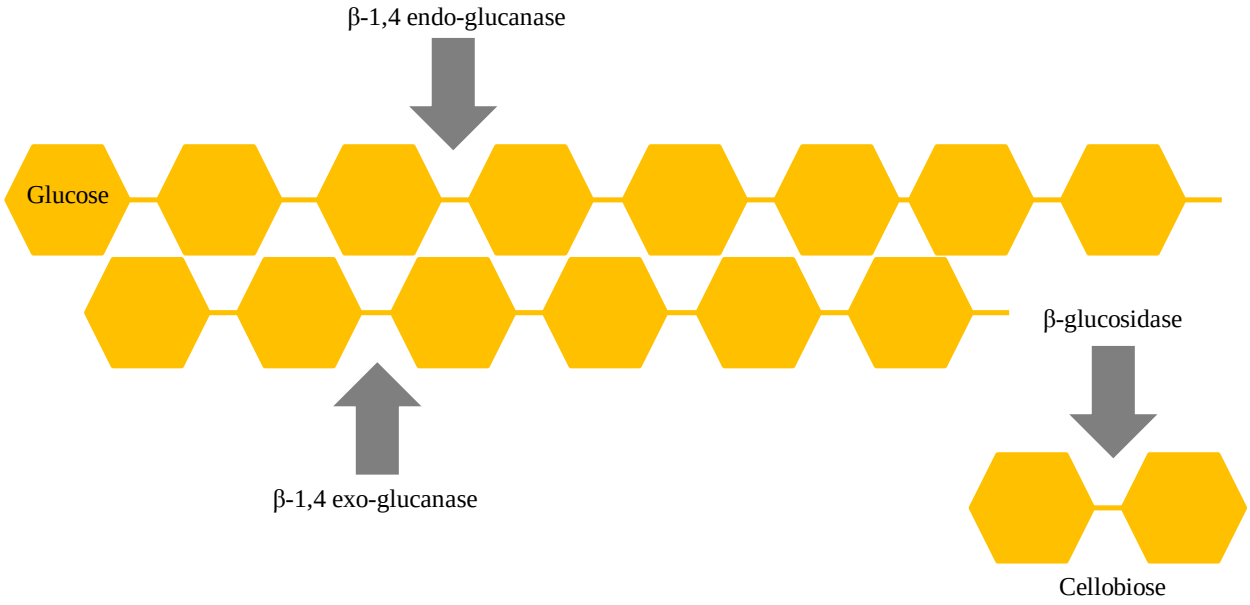

Figure 6. Enzymatic degradation of cellulose to glucose. Endo-glucanases hydrolyze internal bonds while exo-glucanases cleave cellobiose from the reducing ends. Cellobiose is hydrolyzed to glucose by β-glucosidase.

10.2. Biodegradation of Hemi-cellulose

Similar to the degradation of cellulose, the enzymatic hydrolysis of hemi-cellulose requires the action of numerous hemi-cellulases, such as β-1,4-endo-mannanase, β-mannosidases, β-1,4-endo-xylanases and β-xylosidases (Kulkarni *et al.*, 1999; Jeffries *et al.*, 1994). Additional accessory enzymes capable of hydrolysing the hemi-cellulose derived products are also required and include α-arabino-furanosidases, α-xylosidases, α-fucosidases, α-galactosidases, α-glucuronidases, acetyl-glucomannan-esterase, acetyl xylan esterases, *p*-coumaroyl esterases and feruloyl esterases (de Souza *et al.*, 2013; Sakamoto *et al.*, 2011; van den Brink *et al.*, 2011; Pérez *et al.*, 2002; de Vries, 2001; Yoshikawa *et al.*, 1994; 1991).

The enzymes β-1,4-endo-mannanase and β-mannosidases are responsible for the hydrolysis of gluco-galactomannans (Fig 7). Manno-oligosaccharides, mannobiose and mannotriose, are cleaved from the backbone by β-1,4-endo-mannanases and are subsequently hydrolysed into two mannose residues by β-mannosidases. Accessory enzymes associated with gluco-galactomannan hydrolysis include α-galactosidase, acetyl-glucomannan-esterase and β-glucosidase. Structural features of the mannan polymer, such as the number and distribution of side chains, as well as the glucose to mannose ratio, dramatically alter the ability of β-mannanases to

hydrolyze mannan (Mccleary, 1991; Mccleary *et al.*, 1984). The greatest activity is seen on galacto-mannans with few side chains (Civas *et al.*, 1984).

Similarly, xylan is hydrolyzed by β-1,4-endo-xylanases and β-xylosidases (Fig 8) (Kulkarni *et al.*, 1999). The xylan backbone is hydrolyzed by β-1,4-endo-xylanases and the resulting xylose oligosaccharides are cleaved by β-xylosidases. The associated accessory enzymes include acetyl-xylan-esterase, α-glucuronidase and α-arabino-furanosidase (Sakamoto *et al.*, 2011; Yoshikawa *et al.*, 1994).

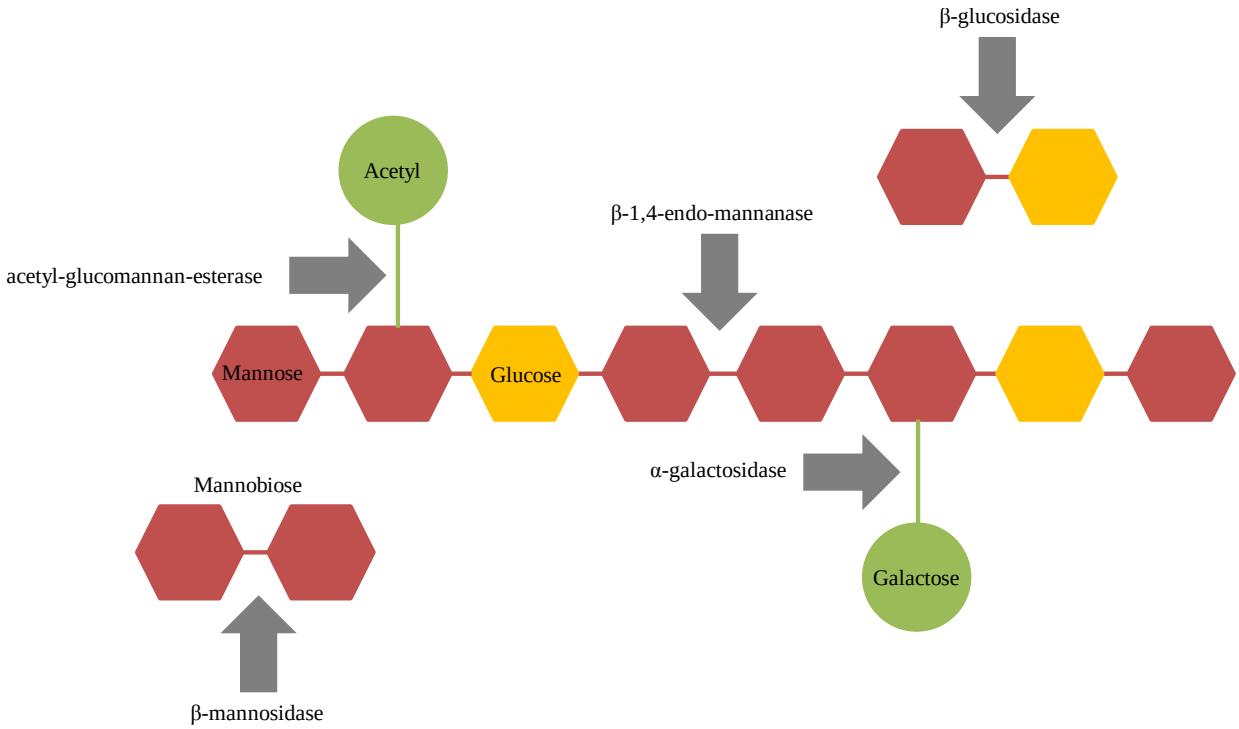

Figure 7. Enzymatic degradation of mannan to mannose and glucose. Endo-mannanases hydrolyze internal bonds while the various manno-oligosaccharides are subsequently hydrolyzed to mannose and glucose by β-mannosidase and β-glucosidase. Various accessory enzymes are required for complete mannan hydrolysis.

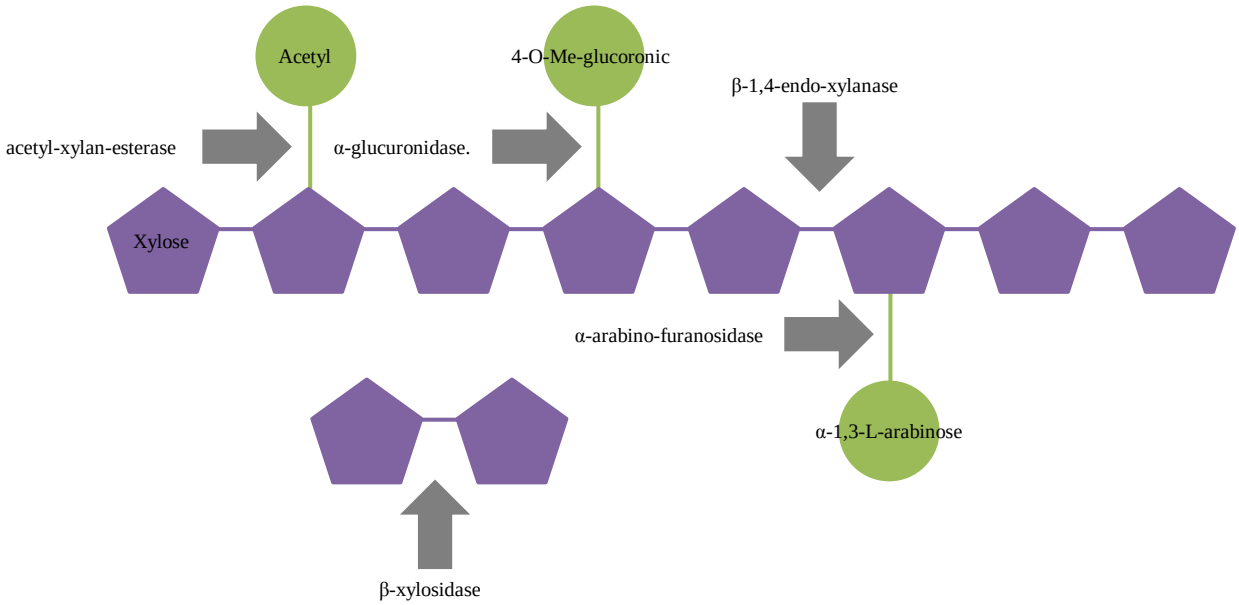

Figure 8. Enzymatic degradation of xylan to xylose. Endo-xylanases hydrolyze internal bonds while β-xylosidase cleaves the xylose disaccharides to xylose. Various accessory enzymes are required for complete xylan hydrolysis.

11. CONCLUSIONS

Cryptococcus neoformans is a pathogenic basidiomycete (Boekhout *et al.*, 1997) responsible for approximately 13 - 44 % of cryptococcosis infections associated with patients already suffering with HIV and AIDS, as well as 5 % of all organ transplant recipients (Park *et al.*, 2009; Vilchez *et al.*, 2003; Husain *et al.*, 2001; Liu *et al.*, 1999; Williamson, 1997; Wang *et al.*, 1996; Chuck *et al.*, 1989). Despite the availability of anti-fungal treatment, mortality rates remain high, ranging between 30 – 60 % in HIV / AIDS patients and up to 50 % in organ transplant patients (Park *et al.*, 2009; Vilchez *et al.*, 2003; Husain *et al.*, 2001). In many cases, cryptococcosis is the first indication of AIDS and is the leading mycological cause of mortality among these patients (Park *et al.*, 2009; Rozenbaum *et al.*, 1994; Kovacs *et al.*, 1985).

With relatively simple nutritional requirements, this yeast is able to reproduce both asexually and sexually, however, *C. neoformans* does not require any animal, plant or protist host in order to complete its life cycle. The identification of cellulolytic enzymes (Loftus *et al.*, 2005), the repeated isolation of *C. neoformans* from decaying wood and the hollows of trees (Randhawa *et al.*, 2011; 2006; 2000; Trilles *et al.*, 2003; Lazéra *et al.*, 1996), as well as recent evidence showing that this yeast is able to grow and complete its life cycle on woody debris in the absence of additional nutrients (Botes *et al.*, 2009), gives a strong indication of a suspected

woody niche for this pathogen. However, little is known regarding the expression of suitable lignocellulosic degrading enzymes.

Studies have shown that within Sub-Saharan Africa, up to 30 % of AIDS patients suffer from cryptococcosis (Park *et al.*, 2009; Powderly, 1993). A survey conducted within the Gauteng Province of South Africa, by the Gauteng Cryptococcal Surveillance Group during 2002 and 2004, were able to identify 2753 cryptococcosis cases (McCarthy *et al*., 2006). Cryptococcal meningitis accounted for 97 % of these cases and the survey determined an in-hospital mortality rate of 27 %. These relatively high mortality rates emphasize the need to further examine this ubiquitous yeast pathogen to determine is true ecological niche. By studying the environmental metabolism of *C. neoformans*, we may gain knowledge into the pathogenesis of this yeast as well as possible treatment options.

12. PROJECT OBJECTIVES

With the above as background, the overall aim of this project was to study the interactions of *C. neoformans* var. *grubii* ATCC H99 with ligneous materials, namely cellulose and hemi-cellulose. The study had a number of objectives:

1. To determine if *C. neoformans* var. *grubii* ATCC H99 was capable of assimilating the various components of lignocellulose, namely cellulose and hemi-cellulose, as well as their associated simple sugars.
2. To phylogenetically identify any putative cellulases, cellobiohydrolases and/or mannanases coded by genes within the genome of *C. neoformans* var. *grubii* ATCC H99.
3. To determine the physical and chemical properties of any identified enzymes, their tertiary structure and cellular localization.
4. To isolate and express the identified enzymes to evaluate their specific activity.
5. To gain an insight into the regulation of these genes in *C. neoformans* var. *grubii* ATCC H99 when the yeast is placed in various environments that range from media containing simple degradation products of lignocellulose, to media containing complex wood polymers.
6. To determine if the presence of mucin would induce the transcription of the identified genes.

Please note the results chapters have been written in a style suitable for publication in a scientific journal. As a result, the repetition of some information could not be avoided.

13. REFERENCES

Abadi J., Nachman S., Kressel A. B., Pirofski L. (1999). Cryptococcosis in children with AIDS. *Clinical Infectious Diseases* **28(2):** 309-313.

Akhter S., McDade H. C., Gorlach J. M., Heinrich G., Cox G. M., Perfect J. R. (2003). Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans. Infection and Immunity* **71(10):** 5794-5802.

Allee W. C., Emerson A. E., Park O., Park T., Schmidt K. P. (1949). Principles of animal ecology. Saunders, Philadelphia.

Alspaugh J. A., Perfect J. R., Heitman J. (1997). *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α-subunit GPA1 and cAMP. *Genes and Development* **11:** 3206-3217.

Alvarez M., Casadevall A. (2006). Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Current Biology* **16(21):** 2161-2165.

Antinori S., Ridolfo A., Fasan M., Magni C., Galimberti L., Milazzo L., Sollima S., Adorni F., Giuliani G., Galli M., Corbellino M., Parravicini C. (2009). AIDS-associated cryptococcosis: a comparison of epidemiology, clinical features and outcome in the pre- and post-HAART eras. Experience of a single centre in Italy. *HIV Medicine* **10:** 6–11.

Armengou A., Pocar C., Mascaro J., Garcia-Bragado F. (1996). Possible development of resistance to fluconazole during suppressive therapy for AIDS-associated cryptococcal meningitis. *Clinical Infectious Diseases* **23:** 1337–1338.

Aro N., Pakula T., Penttila M. (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. *Federation of European Microbiological Society Microbiology Reviews* **29(4):** 719-39.

Atlas R. M., Bartha R. (1998). Interactions among microbial populations**.** In: Atlas R. M., Bartha R. (eds) *Microbial Ecology: Fundamentals and Applications, 4th edn*. California: Benjamin/Cummings Science Publishing, 85-91.

Baroni F. A., Paula C. R., Silva E. G., Viani F. C., Rivera I. N. G., Oliveira M. T., Gambale W. (2006). *Cryptococcus neoformans* strains isolated from church towers in Rio de Janeiro City, RJ, Brazil. *Rev Inst Med Trop. São Paulo* **48:** 71-75.

Bauwens L., Swinne D., De Vroey C., De Meurichy W. (1986). Isolation of *Cryptococcus neoformans* var. neoformans in the aviaries of the Antwerp Zoological Gardens. *Mykosen* **29:** 291–294.

Beguin P. (1990). Molecular-biology of cellulose degradation. *Annual Review of Microbiol**ogy*** **44:** 219-248.

Bell A. A., Wheeler M. H. (1986). Biosynthesis and functions of fungal melanins. *Annual Review of Phytopathol*ogy **24:** 411–451.

Bemis D. A., Krahwinkel D. J., Bowman A., Mondon P., Kwon-Chung K. J. (2000). Temperature-sensitive strain of *Cryptococcus neoformans* producing hyphal elements in a feline nasal granuloma. *Journal of Clinical Microbiology* **38(2):** 926-928.

Bhattacharjee A. K., Kwon-Chung K. J., Glaudemans C. P. (1979a). The structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype D. *Carbohydrate Research* **73:** 183-192.

Bhattacharjee A. K., Kwon-Chung K. J., Glaudemans C. P. (1979b). On the structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype C--II. *Molecular Immunology* **16(7):** 531-532.

Bhattacharjee A. K., Kwon-Chung K. J., Glaudemans C. P. (1980). Structural studies on the major, capsular polysaccharide from *Cryptococcus bacillisporus* serotype B. *Carbohydrate Research* **82(1):** 103-111.

Bhattacharjee A. K., Kwon-Chung K. J., Glaudemans C. P. (1981). Capsular polysaccharides from a parent strain and from a possible, mutant strain of *Cryptococcus neoformans* serotype A. *Carbohydrate Research* **95(2):** 237-248.

Bhattacharjee A. K., Bennett J. E., Glaudemans C. P. (1984). Capsular polysaccharides of *Cryptococcus neoformans. Rev Infect Dis.* **6(5):** 619-24.

Birley H. D., Johnson E. M, McDonald P., Parry C., Carey P. B., Warnock D. W. (1995). Azole drug resistance as a cause of clinical relapse in AIDS patients with cryptococcal meningitis. *International Journal of STD and AIDS* **6:** 353–355.

Blanche P., Gombert B., Ginsburg C., Passeron A., Stubei I., Rigolet A., Salmon D., Sicard D. (1998). HIV combination therapy: immune restitution causing cryptococcal lymphadenitis dramatically improved by anti-inflammatory therapy. *Scandinavian Journal of Infectious Disease* **30:** 615–616.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* **147:** 891-907.

Bose I., Reese A. J., Ory J. J., Janbon G., Doering T. L. (2003). A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryotic Cell* **2(4):** 655-663.

Bott T. L., Kaplan I A. (1991). Selection of surrogates for a genetically engineered microorganism with cellulolytic capability for ecological studies in streams. *Canadian Journal of Microbiology* **37:** 848–857.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Brandt M. E., Hutwagner L. C., Klug L. A., Baughman W. S., Rimland D., Graviss E. A., Hamill R. J., Thomas C., Pappas P. G., Reingold A. L., Pinner R. W. (1996). Molecular subtype distribution of *Cryptococcus neoformans* in four areas of the United States. Cryptococcal Disease Active Surveillance Group. *Journal of Clinical Microbiology* **34:** 912-917.

Brandt M. E., Pfaller M. A., Hajjeh R. A., Hamill R. J., Pappas P. G., Reingold A. L., Rimland D., arnocki D. W. for the Cryptococcal Disease Active Surveillance Group (2001). Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. *Antimicrobial Agents and Chemotherapy* **45(11):** 3065-3069.

Breton G., Seilhean D., Cherin P., Herson S., Benveniste O. (2002). Paradoxical intracranial cryptococcoma in a human immunodeficiency virus–infected man being treated with combination antiretroviral therapy. *American Journal of Medicine* **113:** 155–157.

Briggs M. R., Kadonaga J. T., Bell S. P., Tjian R. (1986). Purification and biochemical characterization of the promoter specific transcription factor, Spl. *Science* **234:** 47-52.

Broekhuyse R. M., Kuhlmann E. D., Winkens H. J., Van Vugt A. H. (1991). Experimental autoimmune anterior uveitis (EAAU), a new form of experimental uveitis. I. Induction by a detergent-insoluble, intrinsic protein fraction of the retinal pigment epithelium. *Experimental Eye Research* **52(4):** 465-474.

Brueske C. H. (1986). Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. *Journal of Clinical Microbiology* **23:** 631–633.

Buchanan K. L., Murphy J. W. (1998). What makes *Cryptococcus neoformans* a pathogen? *Emerging Infectious Diseases* **4(1):** 72-83.

Bui T., Lin X., Malik R., Heitman J., Carter D. (2008). Isolates of *Cryptococcus neoformans* from infected animals reveal genetic exchange in unisexual, alpha mating type populations. *Eukaryotic Cell* **7(10).** 1771-1780.

Busse O. (1984). Ueber parasitärezellen schüsse und thre zuchtung. *Centralbl. Bakt. Parasit.* **16:** 175-180.

Butler M. J., Day A. W. (1998). Fungal melanins: a review. *Canadian Journal of Microbiology* **44(12):** 1115-1136.

Cadena J., Thompson G. R. III, Ho T. T., Medina E., Hughes D. W., Patterson T. F. (2009). Immune reconstitution inflammatory syndrome after cessation of the tumor necrosis factor αblocker adalimumab in cryptococcal pneumonia. *Diagnostic Microbiology and Infectious Disease* **64(3):** 327–330.

Cantarel B. L., Coutinho P. M., Rancurel C., Bernard T., Lombard V., Henrissat B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research* **37:** 233-238.

Casadevall A., Perfect J. R. (1998). *Cryptococcus neoformans*. Washington DC: ASM Press.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Casadevall A. (2005). Fungal virulence, vertebrate endothermy, and dinosaur extinction: is there a connection? *Fungal Genetics and Biology* **42:** 98-106.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Chang Y. C., Kwon-Chung K. J. (1994). Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Molecular and Cellular Biology* **14(7):** 4912–4919.

Chang Y. C., Penoyer L. A., Kwon-Chung K. J. (1996). The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. *Infection and Immunity* **64(6):** 1977-1983.

Chang Y. C., Kwon-Chung K. J. (1998). Isolation of the third capsule-associated gene, CAP60, required for virulence in *Cryptococcus neoformans. Infection and Immunity*. **66(5):** 2230-2236.

Chang Y. C., Kwon-Chung K. J. (1999). Isolation, characterization, and localization of a capsule-associated gene, CAP10, of *Cryptococcus neoformans. Journal of Bacteriology* **181(18):** 5636-5643.

Chang Y. C.,Wickes B. L.,Miller G. F., Penoyer L. A., Kwon-Chung K. J. (2000). *Cryptococcus neoformans STE12alpha* regulates virulence but is not essential for mating. *Journal of Experimental Medicine* **191:** 871-882.

Chang Y. C., Penoyer L. A., Kwon-Chung K. J. (2001). The second *STE12* homologue of *Cryptococcus neoformans* is MAT*a*-specific and plays an important role in virulence. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 3258–3263.

Chang Y. C., Stins M. F., McCaffery M. J., Miller G F., Pare D. R., Dam T., Paul-Satyasee M., Kim K, S., Kwon-Chung K. J. (2004). Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier. *Infection and Immunity* **72(9):** 4985-4995.

Charlier C., Chrétien F., Baudrimont M., Mordelet E., Lortholary O., Dromer F. (2005). Capsule structure changes associated with *Cryptococcus neoformans* crossing of the blood-brain barrier. *American Journal of Pathology* **166(2):** 421-32.

Charlier C., Nielsen K., Daou S., Brigitte M., Chretien F., Dromer F. (2009). Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans. Infection and Immunity* **77(1):** 120-127.

Chayakulkeeree M., Sorrell T. C., Siafakas A. R., Wilson C. F., Pantarat N., Gerik K. J., Boadle R., Djordjevic J. T. (2008a). Role and mechanism of phosphatidylinositol-specific phospholipase C in survival and virulence of *Cryptococcus neoformans. Molecular Microbiology* **69(4):** 809-826.

Chayakulkeeree M., Wilson C., Sorrell T., Djordjevic J. (2008b). Delineating phospholipase C1 (PLC1)-related signalling pathways in Cryptococcus neoformans. In: *77th International Conference of Cryptococcus and Cryptococcosis.* Japan: Nagasaki, Abstract ID 1002.

Chen F., Currie B. P., Chen L. C., Spitzer S. G., Spitzer E. D., Casadevall A. (1995). Genetic relatedness of *Cryptococcus neoformans* clinical isolates grouped with the repetitive DNA probe CNRE-1. *Journal of Clinical Microbiology* **33:** 2818-2822.

Chen L. C., Blank E. S., Casadevall A. (1996). Extracellular proteinase activity of *Cryptococcus neoformans. Clinical Diagnostic and Laboratory Immunology* **3(5):** 570-574.

Chen S. H., Stins M. F., Huang S. H., Chen Y. H., Kwon-Chung K. J., Chang Y., Kim K. S., Suzuki K., Jong A.Y. (2003). *Cryptococcus neoformans* induces alterations in the cytoskeleton of human brain microvascular endothelial cells. *Journal of Medical Microbiology* **52(11):** 961-970.

Cheng V. C., Yuen K. Y., Chan W. M., Wong S. S., Ma E. S., Chan R. M. (2000). Immuno-restitution disease involving the innate and adaptive response. *Clinical Infectious Diseases* **30:** 882–892.

Cheriniak R., Reiss E., Turner S. H. (1982). A galactomannan antigen of *Cryptococcus neoformans* serotype A. *Carbohydrate Research* **103:** 293-250.

Chowdhary A., Randhawa H.S., Prakash, A., Meis, J.F. (2012) Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* in India: An update. *Critical Reviews in Microbiology* **38:** 1–16.

Chowdhary A., Randhawa H. S., Sundar G., Kathuria S., Prakash A., Khan Z., Sun S., Xu J. (2011). *In vitro* antifungal susceptibility profiles and genotypes of 308 clinical and environmental isolates of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* serotype B from north-western India. *Journal of Medical Microbiology* **60(7):** 961-967.

Chrétien F., Lortholary O., Kansua I., Neuville S., Gray F., Dromer F. (2002). Pathogenesis of cerebral *Cryptococcus neoformans* infection after Fungemia. *Journal of Infectious Diseases* **186:** 522-530.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **321:** 794–799.

Civas A., Eberhard R., Ledizet P., Petek F. (1984). Glycosidases induced in *Aspergillus-Tamarii* secreted alpha-D-galactosidase and beta-D-mannanase. *Biochemical Journal* **219(3):** 857-863.

Clarke D. L., Woodlee G. L., McClelland C. M., Seymour T. S., Wickes B. L. (2001). The *Cryptococcus neoformans STE11alpha* gene is similar to other fungal mitogen-activated protein kinase kinase kinase (MAPKKK) genes but is mating type specific. *Molecular Microbiology* **40:** 200-213.

Claus H. (2004). Lacasses: structure, reactions, distribution. *Micron* **35:** 93-96.

Cogliati M., Zamfirova R., TortoranoA., Viviani M. (2011). Population structure of Italian *Cryptococcus neoformans* var. grubii clinical isolates. In: *Proceedings of the 8th International Conference on Cryptococcus and Cryptococcosis*. Charleston: 5.

Cogliati M. (2013). Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: An atlas of the molecular types. *Scientifica* **2013:** 1-23.

Collins H. L., Bancroft G. J. (1991). Encapsulation of *Cryptococcus neoformans* impairs antigen-specific T-cell responses. *Infection and Immunity* **59(11):** 3883-3888.

Cox G. M., Mukherjee J., Cole G. T., Casadevall A., Perfect J. R. (2000). Urease as a virulence factor in experimental cryptococcosis. *Infection and Immunity* **68:** 443–448.

Cox G. M., McDade H. C., Chen S. C., Tucker S. C., Gottfredsson M., Wright L. C., Sorrell T. C., Leidich S. D., Casadevall A., Ghannoum M. A., Perfect J. R. (2001). Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Molecular Microbiology* **39:** 166–175.

Cuenca-Estrella M., Diaz-Guerra T. M. Mellado E., Rodriguez- Tudela J. L. (2001). Flucytosine primary resistance in *Candida* species and *Cryptococcus neoformans. European Journal of Clinical Microbiology and Infectious Diseases* **20:** 276–279.

Currie B. P., Ghannoum., Bessen L., Casadevall A. (1995). Decreased fluconazole susceptibility of a relapse *Cryptococcus neoformans* isolate after fluconazole treatment. *Infectious Diseases in Clinical Practice* **4:** 318–319.

Davidson R. C., Moore T. D., Odom A. R., Heitman J. (2000). Characterization of the MFalpha pheromone of the human fungal pathogen *Cryptococcus neoformans*. *Molecular Microbiology*. **38:** 1017-1026.

Davies G., Henrissat B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure* **3(9):** 853-859.

Decho A.W. (1990). Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. *Oceanography and Marine Biology: Annual Reviews* **28:** 73–153.

de Souza W. R. (2013). Microbial degradation of lignocellulosic biomass In: Chandel A. K., da Silva S. S. (eds.) *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization.* New York: InTech, Chapter 9.

de Vries R. P., Visser J. (2001). *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews* **65(4):** 497-522.

Denning D. W., Tucker R. M., Hanson L. H., Hamilton J. R., Stevens D. A. (1989). Itraconazole therapy for cryptococcal meningitis and cryptococcosis. *Archives of Internal Medicine* **149:** 2301–2308.

de Silva D, Davis-Kaplan S, Fergestad J, Kaplan J. (1997). Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *Journal of Biological Chemistry* **272(22):** 14208-14213.

d'Ischia M., Prota G. (1997). Biosynthesis, structure, and function of neuromelanin and its relation to Parkinson's disease: A critical update. *Pigment Cell Research* **10(6):** 370-376.

Doering T. L., Nosanchuk J. D., Roberts W. K., Casadevall A. (1999). Melanin as a potential cryptococcal defense against microbiocidal proteins. *Medical Mycology* **37:** 175-181.

Dong Z. M., Murphy J. W. (1995). Effects of the two varieties of *Cryptococcus neoformans* cells and culture filtrate antigens on neutrophil locomotion. *Infection and Immunity* **63(7):** 2632-2644.

Dromer F., **Mathoulin S**., **Dupont B**., **Brugiere O**., **Letenneur L. (1996).** Comparison of the efficacy of amphotericin B and fluconazole in the treatment of cryptococcosis in human immunodeficiency virus–negative patients: retrospective analysis of 83 cases. *Clinical and Infectious Diseases* **22:** 154–60.

Dykstra M. A., Friedman L., Murphy J. W. (1977). Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. *Infection and Immunity* **16(1):** 129-135.

Emmons C. W. (1951). Isolation of *Cryptococcus neoformans* from soil. *Journal of Bacteriology* **62:** 685–690.

Emmons C. W. (1955). Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (Columba livia). *American Journal of Hygiene* **62:** 227–232.

Enochs W. S., Sarna T., Zecca L., Riley P. A., Swartz H. M. (1994). The roles of neuromelanin, binding of metal ions, and oxidative cytotoxicity in the pathogenesis of Parkinson's disease: a hypothesis. *Journal of Neural Transmission* **7(2):** 83-100.

Ergin C., Ilkit M., Kaftanoglu O. (2004). Detection of *Cryptococcus neoformans* var. *grubii* in honeybee (*Apis mellifera*) colonies. ***Mycoses*** **47(9-10):** 431-434.

Esser K., Meinhardt F. (1977). A common genetic control of dikaryotic and monokaryotic fruiting in the basidiomycete *Agrocybe aegerita*. *Molecular Genetics and Genomics* **155:** 113-115.

Evans E E. (1950). The antigenic composition of *Cryptococcus neoformans. Journal of Immunology* **64:** 423-430.

Fairall L. R., Bachmann M. O., Louwagie G. M., van Vuuren C., Chikobvu P., Steyn D., Staniland G. H., Timmerman V., Msimanga M., Seebregts C. J., Boulle A., Nhiwatiwa R., Bateman E. D., Zwarenstein M. F., Chapman R. D. (2008). Effectiveness of antiretroviral treatment in a South African program: a cohort study. *Archives of Internal Medicine* **168:** 86–93.

Farhi F., Bulmer G. S., Tacker J. R. (1970). *Cryptococcus neoformans* IV. the not-so-encapsulated yeast. *Infection and Immunity* **1(6): 526-531.**

Feldmesser M., Kress Y., Novikoff P., Casadevall A. (2000). *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infection and Immunity* **68(7):** 4225-4237.

Feldmesser M., Tucker S., Casadevall A. (2001). Intracellular parasitism of macrophages by *Cryptococcus neoformans. TRENDS Microbiology* **9(6):** 273-278.

Fell J. W., Boekhout T., Fonseca A., Scorzetti G., Statzell-Tallman A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International Journal of Systematics and Evolutionary Microbiol*ogy **50:** 1351–1371.

Firacative C., Trilles L., Meyer W. (2012). MALDI-TOF MS enables the rapid identification of the major molecular types within the *Cryptococcus neoformans/C. gattii* species complex. *PLoS ONE* **7(5):** e37566.

Foreman P. K., Brown D., Dankmeyer L., Dean R., Diener S., Dunn-Coleman N. S., Goedegebuur F., Houfek T. D., England G. J., Kelley A. S., Meerman H. J., Mitchell T., Mitchinson C., Olivares H. A., Teunissen P. J., Yao J., Ward M. (2003). Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. *Journal of Biological Chemistry* **278(34):** 31988-31997.

Fortes S. T., Lazéra M. S., Nishikawa M. M., Macedo R. C., Wanke B. (2001). First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. *Mycoses.* **44(5):** 137-40.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global clonal population structure. *Journal of Clinical Microbiology* **35:** 2243-2251.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *Journal of Clinical Microbiology* **36(8):** 2200-2204.

Fraser J. A., Subaran R. L., Nichols C. B., Heitman J. (2003). Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: Implications for an outbreak on Vancouver Island, Canada. *Eukaryotic Cell* **2(5):** 1036-1045.

Fraser J. A., Giles S. S., Wenink E. C., Geunnes-Boyer S. G., Wright J. R., Diezmann S., Allen A., Stajichl J. E., Dietrichl F. S., Perfect J. R., Heitman J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature Letters* **437(27):** 1360-1364.

Freed E. R., Duma R. J., Shadomy H. J., Utz J. P. (1971). Meningoencephalitis due to hyphae-forming *Cryptococcus neoformans. American Journal of Clinical Pathology* **55:** 30-33.

Garcia-Hermoso D., Janbon G., Dromer F. (1999). Epidemiological evidence for dormant *Cryptococcus neoformans* infection. *Journal of Clinical Microbiology* **37:** 3204-3209.

French M. A., Lenzo N., John M., Mallal S. A., McKinnon E. J., James I. R., Price P., Flexman J. P., Tay-Kearney M. L. (2000). Immune restoration disease after the treatment of immunodeficient HIV-infected patients with highly active antiretroviral therapy. *HIV Medicine* **1:** 107–15.

Garcia-Rivera J., Casadevall A. (2001). Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. *Medical Mycology* **39:** 353-357.

Gebler J., Gilkes N. R., Claeyssens M., Wilson D. B., Béguin P., Wakarchuk W. W., Kilburn D. G., Miller R. C. Jr, Warren R. A., Withers S. G. (1992). Stereoselective hydrolysis catalyzed by related beta-1,4-glucanases and beta-1,4-xylanases. *Journal of Biological Chemistry* **267(18):** 12559-12561.

GERMS-SA Annual Report. (2012). Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa. Available from: http://www.nicd.ac.za/units/germs/germs.htm.

Ghannoum M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews* **13:** 122–143.

Ginns J., Malloch D. W. (2003). *Filobasidiella depauperata* (Tremellales): Haustorial branches and parasitism of *Verticillium lecanii***.** *Mycological Progress* **2(2):** 137-140.

Gloster T. M., Turkenburg J. P., Potts J. R., Henrissat B., Davies G. J. (2008). Divergence of catalytic mechanism within a glycosidase family provides insight into evolution of carbohydrate metabolism by human gut flora. *Chemical Biology* **15(10):** 1058-1067.

Goldman D. L., Lee S. C., Mednick A. J., Montella L., Casadevall A. (2000). Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. *Infection and Immunity* **68(2):** 832-838.

Goldman D. L., Khine H., Abadi J., Lindenberg D. J., Pirofski L., Njang R., Casadevall A. (2001). Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics* **107:** 66-73.

Guéhol E., Improvisi L., Christen R., de Hoog G. S. (1993). Phylogenetic relationships of *Cryptococcus neoformans* and some related basidiomycetous yeasts determined from partial large subunit rRNA sequences. *Antonie van Leeuwenhoek* **63:** 175-189.

Guerrero A., Jain N., Goldman D. L., Fries B. C. (2006). Phenotypic switching in *Cryptococcus neoformans. Microbiology* **152:** 3-9.

Haddow L. J., Colebunders R., Meintjes G., Lawn S. D., Elliott J. H., Manabe Y. C., Bohjanen P. R., Sungkanuparph S., Easterbrook P. J., French M. A., Boulware D. R. (2010). Cryptococcal immune reconstitution inflammatory syndrome in HIV-1-infected individuals: proposed clinical case definitions. *The Lancet Infectious Diseases* **10(11):** 791–802.

Hagen F., Illnait-Zaragozi M. T., Meis J. F., Chew W. H. M., Curfs-Breuker I., Mouton J. W., Hopelman A. I., Spanjaard L., Verweij P. E., Kampinga G. A., Kuijper E. J., Boekhout T., Klaassen C. H. W. (2012). Extensive genetic diversity within the Dutch clinical *Cryptococcus neoformans* population. *Journal of Clinical Microbiology* **50(6):** 1918–1926.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. *Journal of Clinical Microbiology* **37:** 2920-2926.

Halliday C. L., Carter D. A. (2003). Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. *Journal of Clinical Microbiology* **41(2):** 703-711.

Harrison T. (2000). *Cryptococcus neoformans* and cryptococcosis. *Journal of Infection.* **41:** 12-17.

Heitman J. (2006). Sexual Reproduction and the evolution of microbial pathogens. *Current Biology* **16:** 711-725.

Henrissat B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 309–316.

Henrissat B., Bairoch A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781–788.

Henrissat B., Callebaut I., Fabrega S., Lehn P., Mornon J. P., Davies G. (1995). Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proceedings of the National Academy of Sciences of the United States of America* **92(15):** 7090-7094.

Henrissat B., Bairoch A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochemical Journal* **316:** 695–696.

Hill H. Z. (1991). Melanins in the photobiology of skin cancer and radiobiology of melanomas. In: Wilson S. H. (ed.) *Cancer Biology and Biosynthesis*. New Jersey: Telford Press, 31-53.

Hill H. Z. (1992). The function of melanin or six blind people examine an elephant. *BioEssays: New and Reviews in Molecualr, Cellular and Developmental Biology* **14:** 49–56.

Hirsch H. H., Kaufmann G., Sendi P., Battegay M. (2004). Immune reconstitution in HIV-infected patients. *Clinical Infectious Diseases* **38:** 1159–66.

Hoeniger J. M. (1985). Microbial decomposition of cellulose in acidifying lakes of south-central Ontario. *Applied and Environmental Microbiology* **50:** 315–322.

Hounsell E. F. (1994). Physicochemical analyses of oligosaccharide determinants of glycoproteins. *Advances in Carbohydrate Chemistry and Biochemistry* **50:** 311–350.

Hsueh Y. P., Xue C., Heitman J. (2009). A constitutively active GPCR governs morphogenic transitions in *Cryptococcus neoformans. European Molecular Biology Organization Journal* **28(9):** 1220-1233.

Hull C. M., Heitman J. (2002). Genetics of *Cryptococcus neoformans*. Annual *Reviews of Genetics* **36:** 557-615.

Hull C. M., Boily M. J., Heitman J. (2005). Sex-specific homeodomain proteins Sxi1 and Sxi2a coordinately regulate sexual development in *Cryptococcus neoformans. Eukaryotic Cell* **4(3):** 526-535.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerging Infectious Disease* **7:** 375-381.

Ibrahim A. S., Filler S. G., Alcouloumre M. S., Kozel T. R., Edwards J. E., Ghannoum M. A. (1995). Adherence to and damage of endothelial cells by *Cryptococcus neoformans In vitro*: Role of the capsule. *Infection and Immunity* **63(11):** 4368-4374.

Ikeda R., Shinoda T., Morita T., Jacobson E. S. (1993). Characterization of a phenol oxidase from *Cryptococcus neoformans* var. *neoformans*. *Microbial Immunology* **37(10):** 759-764.

Ikeda R., Sugita T., Jacobson E. S., Shinoda T. (2003). Effects of melanin upon susceptibility of *Cryptococcus* to antifungals. *Microbiology and Immunology* **47(4):** 271-277.

Illnait-Zaragozi M. T., Martínez-Machín G. F., Fernández-Andreu C. M., Boekhout T., Meis J. F., Klaassen C. H. W. (2010). Microsatellite typing of clinical and environmental *Cryptococcus neoformans* var. grubii isolates from Cuba shows multiple genetic lineages," *PLoS ONE* **5(2):** e9124.

Ingram P. R., Howman R., Leahy M. F., Dyer J. R. (2007). Cryptococcal immune reconstitution inflammatory syndrome following alemtuzumab therapy. *Clinical Infectious Diseases* **44(12):** 115–117.

Ito S. (1993). Biochemistry and physiology of melanin. In: Levine N. (ed.) *Pigmentation and Pigmentary Disorders.* Boca Raton, Florida: CRC Press, 33-59.

Jacobson E. S. M., Tingler J., Quynn L (1989). Effect of hypersolutes upon the polysaccharide capsule in *Cryptococcus neoformans*. *Mycoses* **32:** 14-23.

Jacobson E. S., Compton G. M. (1996). Discordant regulation of phenoloxidase and capsular polysaccharide in *Cryptococcus neoformans. Journal of Medical and Veterinary Mycology* **34(4):** 289-291.

Jacobson E. S., Hong J. D. (1997). Redox buffering by melanin and Fe(II) in *Cryptococcus neoformans. Journal of Bacteriology* **179(17):** 5340-5346.

Jacobson E. S., Goodner A. P., Nyhus K. J. (1998). Ferrous iron uptake in *Cryptococcus neoformans. Infection and Immunity* **66(9):** 4169-4175.

Jain N., B. L. Wickes, Keller S. M., Fu J., Casadevall A., Jain P., Ragan M. A., Banerjee U., Fries B. C. (2005). Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. *Journal of Clinical Microbiology* **43(11):** 5733–5742.

Janbon G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *Federation of European Microbiological Societies Yeast Research* **4:** 765-771.

Jeffries T. W. (1994). Biodegradation of lignin and hemicelluloses. In: Ratledge C. (ed.) *Biochemistry of microbial degradation.* Dordrecht: Kluwer, 233–277.

Jenny-Avital E. R., Abadi M. (2002). Immune reconstitution cryptococcosis after initiation of successful highly active antiretroviral therapy. *Clinical Infectious Diseases* **35:** 128–133.

Jones B., Larsen R. A., Forthal D., Haghighat D., Bozzette S. (1991). Treatment of non-meningeal cryptococcal disease in HIV-infected persons (abstract F4). In: *Proceedings of the 91st annual meeting of the American Society for Microbiology (Dallas, TX).* Washington, DC: American Society for Microbiology.

Kanoh Y., Ohara T., Kanoh M., Akahoshi T. (2008). Serum matrix metalloproteinase-2 levels indicate blood-CSF barrier damage in patients with infectious meningitis. *Inflammation* **31(2):** 99-104.

Karos M., Chang Y. C., McClelland C. M., Clarke D. L., Fu J., Wickes B. L., Kwon-Chung K. J. (2000). Mapping of the *Cryptococcus neoformans MATalpha* locus: presence of mating type-specific mitogen-activated protein kinase cascade homologs. *Journal of Bacteriology* **182:** 6222-6227.

Kaya M., Edward D. P., Tessler H., Hendricks R. L. (1992). Augmentation of intraocular inflammation by melanin. *Investigative Ophthalmology and Visual Science* **33(3):** 522-531.

Kent C. R., Ortiz-Bermúdez P., Giles S. S., Hull C. M. (2008). Formulation of a defined V8 medium for induction of sexual development of *Cryptococcus neoformans. Applied and Environmental Microbiology* **74(20):** 6248-6253.

Kidd S. E., Hagen F., Tscharke R. L., Huynh M., Bartlett K. H., Fyfe M., Macdougall L., Boekhout T., Kwon-Chung K. J., Meyer W. (2004). A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proceedings of the National Academy of Sciences United States of America* **101:** 17258–17263.

Kidd S. E., Guo H., Bartlett K. H., Xu J., Kronstad J. W. (2005). Comparative gene genealogies indicate that two clonal lineages of *Cryptococcus gattii* in British Columbia resemble strains from other geographical areas. *Eukaryotic Cell* **4(10):** 1629-1638

Kidd S. E., Chow Y., Mak S., Bach P. J., Chen H., Hingston A. O., Kronstad J. W., Bartlett K. H. (2007). Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. *Applied and Environmental Microbiology* **73:** 1433–1443.

Klemm D., Philipp B., Heinze T., Heinze U., Wagenknecht W. (1998). Comprehensive cellulose chemistry. Chichester: Wiley VCH.

Kligman A. M., Weidman F. D. (1949). Experimental studies on treatment of human torulosis. *Archives of Dermatology and Syphilol* **60:** 726–741.

Koshland D. E. (1953). Stereochemistry and the mechanism of enzymatic reactions. *Biological Reviews of the Cambridge Philosophical Society* **28:** 416–436.

Kovacs J. A., Kovacs A. A., Polis M., Wright W. C., Gill V. J., Tuazon C. U., Gelmann E. P., Lane H. C., Longfield R., Overturf G., Macher A. M., Fauci A. S., Parrillo J. E., Bennett J. E., Masur H. (1985). Cryptococcosis in the acquired immunodeficiency syndrome. *Annals of Internal Medcines* **103:** 533–538.

Kraus P. R., Boily M. J., Giles S. S., Stajich J. E., Allen A., Cox G. M., Dietrich F. S., Perfect J. R., Heitman J. (2004). Identification of *Cryptococcus neoformans* temperature-regulated genes with a genomic-DNA microarray. *Eukaryotic Cell* **3(5):** 1249-1260.

Kruzel E. K., Giles S. S., Hull C. M. (2012). Analysis of *Cryptococcus neoformans* sexual development reveals rewiring of the pheromone-response network by a change in transcription factor identity. *Genetics* **191:** 435-449.

Kuhlman H. W., Heckmann K. (1985). Interspecific morphogens regulating prey – predator relationships in protozoa. *Science.* **227:** 1347-1349.

Kulkarni N., Shendye A., Rao M. (1999). Molecular and biotechnological aspect of xylanases. *Federation of European Microbiological Societies Microbiology Reviews* **23:** 411–456.

Kumarasamy N., Solomon S., Flanigan T. P., Hemalatha R., Thyagarajan S. P., Mayer K. H. (2003). Natural history of human immunodeficiency virus disease in southern India. *Clinical Infectious Diseases* **36:** 79–85.

Kwon-Chung K. J. (1975). A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. *Mycologia* **61(6):** 1197-1200.

Kwon-Chung K. J. (1976). A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia* **68:** 943- 946.

Kwon-Chung K. J., Bennett J. E. (1978). Distribution of alpha and alpha mating types of *Cryptococcus neoformans* among natural and clinical isolates. *American Journal of Epidemiology* **108(4):** 337-40.

Kwon-Chung K. J. (1980). Nuclear genotypes of spore chains of *Filobasidiella neoformans* (*Cryptococcus neoformans*). *Mycologia* **72:** 418-422.

Kwon-Chung K. J., Hill W. B. (1981). Sexuality and pathogenicity of *Filobasidiella neoformans* (*Cryptococcus neoformans*). In: Vanbreuseghem R., DeVroy C. (eds.) *Sexuality and Pathogenicity of Fungi*. France: Masson, 243-250.

Kwon-Chung K. J., Polacheck I., Popkin T. J. (1982). Melanin lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *Journal of Bacteriology* **150:** 1414-1421.

Kwon-Chung K. J., Edman J. C., Wickes B. L. (1992). Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infection and Immunity* **60:** 602-605.

Kwon-Chung K. J., Chang Y. C., Bauer R., Swann E. C. (1995). The characteristics that differentiate *Filobasidiella depauperata* from *Filobasidiella neoformans. Studies in Mycology* **38:** 67-80.

Kwon-Chung K. J. (1998). *Filobasidiella* Kwon-Chung. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier Science BV, 656-662.

Kwon-Chung K. J. (2010). *Filobasidiella* Olive. In: Kurtzman C. P., Fell J. W., Boekhout T. (eds.) *The Yeasts, a Taxonomic Study*, 5th edn Amsterdam: Elsevier, 1457-1465.

Kuchak A., Afshari S., Shokohi T., Aghili R., Badali H. (2012). Epidemiology and molecular characterization of *Cryptococcus neoformans* isolated from pigeon excreta in Mazandaran province, northern Iran. *Journal de Mycologie Medicale* **22(2):** 160-166.

Lamblin G., Lhermitte M., Klein A., Houdret N., Scharfman A., Ramphal R., Roussel P. (1991). The carbohydrate diversity of human respiratory mucins: A protection of the underlying mucosa? *American Review of Respiratory Disease* **144:** 19–24.

Lamblin G., Degroote S., Perini J. M., Delmotte P., Scharfman A., Davril M., Lo-Guidice J. M., Houdret N., Dumur V., Klein A., Roussel P. (2001). Human airway mucin glycosylation: A

combinatory of carbohydrate determinants which vary in cystic fibrosis. *Glycoconjugate Journal* **18:** 661–684.

Langfelder K., Streibel M., Jahn B., Haase G., Brakhage A. A. (2003). Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genetics and Biology* **38:** 143-158.

Latouche G. N., Huynh M., Sorrell T. C., Meyer W. (2003). PCR-Restriction fragment length polymorphism analysis of the phospholipase B (*PLB1*) gene for sub-typing of *Cryptococcus neoformans* isolates. *Applied and Environnemental Microbiol*ogy **69:** 2080-2086.

London R., Orozco B. S., Mylonakis E. (2006). The pursuit of cryptococcal pathogenesis: heterologous hosts and the study of cryptococcal host pathogen interactions. *Federation of European Microbiological Societies Yeast Research* **6:** 567-573.

López-Martínez R., Castañón-Olivares L. R. (1995). Isolation of *Cryptococcus neoformans* var. *neoformans* from bird droppings, fruits and vegetables in Mexico City. *Mycopathologia* **129(1):** 25-28

Lawn S. D., Bekker L. G., Myer L., Orrell C., Wood R. (2005a). Cryptococcocal immune reconstitution disease: a major cause of early mortality in a South African antiretroviral programme. *AIDS* **19:** 2050–2052.

Lawn S. D., Myer L., Orrell C., Bekker L. G., Wood R. (2005b). Early mortality among adults accessing a community-based antiretroviral service in South Africa: implications for programme design. *AIDS* **19:** 2141–2148.

Lazéra M. S., Pires F. D. A., Camillo-Coura L., Nishikawa M. M., Bezerra C. C. F., Trilles L. & Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34:** 127-131.

Lengeler K. B., Wang P., Cox G. M., Perfect J. R., Heitman J. (2000). Identification of the MAT*a* mating-type locus of *Cryptococcus neoformans* reveals a serotype A MAT*a* strain thought to have been extinct. *Proceedings of the National Academy of Sciences of the United States of America* **97(26):** 14455–14460.

Lengeler K. B., Fox D. S., Fraser J. A., Allen A., Forrester K., Dietrich F. S., Heitman J (2002). Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryotic Cell* **1(5):** 704-718.

Levitz S. M., Nong S., Mansour M. K., Huang C., Specht C. A. (2001). Molecular characterization of a mannoprotein with homology to chitin deacetylases that stimulates T cell responses to

Cryptococcus neoformans. . *Proceedings of the National Academy of Sciences of the United States of America* **98(18):** 10422-10427.

Li X., Gao P. (1997). Isolation and partial properties of cellulose decomposing strain of *Cytophaga* sp. LX-7 from soil. *Journal of Applied Microbiology* **82:** 73–80.

Lin X., Huang J. C., Mitchell T. G. Heitman J. (2006). Virulence attributes and hyphal growth of *C. neoformans* are quantitative traits and the MATa allele enhances filamentation. *Plos Genetics* **2(11):** 1801-1814.

Lin X., Litvintseva A. P., Nielsen K., Patel S., Floyd A., Mitchell T. G., Heitman J. (2007). alpha AD alpha hybrids of *Cryptococcus neoformans*: evidence of same-sex mating in nature and hybrid fitness. *Plos Genetics* **3(10):** 1975-1990.

Littman M. L. (1958). Capsule synthesis by *Cryptococcus neoformans. Transactions of the New York Academy of Sciences* **20:** 623-648.

Litvintseva A. P., Marra R. E., Nielsen K., Heitman J., Vilgalys R., Mitchell T. G. (2003). Evidence of sexual recombination among *Cryptococcus neoformans* Serotype A isolates in Sub-Saharan Africa. *Eukaryotic Cell* **2(6):** 61162-61168.

Liu L., Tewari R. P., Williamson P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity* **67(11):** 6034-6039.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M., Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science.* **307:** 1321-1324.

Lortholary O., Fontanet A., Mémain N., Martin A., Sitbon K., Dromer F. (2005). Incidence and risk factors of immune reconstitution inflammatory syndrome complicating HIV associated cryptococcosis in France. *AIDS* **19:** 1043–1049.

Lum G., Min X. J. (2011). FunSecKB: the Fungal Sectretome KnowlegeBase. *Database - the Journal of Biological Databases and Curation* doi: 10.1093/database/bar001.

Lynd L. R., Weimer P. J., van Zyl W. H., Pretorius I. S. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* **66(3):** 506-577.

Ma H., Croudace J. E., Lammas D. A., May R. C. (2006). Expulsion of live pathogenic yeast by macrophages. *Current Biology* **16(21):** 2156-2160.

Makadzange A. T., Ndhlovu C. E., Takarinda K., Reid M., Kurangwa M., Gona P., Hakim J. G. (2010). Early versus delayed initiation of antiretroviral therapy for concurrent HIV infection and cryptococcal meningitis in sub-Saharan Africa. *Clinical Infectious Disease* **50:** 1532–1538.

Malik R., Krockenberger M. B., Cross G., Doneley R., Madill D. N., Black D., McWhirter P., Rozenwax A., Rose K., Alley M., Forshaw D., Russell-Brown I., Johnstone A. C., Martin P., O'Brien C. R., Love D. N. (2003). Avian cryptococcosis. *Medical Mycology* **41(2):** 115-124.

Marchessault R. H., Sundararajan P. R. (1993). Cellulose. In: Aspinall G. O. (ed.). *The polysaccharides, vol. 2.* New York: Academic Press, Inc., 11–95.

Martinez L. R., Garcia-Rivera J., Casadevall A. (2001). *Cryptococcus neoformans* var. *neoformans* (Serotype D) strains are more susceptible to heat than *C. neoformans* var. *grubii* (Serotype A) strains. *Journal of Clinical Microbiology* **39(9):** 3365-3367.

Mayer A. M., Staples R. C. (2002). Laccase: new functions for and old enzymes. *Phytochemistry* **60:** 551-565.

McCarter J. D., Withers S. G. (1994). Mechanisms of enzymatic glycoside hydrolysis. *Current Opinion in Structural Biology* **4(6):** 885-892.

McCarthy K. M., Morgan J., Wannemuehler K. A., Mirza S. A., Gould S. M., Mhlongo N., Moeng P., Maloba B. R., Crewe-Brown H. H., Brandt M. E., Hajjeh R. A. (2006). Population-based surveillance for cryptococcosis in an antiretroviral-naive South African province with a high HIV seroprevalence. *AIDS* **20:** 2199–2206.

Mccleary B. V., Matheson N. K. (1983). Action patterns and substrate-binding requirements of beta-deuterium-mannanase with mannosaccharides and mannan-type polysaccharides. *Carbohydrate Research* **119:** 191-219.

Mccleary B. V. (1991). Comparison of endolytic hydrolases that depolymerize 1,4-beta-dmannan, 1,5-alpha-L-arabinan, and 1,4-beta-D-galactan. *ACS Symposium Series* **460:** 437-49.

McClelland C. M., Fu J., Woodlee G. L., Seymour T. S., Wickes B. L. (2002). Isolation and characterization of the *Cryptococcus neoformans MATa* pheromone gene. *Genetics* **160:** 935-947.

Meyer W., Mitchell T. G., Freedman E. Z., Vilgalys R. (1993). Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *Journal of Clinical Microbiology* **31(9):** 2274-2280.

Meyer W., Marszewska K., Amimostofian M., Igreja R. P., Hardtke C., Methling K., Viviani M. A., Chindamporn A., Sukroongreung S., John M. A., Ellis D. H., Sorrell T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20:** 1790-1799.

Min K. L., Kim Y. H., Kim Y. W., Jung H. S., Hah Y. C. (2001). Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis. Archives Biochemistry and Biophysics* **392(2):** 279-286.

Mirza S. A., Phelan M., Rimland D., Graviss E., Hamill R., Brandt M. E., Gardner T., Sattah M., de Leon G. P., Baughman W., Hajjeh R. A. (2003). The changing epidemiology of cryptococcosis: an update from population-based active surveillance in 2 large metropolitan areas, 1992-2000. *Clinical Infectious Diseases* **36(6):** 789-794.

Missall T. A., Moran J. M., Corbett J. A., Lodge J. K. (2005). Distinct stress responses of two functional laccases in *Cryptococcus neoformans* are revealed in the absence of the thiol-specific antioxidant Tsa1. *Eukaryotic Cell* **4(1):** 202-208.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* **8(4):** 515-548.

Monari C., Paganelli F., Bistoni F., Kozel T. R., Vecchiarelli A. (2008). Capsular polysaccharide induction of apoptosis by intrinsic and extrinsic mechanisms. *Cellular Microbiology* **10(10):** 2129-2137.

Mondon P., Petter R., Amalfitano G., Luzzati R., Concia E., Polacheck I., Kwon-Chung K. J. (1999). Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans. Antimicrobial Agents and Chemotherapy* **43(8):** 1856-1861.

Montagna M. T., Santacroce M. P., Caggiano G., Tatò D., Ajello L. (2003). Cavernicolous habitats harbouring *Cryptococcus neoformans*: results of a speleological survey in Apulia, Italy, 1999-2000. *Medical Mycology* **41(5):** 451-455

Moore T. D., Edman J. C. (1993). The alpha-mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. *Molecular and Cellular Biology* **13:** 1962-1970.

Mora D. J., Pedrosa A. L., Rodrigues V., Leite Maffei C. M., Trilles L., Dos Santos Lazéra M., Silva-Vergara M. L. (2010). Genotype and mating type distribution within clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from patients with cryptococcal meningitis in Uberaba, Minas Gerais, Brazil. *Medical Mycology* **48(4):** 561-569.

Moss G. P. (2006). Recommendations of the Nomenclature Committee. International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse. Retrieved 2006-03-14.

Murphy J. W. (1998). Protective cell-mediated immunity against *Cryptococcus neoformans*. *Research in Immunology* **149(4-5):** 373-386

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. thesis, University of Delhi, Delhi, India.

Mylonakis E., Ausubel F. M., Perfect J. R., Heitman J., Calderwood S. B. (2002). Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **99(24):** 15675-15680.

Neilson J. B., Ivey M. H., Bulmer G. S. (1978). *Cryptococcus neoformans*: pseudohyphal forms surviving culture with *Acanthamoeba polyphaga. Infection and Immunity* **20:** 262-266.

Nielsen K., Cox G. M., Wang P., Toffaletti D. L., Perfect J. R., Heitman J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic *a* and α isolates. *Infection and Immunity* **71(9):** 4831-4841.

Nielsen K., Marra R. E., Hagen F., Boekhout T., Mitchell T. G., Cox G. M., Heitman J. (2005a). Interaction between genetic background and the mating-type locus in *Cryptococcus neoformans* virulence potential. *Genetics* **171(3):** 975-983.

Nielsen K., Cox G. M., Litvintseva A. P., Mylonakis E., Malliaris S. D., Benjamin D. K. Jr, Giles S. S., Mitchell T. G., Casadevall A., Perfect J. R., Heitman J. (2005b). *Cryptococcus neoformans* {alpha} strains preferentially disseminate to the central nervous system during coinfection. *Infection and Immunity* **73(8):** 4922-4933.

Nielsen K., De Obaldia A. L., Heitman J. (2007). *Cryptococcus neoformans* Mates on Pigeon Guano: Implications for the Realized Ecological Niche and Globalization. *Eukaryotic Cell* **6(6):** 949–959.

Nessa K., Gross N. T., Jarstrand C., Johansson A., Camner P. (1997). *In vivo* interaction between alveolar macrophages and *Cryptococcus neoformans*. *Mycopathologia* **139:** 1-7.

Ngamskulrungroj P., Gilgado F., Faganello J., Litvintseva A. P., Leal A. L., Tsui K. M., Mitchell T. G., Vainstein M. H., Meyer W. (2009). Genetic diversity of the *Cryptococcus* species complex suggests that *Cryptococcus gattii* deserves to have varieties. *PLoS ONE* **4(6):** e5862.

Noverr M. C., Cox G. M., Perfect J. R., Huffnagle G. B. (2003). Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. *Infection and Immunity* **71(3):** 1538-1547.

Olszewski M. A., Noverr M. C., Chen G. H., Toews G. B., Cox G. M., Perfect J. R., Huffnagle G. B. (2004). Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *American Journal of Pathology* **164(5):** 1761-1771.

Othman N., Abdullah N. A., Wahab Z. A. (2004). Cryptococcal meningitis in an immunocompetent child: a case report and literature review. *Southeast Asian Journal of Tropical Medicine and Public Health* **35(4):** 930-934.

Pappas P. G., Perfect J., Larsen R. A. (1998). Cryptococcosis in HIV-negative patients: analysis of 306 cases (abstract 101). In: *36th annual meeting of the Infectious Diseases Society of America (Denver, CO).* Alexandria, VA: Infectious Diseases Society of America.

Park B. J., Wannemuehler K. A., Marston B. J., Govender N., Pappas P. G., Chiller T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Pérez C., Dolande M., Moya M., Roselló A., Hartung de Capriles C. R., Landaeta M. E., Mata-Essayag S. (2008). *Cryptococcus neoformans*, *Cryptococcus gattii*: Serotypes in Venezuela. *Mycopathologia* **166:** 149–153.

Perfect J. R. (1995). *Cryptococcus neoformans*: A sugar-coated killer with designer genes. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **45(3):** 365-404.

Perfect J. R. (2006). *Cryptococcus neoformans*: the yeast that likes it hot. *Federation of European Microbiological Societies Yeast Research* **6:** 463-468.

Pfaller M. A., Messer S. A., Boyken L., Rice C., Tendolkar S., Hollis R. J., Doern G. V., Diekema D. J. (2005). Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004). *Journal of Clinical Microbiology* **43(5):** 2163-2167.

Polacheck I., Hearing V. J., Kwon-Chung K. J. (1982). Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *Journal of Bacteriology* **150(3):** 1212-1220.

Polacheck I., Kwon-Chung K. J. (1988). Melanogenesis in *Cryptococcus neoformans. Journal of General Microbiology* **134(4):** 1037-1041.

Posteraro B., Vella A., Cogliati M., De Carolis E., Florio A. R., Posteraro P., Sanguinetti M., Tortorano A. M. (2012). Matrix-assisted laser desorption ionization-time of flight mass spectrometry based method for discrimination between molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii*. *Journal of Clinical Microbiology* **50(7):** 2472–2476.

Powderly W. G., **Saag M. S**., **Cloud G. A**., **Robinson P**., **Meyer R. D**., **Jacobson J. M**., **Graybill J. R**., **Sugar A. M**., **McAuliffe V. J**., **Follansbee S. E**. **(1992).** A controlled trial of fluconazole or amphotericin B to prevent relapse of cryptococcal meningitis in patients with the acquired immunodeficiency syndrome. *New England Journal of Medicine* **326:** 793–8.

Powderly W. G. (1993). Cryptococcal meningitis and AIDS. *Clinical Infectious Diseases* **17:** 837–842.

Race E. M., Adelson-Mitty J., Kriegel G. R., Barlam T. F., Reimann K. A., Letvin N. L., Japour A. J. (1998). Focal mycobacterial lymphadenitis following initiation of protease-inhibitor therapy in patients with advanced HIV-1 disease. *Lancet* **351:** 252–255.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Rebers P. A., Barker S. A., Heidelberger M., Dische Z., Evans E. E. (1958). Precipitation of the specific polysaccharide of *Cryptococcus neoformans* A by Types II and XIV antipneumococcal sera *Journal of American Chemical Society* **80(5):** 1135–1137.

Reese E. T., Sui R. G. H, Levinson H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bacteriology* **59:** 485–497.

Reese E. T. (1956). A microbiological process report: enzymatic hydrolysis of cellulose. *Applied Microbiology* **4:** 39–45.

Reese E. T., Mandels M. (1971). Enzymatic degradation. In: Bikales N. M., Segal L. (eds.) *Cellulose and cellulose derivatives.* New York: Wiley Interscience, 1079–1094.

Rhodes J. C., Kwon-Chung K. J. (1985). Production and regeneration of protoplasts from *Cryptococcus. Sabouraudia* **23(1):** 77-80.

Riley P. A. (1991). Melanogenesis: a realistic target for antimelanoma therapy? *European Journal of Cancer* **27(9):** 1172-1177.

Rittenberg S. C. (1983). *Bdellovibrio*: Attack, penetration and growth on its prey. *Amercian Society of Microbiology News*. **49:** 435-439.

Rivera J., Feldmesser M., Cammer M., Casadevall A. (1998). Organ-dependent variation of capsule thickness in *Cryptococcus neoformans* during experimental murine. *Infection and Immunity* **66(10):** 5027-5030.

Roberts P. (1997). New heterobasidiomycetes from Great Britian. *Mycotaxon* **63:** 195-216.

Romeo O., Scordino F., Chillemi V., Criseo G. (2012). *Cryptococcus neoformans*/ *Cryptococcus gattii* species complex in southern Italy: an overview on the environmental diffusion of serotypes, genotypes and mating-types. *Mycopathologia* **174(4):** 283-291.

Rosas A. L, Casadevall A. (1997). Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. *Federation of European Microbiological Societies Microbiology Letters* **153:** 265-272.

Rosas A. L., Casadevall A. (2001). Melanin decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. *Mycopathologia.***151:** 53-56.

Rose M. C., Voynow J. A. (2006). Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiological Reviews* **86:** 245–278.

Rozenbaum R., Rios Goncalves A. J. (1994). Clinical epidemiological study of 171 cases of cryptococcosis. *Clinical Infectious Diseases* **18:** 369–380.

Ruma-Haynes P., Brwonlee A. G., Sorrell T. C. (2000). A rapid method for detecting extracellular proteinase activity in Cryptococcus neoformans and survey of 63 isolates. *Journal of Medical Microbiology* **49:** 733-737.

Saag M. S., **Cloud G. A**., **Graybill J. R**., **Sobel J. D**., **Tuazon C. U**., **Johnson P. C**., **Fessel W. J**., **Moskovitz B. L**., **Wiesinger B**., **Cosmatos D**., **Riser L**., **Thomas C**., **Hafner R**., **Dismukes W. E**. **(1999).** A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated cryptococcal meningitis. *Clinical Infectious Diseases.* **28:** 291–6.

Saag M. S., Graybill R. J., Larsen R. A., Pappas P. G., Perfect J. R., Powderly W. G., Sobel J. D., Dismules W. E. for the Mycoses Study Group Cryptococcal Subproject (2000). Practice guidelines for the management of cryptococcal disease. . *Clinical Infectious Diseases* **30:** 710–718.

Sakamoto T., Ogura A., Inui M., Tokuda S., Hosokawa S., Ihara H, Kasai N. (2011). Identification of a GH62 alpha-l-arabinofuranosidase specific for arabinoxylan produced by *Penicillium chrysogenum. Applied Microbiology and Biotechnology* **90(1):** 137-146.

Salas S. D., Bennett J. E., Kwon-Chung K. J., Perfect J. R., Williamson P. R. (1996). Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans. Journal of Experimental Medicine* **184:** 377-386.

Sanchez C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advances* **27:** 185-194.

Sethi K. K., Randhawa H. S., Abraham S., Mishra S. K., Damodaran V. N. (1966). Occurrence of *Cryptococcus neoformans* in pigeon excreta in Delhi. *Indian Journal of Chest Disseases* **8:** 207–210.

Sethi K. K., Randhawa H. S. (1968). Survival of *Cryptococcus neoformans* in the gastrointestinal tract of pigeons following ingestion of the organism. *Journal of Infectious Diseases* **118:** 135–138.

Shadomy H. J., Utz J. P. (1966). Preliminary studies on a hypha forming mutant of *Cryptococcus neoformans. Mycologia* **58:** 383-390.

Shadomy H. J., Lurie H. I. (1971). Histopathological observations in experimental cryptococcosis causedby a hypha-producing strain of *Cryptococcus neoformans* (Cowardstrain) in mice. *Sabouraudia* **9:** 6-9.

Shelburne S. A. III, Hamill R. J., Rodriguez-Barradas M.C, Greenberg S. B., Atmar R. L., Musher D. W., Gathe J. C. Jr, Visnegarwala F., Trautner B. W. (2002). Immune reconstitution inflammatory syndrome: emergence of a unique syndrome during highly active antiretroviral therapy. *Medicine* **81:** 213–227.

Shen W.C., Davidson R. C., Cox G. M., Heitman J. (2002). Pheromones stimulate mating and differentiation via paracrine and autocrine signaling in *Cryptococcus neoformans. Eukaryotic Cell* **1:** 366–377.

Singh N., Lortholary O., Alexander B. D., Gupta K. L., John G. T., Pursell K., Munoz P., Klintmalm G. B., Stosor V., del Busto R., Limaye A. P., Somani J., Lyon M., Houston S., House A. A., Pruett T. L., Orloff S., Humar A., Dowdy L., Garcia-Diaz J., Kalil A. C., Fisher R. A., Husain S. (2005). An immune reconstitution syndrome-like illness associated with Cryptococcus neoformans infection in organ transplant recipients. *Clinical Infectious Diseases* **40(12):** 1756–1761.

Sinnott M. L. (1990). Catalytic mechanisms of enzymatic glycosyl transfer. *Chemical Reviews* **90:** 1171-1202.

Skiest D. J., Hester L. J., Hardy R. D. (2005). Cryptococcal immune reconstitution inflammatory syndrome: report of four cases in three patients and review of the literature. *Journal of Infection* **51(5):** 289-297.

Slobodkin L. B. (1968). How to be a predator. *American Zoolog.* **8**: 43-51.

Sorrell T. C., Chen S. C. A., Ruma P., Meyer W., Pfeiffer T. J., Ellis D. H., Brownlee A. G. (1996). Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *Journal of Clinical Microbiology* **34(12):** 1253-1260.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans. Revista Iberoamericana de Micologia* **14:** 42-43.

Staib F. (1962). *Cryptococcus neoformans* and *Guizotia abyssinica* (syn. G *Oleifera* D. C.) *Zeitschrift fur Hygiene und Infektionskrankheiten* **148:** 466-475.

Steen B. R., Lian T., Zuyderuyn S., MacDonals W. K., Marra M., Jones S. J. M., Kronstad J. W. (2002). Temperature-regulated transcription in the pathogenic fungus *Cryptococcus neoformans. Genome Research* **12:** 1386-1400.

Steenbergen J. N., Shuman H. A., Casadevall A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **98(26):** 15245-15250.

Steenbergen J. N., Casadevall A. (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes and Infection* **5:** 667-675.

Sungkanuparph S., Vibhagool A., Mootsikapun P., Chetchotisakd P., Tansuphaswaswadikul S., Bowonwatanuwong C. (2003). Opportunistic infections after the initiation of highly active antiretroviral therapy in advanced AIDS patients in an area with a high prevalence of tuberculosis. *AIDS* **17:** 2129–2131.

Sweeney D. A., Caserta M. T., Korones D. N., Casadevall A., Goldman D. L. (2003). A ten-year-old boy with a pulmonary nodule secondary to *Cryptococcus neoformans*: case report and review of the literature. The Paediatric Infectious Disease Journal **22(12):** 1089-1093.

Tartar A., Wheeler M. M., Zhou X., Coy M. R., Boucias D. G., Scharf M. E. (2009). Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite *Reticulitermes flavipes*. *Biotechnology for Biofuels* **2(25):** 1-19.

Taylor M., Chadwick D., Barkham T. (2002). First reported isolation of *Cryptococcus neoformans* var. *gattii* from a patient in Singapore. *Journal of Clinical Microbiology* **40:** 3098-3099.

Thurston C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140:** 19–26.

Tortorano A. M., Roverselli A., Cogliati M., Brasca M. (1993). Survey of *Cryptococcus neoformans* susceptibility to antifungals in Northern Italy. (Abstract P6-7), 2nd International Conference on Cryptococcusand Cryptococcosis, Milano.

Trevenzoli M., Cattelan A. M., Rea F., Sasset L., Semisa M., Lanzafame M., Meneghetti F., Cadrobbi P. (2002). Mediastinitis due to cryptococcal infection: a new clinical entity in the HAART era. *Journal of Infection* **45:** 173–179.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout, T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Medical Mycology* **41:** 383-390.

Tscharke R. L., Lazéra M., Chang Y. C., Wickes B. L., Kwon-Chung K. J. (2003). Haploid fruiting in *Cryptococcus neoformans* is not mating type α-specific. *Fungal Genetics and Biology* **39:** 230-237.

Tucker S. C., Casadevall A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proceedings of the National Academy of Sciences of the United States of America* **99(5):** 3165-70.

Turner S. H., Cheriniak R., Reiss E. (1984). Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans. Carbohydrate Research* **125(2):** 343-349.

Vaishnav V. V., Bacon B. E., O'Neill M., Chriniak R. (1998). Structural characterization of galactoxylomannan of *Cryptococcus neoformans* Cap67. *Carbohydrate Research* **306:** 315-330.

Vajpayee M., Kanswal S., Seth P., Wig N. (2003). Spectrum of opportunistic infections and profile of CD4+ counts among AIDS patients in North India. *Infection* **31:** 336–340.

Valderrama B., Oliver P., Medrano-Soto A., Vazquez-Duhalt R. (2003). Evolutionary and structural diversity of fungal laccases. *Antonie van Leeuwenhoek.* **84:** 289–299.

van den Bosch J. S. G., Smals A. G. H., Valk I. M., Kloppenborg P. W. C. (1982). Lack of difference in the growth stimulating effect between weekly single and multiple human chorionic gonadotrophin administration in boys with delayed puberty. *Clinical Endocrinology* **16(1):** 1-9.

van den Brink J., de Vries R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology* **91(6):** 1477-14792.

van der Horst C. M., **Saag M. S.**, **Cloud G. A.**, **Hamill R. J.**, **Graybill J. R.**, **Sobel J. D.**, **Johnson P. C.**, **Tuazon C. U.**, **Kerkering T.**, **Moskovitz B. L.**, **Powderly W. G.**, **Dismukes W. E. (1997).** Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. *New England Journal of Medicine* **337:** 15–21.

Van der Walt J. P., Scott D. B., van der Klift W. C. (1972). Six new *Candida* species from South African insect sources. *Mycopathologia et mycologia applicata* **47(3):** 221-236.

van Duin D., Casadevall A., Nosanchuk J. D. (2002). Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. *Antimicrobial Agents and Chemotherapy* **46(11):** 3394-3400.

van Heerden A., van Zyl W. H., Cruywagen C. W., Mouton M., Botha A. (2011). The lignicolous fungus *Coniochaeta pulveracea* and its interactions with syntrophic yeasts from the woody phylloplane. *Microbial Ecology* **62(3):** 609-619.

van Soest P. J. (1994). Nutritional ecology of the ruminant, 2nd edition. New York: Cornell University Press.

Varma A., Kwon-Chung K. J. (1992). DNA probe for strain typing of *Cryptococcus neoformans*. *Journal of Clinical Microbiology* **30(11):** 2960–2967.

Varma A., Swinne D., Staib F., Bennett J. E., Kwon-Chung K. J. (1995). Diversity of DNA fingerprints in *Cryptococcus neoformans. Journal of Clinical Microbiology* **33(7):** 1807-1814.

Vartivarian S. E., Anaissie E. J., Cowart R. E., Sprigg H. A., Tingler M. J., Jacobson E. S. (1993). Regulation of cryptococcal capsular polysaccharide by iron. *Journal of Infectious Diseases* ***167*****(1):** 186-190.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transplant International* **16:** 336-340.

Viviani M. A. (1996). Cryptococcal meningitis: Diagnosis and treatment. *International Journal of Antimicrobial Agents* **6:** 169-173.

Wang Y., Casadevall A. (1994). Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. *Infection and Immunity* **62(7):** 3004-3007.

Wang Y., Aisen P., Casadevall A. (1995). *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infection and Immunity* **63:** 3131-3136.

Wang Y., Aisen P., Casadevall A. (1996). Melanin, melanin "ghosts," and melanin composition in *Cryptococcus neoformans. Infection and Immunity* **64(7):** 2420-2424.

Wang P., Heitman J. (1999). Signal transduction cascades regulating mating, filamentation and virulence in *Cryptococcus neoformans*. *Current Opinion in Microbiology* **2:** 358-362.

Wang P., Perfect J. R., Heitman J. (2000). The G-protein beta subunit *GPB1* is required for mating and haploid fruiting in *Cryptococcus neoformans*. *Molecular and Cellular Biology* **20:** 352-362.

Wang P., Nichols C. B., Lengeler K. B., Cardenas M. E., Cox G. M., Perfect J. R., Heitman J. (2002). Mating-type-specific and nonspecific PAK kinases play shared and divergent roles in *Cryptococcus neoformans*. *Eukaryotic Cell* **1(2):** 257-272.

Warren R. A. J. (1996). Microbial hydrolysis of polysaccharides. *Annual Review of Microbiol**ogy*** **50:** 183–212.

Wassermann H. P., van der Walt J. J. (1973). Antibodies against melanin. The significance of negative results. *South African Medical Journal* **47:** 7-9.

Webb E. C. (1992). Enzyme nomenclature 1992: recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes. San Diego: Academic Press.

Wickes B. L., Mayorga M. E., Edman U., Edman J. C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α-mating type. *Microbiology* **93:** 7327-7331.

Wickes B. L., Edman U., Edman J. C. (1997). The *Cryptococcus neoformans STE12alpha* gene: a putative *Saccharomyces cerevisiae STE12* homologue that is mating type specific. *Molecular Microbiology* **26:** 951-960.

Wickes B. L. (2002). The role of mating type and morphology in *Cryptococcus neoformans* pathogenesis. *International Journal of Medical Microbiology* **292:** 313-329.

Williamson J. D., Silverman J. F., Mallak C. T., Christie J. D. (1996). Atypical cytomorphologic appearance of *Cryptococcus neoformans*: a report of five cases. *ACTA Cytologica* **40:** 363-370.

Williamson P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* **2:** 99-107.

Woods M. L. II, MacGinley R., Eisen D. P., Allworth A. M. (1998). HIV combination therapy: partial immune restitution unmasking latent cryptococcal infection. *AIDS* **12(12):** 1491–1494.

Xie L., Zhang L., Zhong Y., Liu N., Long Y., Wang S., Zhou X., Zhou Z., Huang Y., Wang Q. (2012). Profiling the metatranscriptome of the protistan community in *Coptotermes formosanus* with emphasis on the lignocellulolytic system. *Genomics* **99:** 246–255.

Xu J., Ali R. Y., Gregory D. A., Amick D., Lambert S. E., Yoell H. J., Vilgalys R. J., Mitchell T. G. (2000). Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans*. *Current Microbiology* **40:** 269–273.

Xue C., Tada Y., Dong X., Heitman J. (2007). The human fungal pathogen *Cryptococcus* can complete its sexual cycle during a pathogenic association with plants. *Cell Host and Microbe* **1(4):** 263-273.

Yan Z, Li X., Xu J. (2002). Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. *Journal of Clinical Microbiology* **40(3):** 956-972.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier, 77-98.

Yip V. L., Thompson J., Withers S. G. (2007). Mechanism of GlvA from *Bacillus subtilis*: a detailed kinetic analysis of a 6-phospho-alpha-glucosidase from glycoside hydrolase family 4. *Biochemistry* **46(34):** 9840-9852.

Yoshikawa K., Yamamoto K., Okada S. (1991). Isolation of *Aspergillus flavus* MO-5 producing two types of intracellular alpha-D-xylosidases: purification and characterization of alpha-D-xylosidase I. *Bioscience Biotechnology and Biochemistry* **57(8):** 1275-1280.

Yoshikawa K., Yamamoto K., Okada S. (1994). Classification of some alpha-glucosidases and alpha-xylosidases on the basis of substrate specificity. *Bioscience Biotechnology and Biochemistry* **58(8):** 1392-1398.

Yue C., Cavallo L. M., Alspaugh J. A., Wang P., Cox G. M., Perfect J. R., Heitman J. (1999). The *STE12alpha* homolog is requiredfor haploid filamentation but largely dispensable for mating and virulence in *Cryptococcus neoformans*. *Genetics* **153:** 1601-1615.

Zaragoza O., Cuenca-Estrella M., Regadera J., Rodríguez-Tudela J. L. (2008a). The capsule of the fungal pathogen *Cryptococcus neoformans* paradoxically inhibits invasive growth. *The Open Mycology Journal* **1(1):** 29-39.

Zaragoza O, Chrisman C. J., Castelli M. V., Frases S., Cuenca-Estrella M., Rodríguez-Tudela J. L., Casadevall A. (2008b). Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cellular Microbiology* **10(10):** 2043-2057.

Zhang S., Varma A., Williamson P. R. (1999). The yeast *Cryptococcus neoformans* uses 'mammalian' enhancer sites in the regulation of the virulence gene, *CNLAC1*. *Gene.* **227:** 231-240.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infection and Immunity* **69(9):** 5589-5596.

Zhu X., Gibbon J., Zhang S., Williamson P. (2003). Copper mediated reversal of defective laccase in a *vph1* avirulent mutant of *Cryptococcus neoformans*. *Molecular Microbiology* **47:** 1007–1014.

Zhu X., Williamson P. R. (2004). Role of laccase in the biology and virulence of *Cryptococcus neoformans. Federation of European Microbiological Societies Yeast Research* **5:** 1-10.

CHAPTER 2

The assimilation of complex lignocellulosic substrates and their associated monosaccharides by the yeast pathogen *Cryptococcus neoformans* var. *grubii*

The assimilation of complex lignocellulosic substrates and their associated monosaccharides by the yeast pathogen *Cryptococcus neoformans* var. *grubii*

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Cryptococcus neoformans (Sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative opportunistic fungal pathogen known to cause cryptococcosis and cryptococcal meningitis in immuno-suppressed individuals. Previous research has determined that *C. neoformans* var. *grubii* is capable of growth on woody material as well as carboxymethyl cellulose (CMC) during plate assays, however, it is not known if the yeast is capable of assimilating other lignocellulosic components such as hemi-cellulose. Thus, the objective was to determine if *C. neoformans* var. *grubii* ATCC H99 was capable of utilizing cellulose (CMC) and hemi-cellulose (gluco- or galactomannan) as sole carbon source and to compare the growth of this pathogen on the various monosaccharides associated with lignocellulose, particularly mannose. *Cryptococcus neoformans* var. *grubii* ATCC H99 was found to be capable of growth on both cellulose and hemi-cellulose. Similarly, growth was observed on all the simple sugars, namely L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose. The highest maximum specific growth rate (μ_{max} = 0.414 h^{-1} ± 0.003) was achieved on D-mannose, an important structural component of hemi-cellulose. The results of this study provide further evidence that woody habitats act as a natural habitat for *Cryptococcus neoformans* var. *grubii*.

1. INTRODUCTION

Cryptococcus neoformans is a facultative intracellular pathogen causing cryptococcal meningitis primarily in immune compromised patients (Casadevall *et al*., 2003; Franzot *et al*., 1998; Mitchell *et al.*, 1995). Currently, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are still regarded as being strongly associated with pigeon guano, while their sister species, *C. gattii*, displays a great prevalence for eucalyptus trees (Sorrell *et al.*, 1997; Sorrell *et al.*, 1996). However, previous and on-going research suggests that similar to *C. gattii*, *C. neoformans* prefers a woody environment and has been consistently isolated from various tree species and habitats associated with decaying woody material (Randhawa *et al.*, 2011; 2008; 2006; 2000; Trilles *et al*., 2003; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996). Our own research demonstrated that both clinical and environmental strains of *C. neoformans* var. *grubii* are capable of growth when cultured in a microcosm comprised solely of woody debris of either *Acacia mearnsii* or *Eucalyptus camaldulensis* (Botes *et al.*, 2009). Similarly, these cryptococcal strains displayed both dikaryotic and monokaryotic fruiting when mated on a solid medium containing woody debris.

The above-mentioned findings highlight the role of lignocellulose in the growth and survival of *C. neoformans* var. *grubii* within the natural environment. It has been known for decades that *C. neoformans* is characterized by the ability to assimilate some of the degradation products of cellulose, such as glucose and cellobiose, as well as hemi-cellulose that includes arabinose, galactose, glucose, rhamnose and xylose (Kwon-Chung, 1998). Mannose is a major monomeric component of hemi-cellulose (Fengel *et al.*, 1989) and forms the structural back-bone of the polysaccharide capsule (Janbon, 2004; Bose *et al*., 2003; Steenbergen *et al*., 2003), however, little information exists on the assimilation of mannose by representatives of *C. neoformans* var. *grubii* in terms of biomass production and growth rate.

Similar to the findings on the ability of *C. neoformans* to utilize the above-mentioned simple carbohydrates, evidence exists that the ability to degrade cellulosic material resides within *C. neoformans* var. *grubii*, as it was found that representatives of *C. neoformans* var. *grubii* are capable of producing colonies on agar plates containing carboxymethyl cellulose (CMC) as sole carbon source (Botes *et al.*, 2009). More recently, it was demonstrated that growth of *C. neoformans* var. *grubii* ATCC

H99 on CMC plates can be enhanced by the cellulolytic activity of the lignicolous fungus *Coniochaeta pulveracea* (van Heerden *et al.*, 2011). The presence of the latter resulted in the increased release of carbon sources, such as cellobiose and glucose, from the CMC, which in turn increased growth of the cryptococcal strain. The results therefore point to a potential symbiotic interaction regarding cellulose utilization between pathogenic cryptococci and lignicolous fungi occurring in the same woody habitat. However, it is still unclear whether *C. neoformans* var. *grubii* can utilize mannose and grow on hemi-cellulosic substrates rich in this hexose.

With the above as background, we wanted to test the hypothesis that *C. neoformans* var. *grubii* ATCC H99 is adapted to grow on cellulosic and hemi-cellulosic materials. Thus, the first goal of this study was to test whether *C. neoformans* var. *grubii* ATCC H99 is able to grow on complex lignocellulosic substrates such as CMC, galactomannan (Bean gum locust) and glucomannan. The second aim was to compare the growth rate of this yeast on the degradation products of both cellulose and hemi-cellulose.

2. MATERIALS AND METHODS

2.1 Strain and Culture Conditions

Cryptococcus neoformans var. *grubii* ATCC H99 (CBS 10515) was obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and maintained by periodic transfer to yeast peptone glucose (YPG, pH 5.5) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Gauteng, South Africa) and incubated at 22 °C. All media was prepared as per the manufacturer's instructions.

2.2 Growth on Complex Lignocellulosic Substrates

A pre-inoculum of *C. neoformans* var. *grubii* ATCC H99 was prepared at 30 °C for 48 hours in 250 mL conical flasks, containing 25 mL YPG broth (pH 5.5) on a rotary shaker (150 rpm; New Brunswick Scientific G53, Canada). A total of 2 mL of culture was centrifuged (Biofuge fresco, Heraeus Instruments, Hanua, Germany) for 5 min at 13 793 RCF (Beckman Coulter, Avanti J-E, California, USA) and the pellet was re-suspended in 2 mL distilled water. Total cell counts of the resulting suspensions that

served as final inoculums were done using a Neubauer haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

Series of complex lignocellulosic substrates, namely carboxymethyl cellulose (Sigma), galactomannan (Bean gum locust; Sigma) and glucomannan (Konjak, Patrick Holfords, Birmingham, United Kingdom); were prepared in 250 mL conical flasks. The flasks, each containing 25 mL substrate (0.1 % w/v; pH 6) and 0.1 % glucose were autoclaved (121 °C, 15 min) and subsequently supplemented with yeast nitrogen base (YNB, Becton Dickinson, Gauteng, South Africa). Each substrate was inoculated with *C. neoformans* var. *grubii* ATCC H99 to a final concentration of 1 x 10^6 yeast cells / mL substrate and incubated at 30 °C on a rotary shaker (setting of 4). After 1 hour a total of 500 µL was sampled from each of the inoculated flasks and yeasts were enumerated using dilution plates with malt extract agar (MEA; Merck, Gauteng, South Africa). Plates were incubated at 30 °C for 48 hours before the colonies were counted. Enumeration of yeasts was repeated every 12 hours for 7 days. All experiments were conducted in triplicate and the data were plotted on a log graph using Microsoft Office Excel 2010.

2.3 Growth on the Degradation Products of Lignocellulose – Simple Sugars

Growth curves were compiled in order to compare the growth rate of *C. neoformans* var. *grubii* ATCC H99 on the simple sugars released during the degradation of lignocellulose. A pre-inoculum of *C. neoformans* var. *grubii* ATCC H99 was prepared prior to growth evaluation on specific media. The strain was cultured at 30 °C for 48 hours in 250 mL conical flasks, containing 25 mL YPG broth (pH 5.5) on a rotary shaker (setting 4). Cells were collected via centrifugation (13 793 RCF for 5 min), washed twice in distilled water and re-suspended in 100 mL yeast peptone broth (pH 5.5; Merck, Gauteng, South Africa) and incubated at 30 °C on a rotary shaker (150 rpm; New Brunswick Scientific G53) for a further four hours. A total of 2 mL of each culture was centrifuged (Biofuge fresco, Heraeus Instruments) for 5 min at 13 793 RCF (Beckman Coulter, Avanti J-E) and the pellet was re-suspended in 2 mL distilled water. Total cell counts of the resulting suspensions that served as final inoculums were done using a Neubauer haemocytometer.

Series of 250 mL conical flasks containing 25 mL YNB (pH 6) were supplemented with one of six carbon sources (2 % w/v) namely, L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose. Each substrate was

inoculated with *C. neoformans* var. *grubii* ATCC H99 to a final concentration of 1 x 10^6 yeast cells / mL substrate and incubated at 30 °C on a rotary shaker (setting 4). Cell growth was monitored over a period of 54 hours spectrophotometrically (600 nm, BioRad SmartSpec Plus, 273 BR 04238, California, USA). All experiments were conducted in triplicate and the data were plotted on a log graph using Microsoft Office Excel 2010.

3. RESULTS AND DISCUSSION

3.1 Growth on Complex Lignocellulosic Substrates

Repeated isolation of *C. neoformans* from habitats rich in lignocellulosic material, coupled to the ability of this pathogen to undergo both dikaryotic and monokaryotic fruiting on woody debris (Randhawa *et al.*, 2011; 2008; 2006; 2000; Botes *et al*., 2009; Trilles *et al*., 2003; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996), led us to further evaluate the growth of *C. neoformans* with regards to the specific components of lignocellulose, specifically cellulose and hemi-cellulose. Cellulose is comprised of numerous β-1,4 coupled D-glucose residues resulting in a crystalline structure (Klemm *et al.*, 1998; Fengel *et al.*, 1989). The hemi-cellulose backbone can be comprised of either β-1,4 coupled D-xylose residues (xylan) or β-1,4 D-mannose/ D-glucose residues (mannans/glucomannans) (Fengel *et al.*, 1989). In the case of hemi-celluloses, the structural diversity of the carbohydrate side chains adds to the overall complexity of this material.

In this study we found that *C. neoformans* var. *grubii* ATCC H99 is able to grow in media containing a cellulosic carbon source such as carboxymethyl cellulose (CMC), as well as in media containing the hemi-cellulosic substrates, glucomannan and bean gum locust (galactomannan) (Fig 1). Growth on the hemi-cellulosic carbon source, xylan, was not evaluated as previous research has shown that *C. neoformans* strains test negative for xylanase activity on plate assays (Botes *et al.*, 2009).

Cryptococcus neoformans var. *grubii* ATCC H99 displayed a shorter lag phase on glucomannan and galactomannan when compared to growth on CMC, which may be due to the more crystalline nature of cellulose that renders it less readily degraded. However, this did not impact on cell biomass as similar cell numbers were observed for all three components after 48 hours (Fig 1).

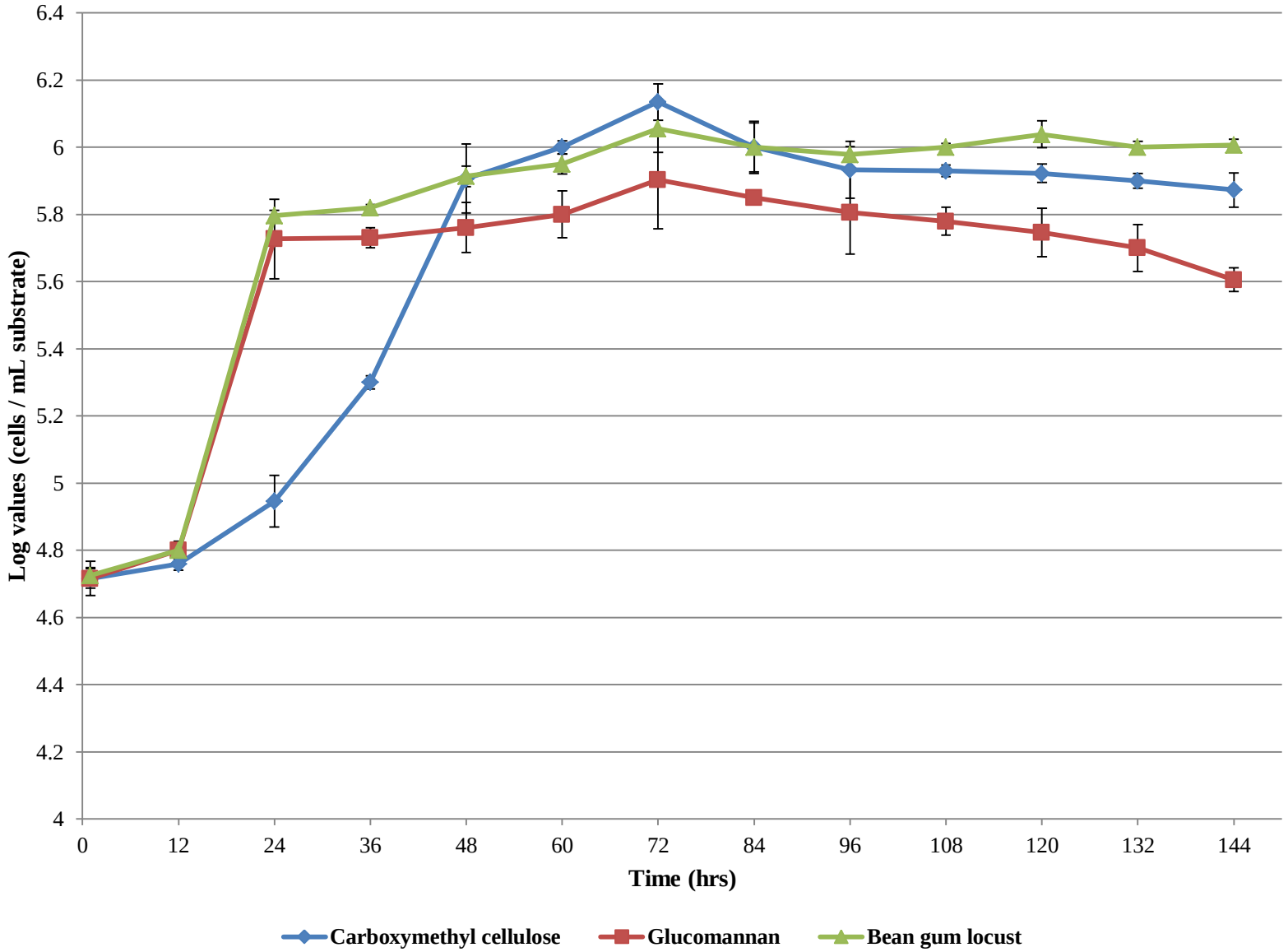

Figure 1 Growth curves of *C. neoformans* var. *grubii* ATCC H99 in liquid cultures containing either carboxymethyl cellulose (CMC), glucomannan (GM) or bean gum locust (LBG) at a concentration of 0.1 % w/v. Values represent the mean of three repetitions while the bars denote standard errors.

3.2 Growth on the Degradation Products of Lignocellulose – Simple Sugars

As mentioned previously, *C. neoformans* is able to assimilate some of the degradation products of cellulose and hemi-cellulose, namely, arabinose, galactose, glucose, rhamnose and xylose (Kwon-Chung, 1998). However, little is known regarding the growth rate of *C. neoformans* on the hemi-cellulosic monomer mannose. Growth curves were done in order to determine if *C. neoformans* var. *grubii* ATCC H99 is capable of assimilating mannose and if so to compare the maximum specific growth rate (μ_{max}) with other lignocellulosic-associated sugars.

The growth curves of the yeast on the degradation products of cellulose and hemi-cellulose were notably different from each other (Fig 2). Growth on D-glucose resulted in the shortest lag phase and a μ_{max} of 0.317 h^{-1} (± 0.046). Despite a slightly longer lag phase, growth on D-mannose resulted in the highest μ_{max} (0.414 h^{-1} ± 0.003). This longer lag phase may be attributed to an adaptation period that is commonly seen amongst fungi when faced with a carbon source other than glucose (Griffin, 1996). Growth on the hexose D-galactose (μ_{max} = 0.238 h^{-1} ± 0.014) and the pentose L-rhamnose (μ_{max} = 0.243 h^{-1} ± 0.024) produced similar lag phases, however cells numbers were significantly higher when *C. neoformans* var. *grubii* ATCC H99 was cultured on D-galactose.

Interestingly, growth on the pentose sugars L-arabinose, (μ_{max} = 0.073 h^{-1} ± 0.009) and D-xylose (μ_{max} = 0.090 h^{-1} ± 0.007) resulted in a much longer lag phase and lower maximum specific growth rates when compared to L-rhamnose. This may be as a result of differences with regards to how these pentose sugars are metabolized. L-Arabinose and D-xylose are both converted to D-xylulose-5-phosphate, which then enters the pentose phosphate pathway and ultimately glycolysis (Kanehisa *et al.*, 2012; Kanehisa *et al.*, 2000). The metabolism of L-rhamnose does not require the pentose phosphate pathway; L-rhamnose is instead converted directly into glyceraldehyde-3-phosphate and finally pyruvate. By-passing the pentose phosphate pathway, growth on L-rhamnose may reduce the metabolic burden on the cell and thereby allow for higher growth rates and cell numbers.

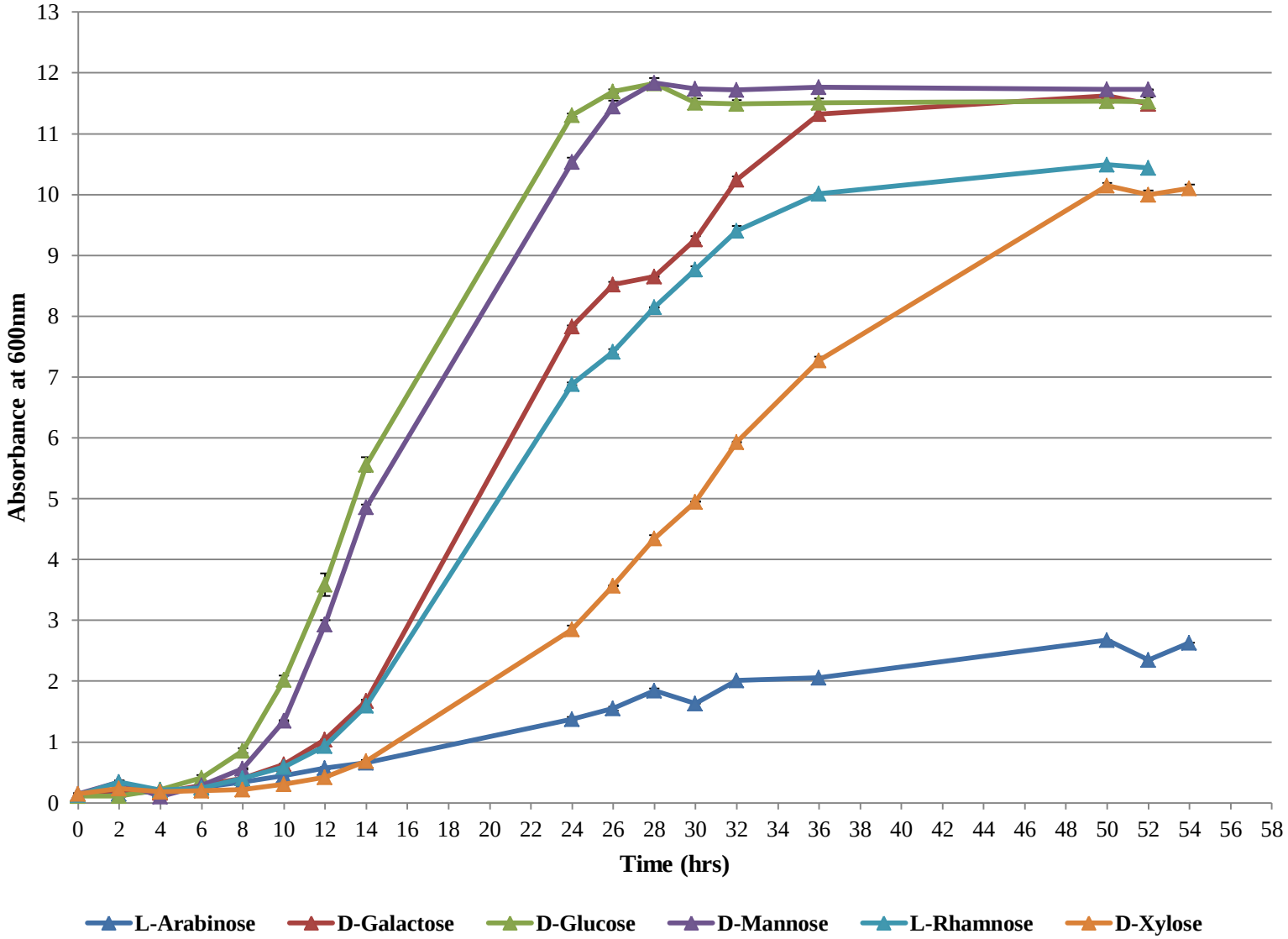

Figure 2 Growth curves of *C. neoformans* var. *grubii* ATCC H99 in liquid cultures containing either L-arabinose, D-galactose, D-glucose D-mannose, L-rhamnose or D-xylose at a concentration of 2 % w/v. Values represent the mean of three repetitions while the bars denote standard errors.

4. CONCLUSIONS

We confirmed that *C. neoformans* var. *grubii* ATCC H99 is capable of growth on various lignocellulosic components, specifically cellulose (carboxymethyl cellulose) and hemi-cellulose (gluco- and galacto-mannan), as well as their associated monosaccharides, L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose. Since the highest specific growth rate was obtained on mannose, and a shorter lag phase was observed when the yeast was cultivated on mannose containing polymers than on CMC, and given that this monosaccharide is a structural component of the cryptococcal capsule, it may indicate that mannose is important in the physiology of *C. neoformans* var. *grubii*.

To conclude, the ability of *C. neoformans* var. *grubii* ATCC H99 to utilize lignocellulosic materials, as well as their monomeric components, further supports previous research that identified woody habitats as the ecological niche of this yeast pathogen.

5. REFERENCES

Bose I., Reese A. J., Ory J. J., Janbon G., Doering T. L. (2003). A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryotic Cell* **2(4):** 655-663.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready-made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. gattii from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Fengel D., Wegener G. (1989). Wood: Chemistry, ultrastructure, reaction. New York: Walter de Gruyter.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *Journal of Clinical Microbiology* **36(8):** 2200-2204.

Griffin D. H. (1996). Fungal Physiology. New York: John Wiley & Sons.

Janbon G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *Federation of European Microbiological Societies Yeast Research* **4:** 765-771.

Kanehisa M. Goto S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research* **28:** 27-30.

Kanehisa M., Goto S., Sato Y., Furumichi M., Tanabe M. (2012). KEGG for integration and interpretation of large-scale molecular datasets. *Nucleic Acids Research* **40:** 109-114.

Klemm D., Philipp B., Heinze T., Heinze U., Wagenknecht W. (1998). Comprehensive cellulose chemistry. Chichester: Wiley VCH.

Kwon-Chung K. J. (1998). Filobasidiella Kwon-Chung. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier Science BV, 656-662.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans. Clinical Microbiology Reviews* **8(4):** 515-548.

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. dissertation, University of Delhi, Delhi, India.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus* neoformans in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Sorrell T. C., Chen S. C. A., Ruma P., Meyer W., Pfeiffer T. J., Ellis D. H., Brownlee A. G. (1996). Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *Journal of Clinical Microbiology* **34(12):** 1253-1260.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans. Revista Iberoamericana de Micologia* **14:** 42-43.

Steenbergen J. N., Casadevall A. (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes and Infection* **5:** 667-675.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Medical Mycology* **41(5):** 383-390.

van Heerden A., van Zyl W. H., Cruywagen C. W., Mouton M., Botha A. (2011). The lignicolous fungus *Coniochaeta pulveracea* and its interactions with syntrophic yeasts from the woody phylloplane. *Microbial Ecology* **62(3):** 609-619**.**

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier., 77-98.

CHAPTER 3

Identification and phylogenetic analyses of three putative cellulase genes within the genome of *Cryptococcus neoformans* var. *grubii*

Identification and phylogenetic analyses of three putative cellulase genes within the genome of *Cryptococcus neoformans* var. *grubii*

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

*Cryptococcus neoformans (*sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis and cryptococcal meningitis in immuno-suppressed individuals. Despite the repeated isolation of *C. neoformans* from a variety of different sources rich in lignocellulosic material as well as recent research indicating that this yeast is capable of using woody debris as a sole carbon and nitrogen sources the lignocellulosic physiology of this pathogen has not yet been identified. Thus, the objective of this study was to determine the presence of genes coding for wood degrading enzymes in the genome of *C. neoformans* var. *grubii* ATCC H99. Screening of the genome identified three such genes and their putative protein products, namely an endo-glucanase (EC7) and two cellulases (CC1 and CC6). Analysis of the amino acid sequences indicated that all three enzymes belong to the largest glycoside hydrolase family (GHF), namely GHF5. This family is noted for its large representation of cellulases and mannanases particularly from various fungal species. Phylogenetic analyses revealed that the three cryptococcal enzymes grouped in distinct clades with other GHF5 members and could be distinguished on the basis of conserved amino acid regions associated with known catalytic sites. Both EC7 and CC6 clustered with other basidiomycete and ascomycete species known to hydrolyze lignocellulose. The putative cellulase CC1, however, clustered with cellulases isolated from various prokaryotes known to be associated with woody environments. These results suggest that horizontal gene transfer (HGT) may have occurred between the human pathogen *C. neoformans* var. *grubii* and other microbes present within its ecological niche.

1. INTRODUCTION

Cryptococcus neoformans (sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis and cryptococcal meningitis in immuno-suppressed individuals (Casadevall *et al*., 2003; Franzot *et al*., 1997; Boekhout *et al*., 1997; Mitchell *et al*., 1995). It belongs to the order *Tremellales*, also known as the jelly fungi and is predominantly comprised of basidiomycetous yeasts (Boekhout *et al*., 2001; Fell *et al*., 2000). Two main varieties are known, namely *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), with a rare hybrid variety, *C. neoformans* (serotype AD) also reported (Boekhout *et al.*, 2001).

The two main variants, C. *neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, have a worldwide distribution and were originally thought to be associated primarily with avian guano or avian guano contaminated soil (Sorrell *et al*., 1997). However, both varieties have been isolated from a number of different habitats including soil, vegetative debris and particularly decaying wood and tree hollows (Randhawa *et al.*, 2011; 2008; 2006; 2000; Baroni *et al.*, 2006; Ergin *et al.*, 2004; Montagna *et al.*, 2003; Trilles *et al*., 2003; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996; López *et al.*, 1995; Bauwens *et al.*, 1986; Emmons, 1955; 1951). Recent research has demonstrated that this yeast is able to grow and complete its life cycle on woody debris in the absence of additional nutrients (Botes *et al*., 2009).

It has been known for decades that *C. neoformans* is able to assimilate the carbohydrate degradation products of woody material, namely arabinose, cellobiose, galactose, glucose, rhamnose and xylose (Kwon-Chung, 1998). Research described previously (Chapter 2) demonstrated that *C. neoformans* var. *grubii* ATCC H99 is able to grow in a medium containing carboxymethyl cellulose (CMC) as carbon source, assimilate mannose, and grow on complex hemi-cellulosic carbon sources rich in this hexose. However, nothing is known about the lignocellulosic physiology of this pathogen, including the enzymes responsible for the degradation of complex woody polymers.

The ability to utilize lignocellulose is distributed throughout the entire fungal kingdom (de Souza, 2013) however the basidiomycetes are often regarded as being the most potent wood degraders due to their ability to hydrolyze the complex polymer lignin (Eriksson *et al.*, 1990). Degradation of lignocellulose is achieved through the combination of various glycoside hydrolases (GHs) (de Souza, 2013; Lum *et al.*,

2011; Sanchez, 2009). These carbohydrate active enzymes hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety and include cellulases, mannanases, cellobiohydrolases and xylanases, to name a few.

Glycoside hydrolases are currently grouped into 131 families (Cantarel *et al.*, 2009) on the basis of their amino acid sequence (Hessrinat *et al.*, 1993; 1991). Glycoside hydrolase family 5 (GHF5) is one of the largest families and is noted for the inclusion of numerous endo-glucanase and endo-mannanase species from a variety of fungi. Other activities include 1,6-galactanase, 1,3-mannanase, 1,4-xylanase, endo-glycoceramidase, as well as high-specificity xyloglucanases. Cellulases have been isolated from a variety of organisms such as bacteria, plant and human fungal pathogens, insects, nematodes and more recently mollusks. Given the wide variety of cellulase producers it is no surprise that these enzymes are classified into a number of glycoside hydrolase families (GHF) including GHF5, 6, 7, 8, 9, 10, 12, 16, 18, 19, 26, 44, 45, 48, 51, 74, and 124.

With the above as background, the objective of this study was to screen the genome of *C. neoformans* var. *grubii* ATCC H99 and identify putative cellulase and/or mannanase encoding genes. The deduced amino acid sequences of these putative enzymes would then be used for phylogenetical classification into the appropriate glycoside hydrolase families.

2. MATERIALS AND METHODS

2.1 Phylogenetic Analysis

Putative cellulase amino acid sequences were obtained from the annotated genome of *C. neoformans* var. *grubii* available on the Broad Institute database (www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/). Closely related protein sequences were identified with BlastN and BlastP on the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov.). Selected cryptococcal amino acid sequences, their highest matches from the Blast analyses as well as some selected representatives from glycoside hydrolases were aligned for phylogenetic analyses using ClustalW (Thompson *et al.*, 1994). The phylogenetic tree was generated using MEGA5.2.1 (Tamura *et al.*, 2011) employing the Neighbour-Joining (NJ) method with 1000 bootstrap iterations. The amino acid

sequences of the identified cellulases as well as other glycoside hydrolase family 5 enzymes used to compile the phylogenetic tree are listed in Appendix A.

3. RESULTS AND DISCUSSION

By comparing the genome sequence of *C. neoformans* var. *grubii* (Broad Institute database) with known sequences on the NCBI database (www.ncbi.nlm.nih.gov.), we were able to identify three putative lignocellulose hydrolyzing proteins, namely an endo-glucanase (EC7) and two cellulases (CC1 and CC6). Based on their amino acid sequences, all three proteins are members of GHF5 (Hessrinat *et al.*, 1993; 1991). This classification was further confirmed by the construction of a Neighbour-Joining (NJ) phylogenetic tree (Tamura *et al.*, 2011) where all three proteins clustered with other known GHF5 members (Fig 1).

Glycoside hydrolase family 5 is the largest family and is noted for including numerous cellulases and mannanases originating from various fungal species (Hessrinat *et al.*, 1989). Members of GHF5 are all retaining enzymes that employ the Koshland double displacement mechanism (Barras *et al.*, 1992; Koshland., 1953) and possess the classical $(\alpha/\beta)_8$ TIM barrel fold (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). The two catalytic residues are conserved glutamic acids (Glu – E) located at the C-terminal ends of β-strands 4 (acid/base) and 7 (nucleophile) (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). ClustalW alignment (Thompson *et al.*, 1994) of the protein sequences allowed for the identification of both the acid/base and nucleophile catalytic glutamic acid residues for each putative protein (Table 1). The complete amino acid sequence for each protein as well as all the relevant amino acid abbreviations are available in Appendix A and B, respectively.

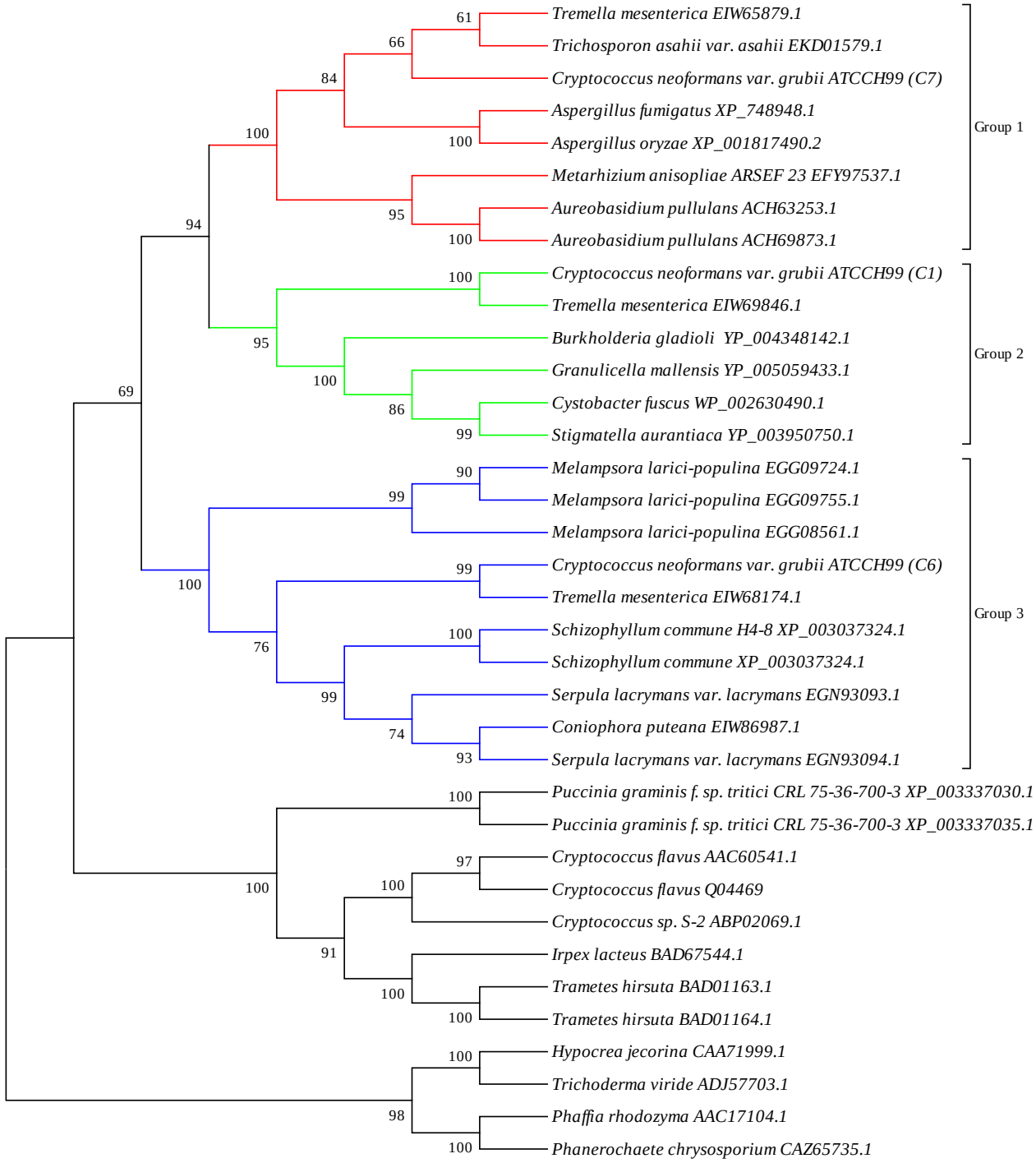

Figure 1 The phylogeny of cellulases within GHF5- based on their amino acid sequences. The evolutionary history was inferred using the Neighbour-Joining method (Saitou *et al.*, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei, 2000) and are in the units of the number of amino acid differences per site. The analysis involved 36 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 113 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.2 (Tamura *et al.*, 2011). The amino acid sequences of the identified cellulases are listed in Appendix A

Table 1 Conserved acid/base and nucleophile catalytic residues (glutamic acid – E) of the putative glycoside hydrolase family 5 enzymes identified within the genome of *C. neoformans* var. *grubii* ATCC H99. Amino acid sequence alignments were done using ClustalW (Thompson *et al.*, 1994). All amino acids and their relative abbreviations are listed in Appendix B.

Putative protein	**Conserved catalytic glutamic acid (E) residue**	
	Acid / Base	**Nucleophile**
CC1	578	644
CC6	215	312
EC7	203	284

3.1 *Cryptococcus neoformans* var. *grubii* - CC1

A gene encoding a putative cellulase (CC1) was identified within the *C. neoformans* var. *grubii* genome. The gene is located on chromosome one and is flanked by a gene coding for a hypothetical protein of unknown function and a gene coding for nicotinamidase (Fig 2) which is involved in the metabolism of nicotinate and nicotinamide (Petrack *et al*., 1965; Sarma *et al*., 1964).

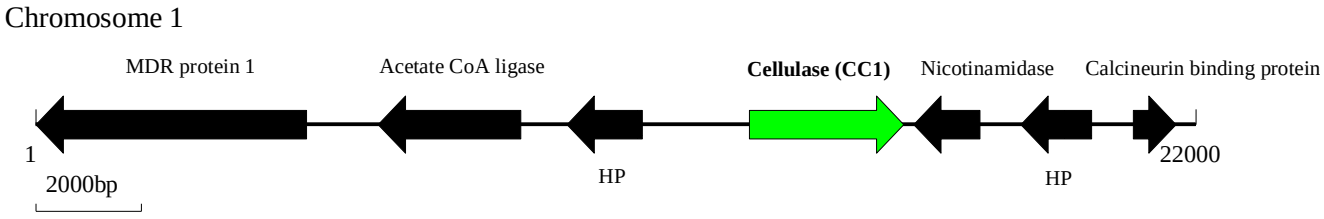

Figure 2 Genome map of the putative cellulase C1 (CC1) located on chromosome 1 of *C. neoformans* var. *grubii* ATCC H99. HP = Hypothetical protein.

The Neighbour-Joining tree of GHF5 cellulases revealed that proteins occurring within the Group 2 (Fig 1) displayed the least degree of conservation relative to Groups 1 and 3 with regards to both the acid/base and nucleophile catalytic sites. Only the active glutamic acid residue was conserved at the nucleophile catalytic site in Group 2, while the acid/base region displayed a conserved continuous sequence of only four amino acid residues, isoleucine-asparagine-glutamic acid-proline (I-N-E-P) (Fig 3).

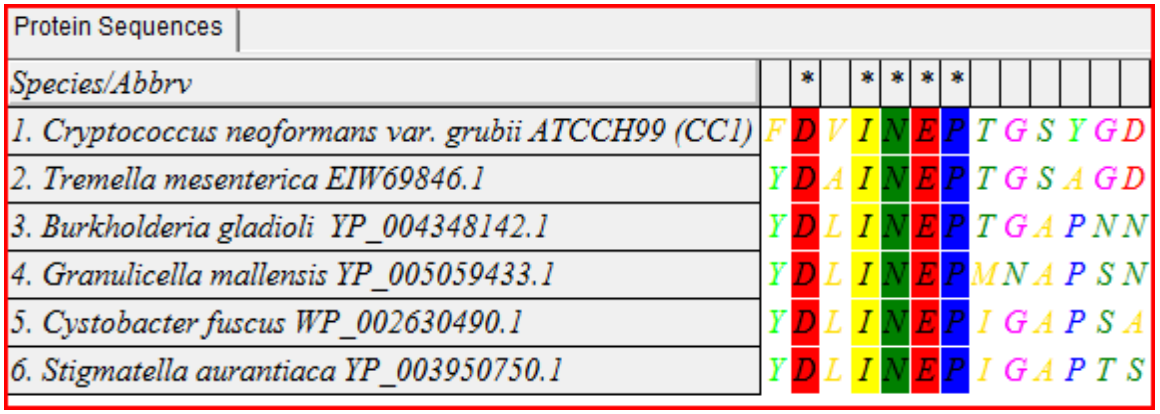

Figure 3 ClustalW alignment of amino acid sequences from Group 2 conducted using MEGA 5.2.1 (Tamura *et al.*, 2011) showing limited sequence conservation with regard to the acid/base catalytic region. The conserved glutamic acid (E) residue is known to be the active residue (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). All amino acids and their relative abbreviations are listed in Appendix B.

Phylogenetic analyses revealed that although CC1 was closely related to a cellulase isolated from *Tremella mesenterica*, the protein also grouped with a number of cellulases originating from prokaryotic genera, such as *Burkholderia*, *Cystobacter* and *Stigmatella*.

Members of the genus *Burkholderia* are known to be very active in the recycling of organic material (Stoyanova *et al.*, 2007). *Burkholderia gladioli* is recognized as both a plant pathogen and human pathogen usually associated with patients suffering from cystic fibrosis (Vandamme *et al.*, 2007; Graves *et al.*, 1997). *Cystobacter* and *Stigmatella* belong to the order *Myxococcales* and are known for their swarming predatory behaviour towards both bacterial and yeast species (Dworkin, 1996; Reichenbach *et al.*, 1992; Dworkin, 1985; Burchard *et al.*, 1977). *Stigmatella aurantiaca* is found on rotting wood and is known to produce enzymes capable of hydrolyzing a wide selection of peptidoglycans, polysaccharides, proteins and other cellular detritus (Reichenbach *et al.*, 1992; Singh *et al.*, 1971).

The jelly fungus, *T. mesenterica*, is closely related to *C. neoformans* according to both D1/D2 and ITS sequence analysis (Fell *et al.*, 2000). Similar to *C. neoformans*, the life-cycle of *T. mesenterica* has both an asexual yeast phase and a sexual filamentous phase (Bandoni, 1965). *Tremella* species, however, are not known as wood degraders, but rather act as parasites on other wood degrading fungi such as members of the genus *Peniophoro* (Bandoni, 1995; Zugmaier et al., 1994).

Given the common ecological habitat of these microbes, the possibility of horizontal gene transfer (HGT) events seems plausible (Mayer *et al.,* 2011, Dunning Hotopp *et al.,* 2007). Horizontal gene transfer involves the movement of genetic material from one organism to another by mechanisms other than inheritance. The phenomenon is wide spread amongst prokaryotes, but regarded as rare in eukaryotes given their ability to recombine genetic material during meiosis. However, it has been demonstrated that bacterial and fungal cellulases have been incorporated into the genome of plant-parasitic nematodes and termites via HGT (Dunning Hotopp *et al.,* 2007; Jones *et al.,* 2005). Interestingly, phylogenetic analysis of these acquired cellulases indicates that HGT occurred between several distinct bacterial donor species and the plant parasitic nematodes (Mayer *et al.,* 2011; Jones *et al.,* 2005). This would suggest that the event of HGT amongst prokaryotes and eukaryotes is more common than previously believed.

Further analysis of the amino acid sequence of CC1 revealed the presence of a ricin-type B lectin (Fig 4), which belongs to the R-type lectin family (Cummings *et al.,* 2009), as well as putative sugar binding sites (Fig 5). Ricin is a naturally occurring plant lectin (carbohydrate binding protein) that is poisonous when inhaled or ingested (Lord *et al.,* 2005; Rauber *et al.,* 1985; Olsnes *et al.,* 1973). Structural analysis revealed that ricin is a hetero-dimer comprised of two chains, ricin A chain (RTA) and ricin B chain (RTB). The ricin A chain is responsible for the deactivation of the 60s subunit of ribosomal ribonucleic acid (rRNA) found within eukaryotes, thereby inhibiting protein synthesis (Weston *et al.,* 1994).

The ricin B chain is comprised of three homologous sub-domains believed to have arisen as a result of gene triplication from a primitive galactose binding protein and is common in bacteria, animals and plants, while only a few have been identified in fungi (Candy *et al.,* 2001). The most characteristic feature of this domain is the presence of a $(QxW)_3$ (glutamine – any residue – tryptophan) motif, although some other amino acid preferences do exist. The RTB is able to bind to terminal galactose residues and mannose receptors on the cell surface (Wales *et al.,* 1991).

Currently the R-type domain has been placed within the carbohydrate binding module (CBM) family 13 on the basis of its amino acid sequence (Cummings *et al.,* 2009; Tomme *et al.,* 1995). Interestingly, similar ricin-type B lectins have also been observed within the genomes of the *Burkholderia pseudomallei* (Winsor *et al.,* 2008) and *S. aurantiaca* (Huntely *et al.,* 2011).

The association of a carbohydrate binding protein with a cellulase is not uncommon (Gilkes *et al.*, 1998; Gilkes *et al.*, 1988; Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986). Cellulose binding domains (CDBs) were first identified in the 1940s (Reese *et al.*, 1950), however, the continued isolation of carbohydrate active enzymes that did not bind cellulose led to their renaming as carbohydrate binding modules (CBMs) (Boraston *et al.*, 1999). Similar to glycoside hydrolases (GHs), CBMs are grouped according to their amino acid sequence (Tomme *et al.*, 1995) and there are currently 67 CBM families (Cantarel *et al.*, 2009). Additionally, CBMs are also grouped according to their tree-dimensional structure (seven families) as well as their type of sugar recognition (three types). Carbohydrate binding modules are usually located at either the C- or N-terminal, but some have been shown to be located centrally within the protein (Tomme *et al.*, 1995). Carbohydrate binding modules are thought to serve as an advantage to the enzymes as they bind to the substrate and thereby prolong the association between the enzyme and substrate increasing the rate of hydrolysis (Teeri *et al.*, 1998; Tomme *et al.*, 1998; Linder *et al.*, 1997). This notion is strengthened by studies demonstrating that removal of the CBM from certain enzymes dramatically decrease enzyme activity (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986).

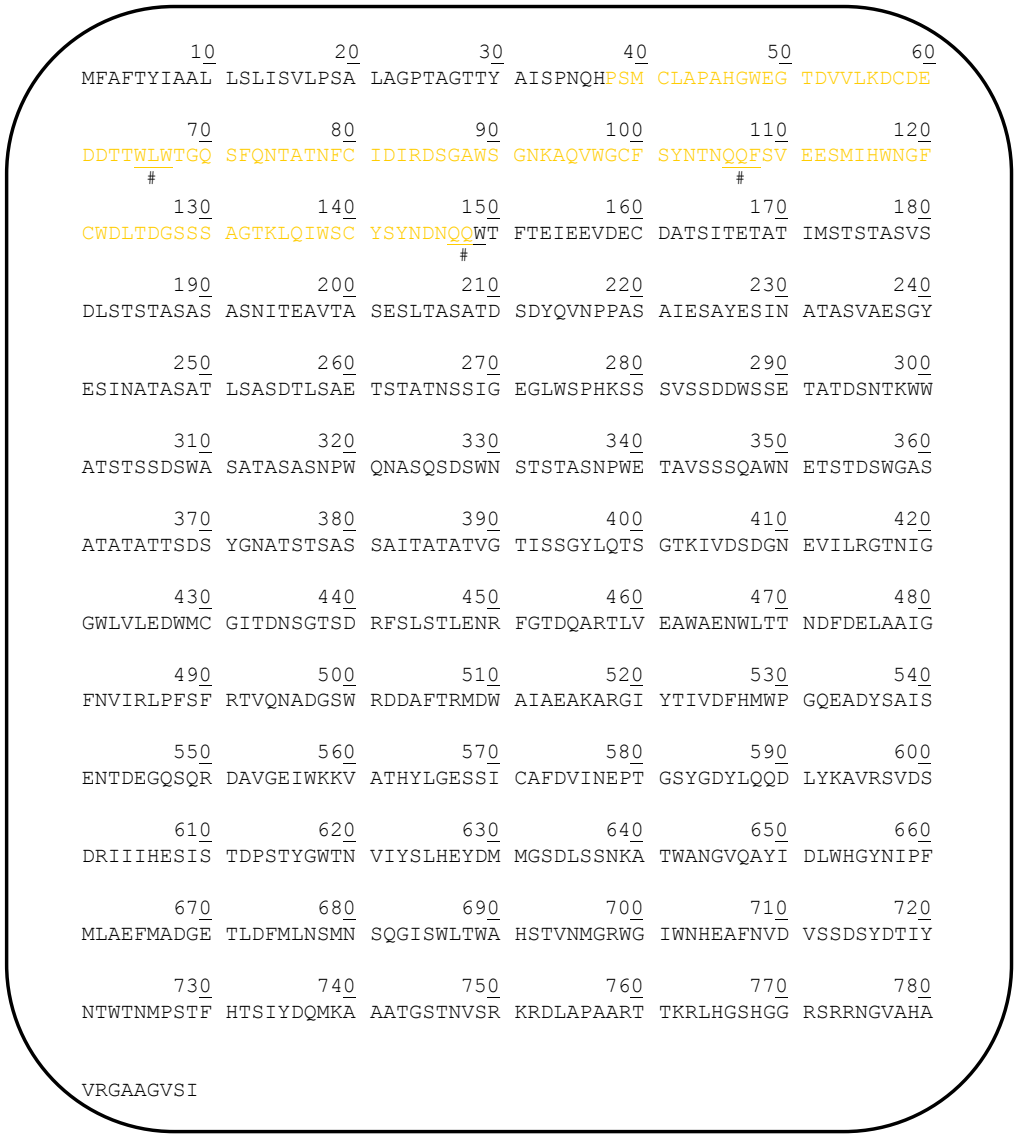

Figure 4 Amino acid sequence of CC1 highlighting the ricin B chain (RTB – yellow) and the classic $(QxW)_3$ motif (# and underlined). All amino acids and their relative abbreviations are listed in Appendix B.

Figure 5 Amino acid sequence of CC1 highlighting the putative sugar binding residues (red). All amino acids and their relative abbreviations are listed in Appendix B.

Until recently, lectins and CBMs were considered to be distinct groups (Boraston *et al.*, 2004). Lectins are generally referred to as non-enzymatic proteins that are specific for saccharide motifs in glycans or glycoproteins, while CBMs are regarded as being associated with polysaccharide-degrading enzymes. On-going research has demonstrated that this distinction is becoming blurred. It was found that CBMs of bacterial xylanases (CBM13) display similarity to the classical β-trefoil lectins and are capable of binding xylose (Fujimoto *et al.*, 2002). Similarly, family 9 CBMs have a lectin-like specificity for monosaccharides and display a β-sandwich fold characteristic of many lectins produced by eukaryotes. It therefore seems plausible that the ricin-type B lectin, containing the R-type domain, identified in the CC1 amino acid sequence may perform the role of a CBM by binding to the mannose or galactose residues present within the hemi-cellulose fraction of woody material.

3.2 *Cryptococcus neoformans* var. *grubii* – CC6

A gene encoding a putative cellulase (CC6) was identified within the *C. neoformans* var. *grubii* genome. The gene is located on chromosome six and is flanked by two hypothetical proteins of unknown function (Fig 6).

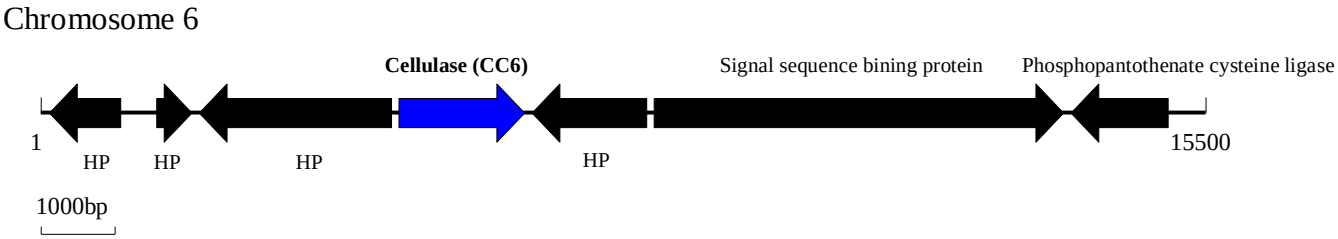

Figure 6 Genome map of the putative cellulase C6 (CC6) located on chromosome 6 of *C. neoformans* var. *grubii* ATCC H99. HP = Hypothetical protein.

Sequences within the CC6 group (Fig 1; Group 3) displayed an increased level of amino acid sequence conservation when compared to the CC1 group. Both the acid/base and nucleophile catalytic regions displayed a conserved continuous sequence of five, leucine-asparagine-glutamic acids-proline-alanine (L-N-E-P-A), and two, glycine-glutamic acid (GE), amino acid residues respectively (Figs 7 and 8).

Similar to CC1, phylogenetic analysis revealed that CC6 was closely related to a cellulase isolated from *T. mesenterica* (Fig 1). Included in this group were other well-known wood degrading basidiomycetes, namely *Coniophora puteana*, *Melampsora*

larici-populina, *Schizophyllum commune* and *Serpula lacrymans* var. *lacrymans*. All four fungi are known to occur in woody environments and display varying degrees of lignocellulosic hydrolysis (Floudas *et al.*, 2012; Xhaard *et al.*, 2011; Ohm *et al.*, 2010; Kirk *et al.*, 2008; Schmidt, 2006; Palfreyman, 2001; Guarro *et al.*, 1999; Cooke, 1961). *Coniophora puteana* is regarded as a brown rot fungus and produces cellulases belonging to GHF6 and 7 (Floudas *et al.*, 2012). *Melampsora larici-populina* is classified as a rust fungus and is the most devastating pathogen in poplar plantations grown for the pulp industry (Xhaard *et al.*, 2011). *Serpula lacrymans* is considered the most damaging degrader of wooden construction materials despite its ability to only hydrolyze cellulose (Schmidt, 2006). The white rot fungus, *S. commune* has been isolated on every continent, except Antarctica, and is known as a potent wood degrader (Ohm *et al.*, 2010; Schmidt, 2006; Guarro *et al.*, 1999; Cooke, 1961). Interestingly, *S. commune* is known to produce a number of medically important compounds; such as Schizophyllan that is known to possess anti-cancer properties (Kony *et al.*, 2007); however, this filamentous fungus is one of the few basidiomycetes known to cause severe infections in immune-competent individuals (Guarro *et al.*, 1999).

Protein Sequences

Species/Abbrv	*	*	*	*	*						
1. Cryptococcus neoformans var. grubii ATCCH99 (CC6)	L	L	N	E	P	A	T	Y	L	N	-	-
2. Tremella mesenterica EIW68174.1	L	L	N	E	P	A	T	Y	L	N	-	-
3. Schizophyllum commune H4-8 XP_003037324.1	P	L	N	E	P	A	G	F	H	G	-	-
4. Schizophyllum commune XP_003037324.1	P	L	N	E	P	A	G	F	H	G	-	-
5. Serpula lacrymans var. lacrymans EGN93093.1	P	L	N	E	P	A	G	F	D	G	-	-
6. Coniophora puteana EIW86987.1	P	L	N	E	P	A	G	Y	D	G	-	-
7. Serpula lacrymans var. lacrymans EGN93094.1	P	L	N	E	P	A	G	Y	D	G	-	-
8. Melampsora larici-populina EGG08561.1	S	L	N	E	P	A	G	F	A	N	-	-
9. Melampsora larici-populina EGG09724.1	A	L	N	E	P	A	G	F	A	N	D	G
10. Melampsora larici-populina EGG09755.1	A	L	N	E	P	A	G	F	A	N	D	G

Figure 7 ClustalW alignment of amino acid sequences from group 3 conducted using MEGA 5.2.1 (Tamura *et al.*, 2011) showing sequence conservation with regard to the acid/base catalytic region. The conserved glutamic acid (E) residue is known to be the active residue (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). All amino acids and their relative abbreviations are listed in Appendix B.

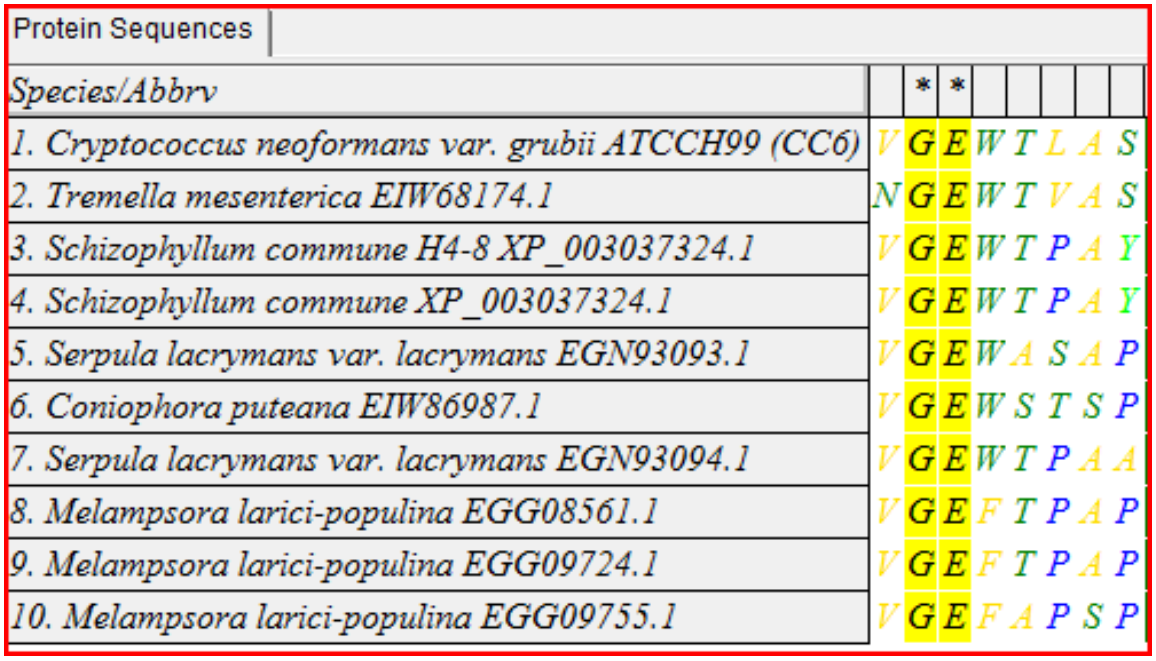

Figure 8 ClustalW alignment of amino acid sequences from group 3 conducted using MEGA 5.2.1 (Tamura *et al.*, 2011) showing sequence conservation with regard to the nucleophile catalytic region. The conserved glutamic acid (E) residue is known to be the active residue (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). All amino acids and their relative abbreviations are listed in Appendix B.

3.4 *Cryptococcus neoformans* var. *grubii* – EC7

A gene encoding a putative endo-glucanase (EC7) was identified within the *C. neoformans* var. *grubii* genome. The gene is located on chromosome seven and is flanked by an ubiquitin-conjugating enzyme and a hypothetical protein of unknown function (Fig 9). Ubiquitination involves the post-translation modification of eukaryotic proteins via a small regulatory protein, ubiquitin (Wilkinson *et al.*, 2005; Pickart *et al.*, 2004; Goldstein et al., 1975), which targets the protein for degradation via the proteasome. Ubiquitin-conjugating enzymes (E2 enzymes) catalyze the second step of this three-step process. These enzymes are characterized by their approximately 150 amino acid core that exhibit at least 25 % sequence identity across the eukaryotic kingdom.

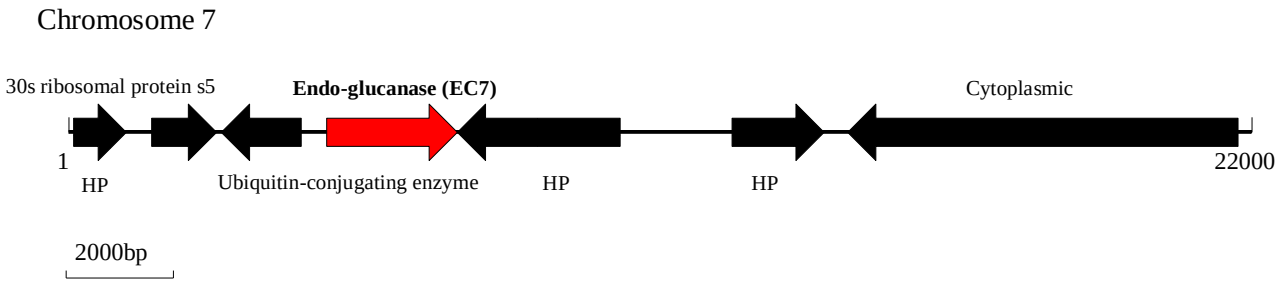

Figure 9 Genome map of the putative endo-glucanse 7 (EC7) located on chromosome 7 of *C. neoformans* var. *grubii* ATCC. HP = Hypothetical protein.

Sequences within the Group 1 (Fig 1) displayed the greatest degree of amino acid sequence conservation when compared with Groups 2 and 3 with regards to the catalytic sites. The acid/base site displayed a conserved continuous sequence of nine amino acid residues, alanine- glycine- tyrosine- asparagine- proline- leucine- asparagine- glutamic- acid-proline (A-G-Y-N-P-L-N-E-P) (Fig 10), while the nucleophile catalytic site was composed of a conserved six amino acid region, glycine-glutamic acid-phenylalanine-glycine-proline-valine (G-E-F-G-P-V) (Fig 11). These highly conserved amino acid regions may be an indication of increased substrate specificity (Wu *et al.*, 2009; Shmara *et al.*, 2001).

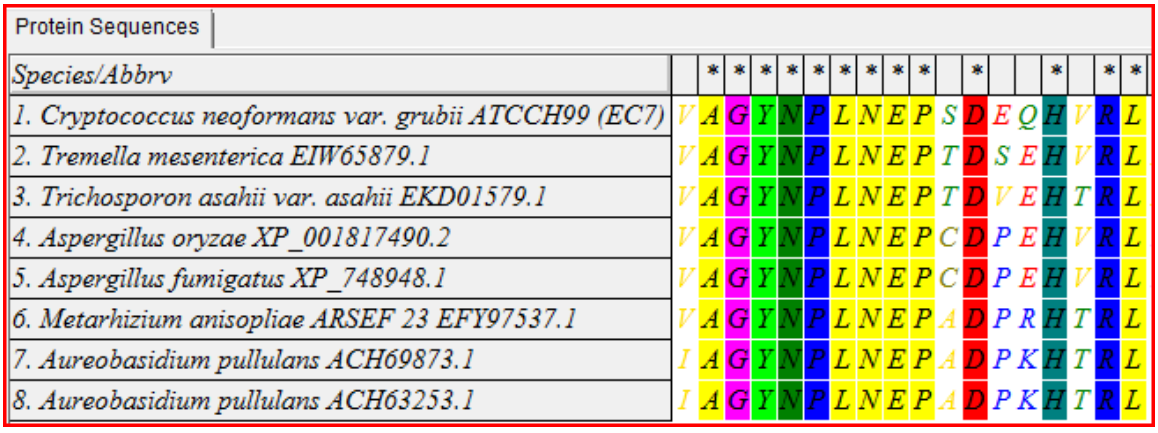

Figure 10 ClustalW alignment of amino acid sequences from Group 1 conducted using MEGA 5.2.1 (Tamura *et al.*, 2011) showing sequence conservation with regard to the acid/base catalytic region. The conserved glutamic acid (E) residue is known to be the active residue (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). All amino acids and their relative abbreviations are listed in Appendix B.

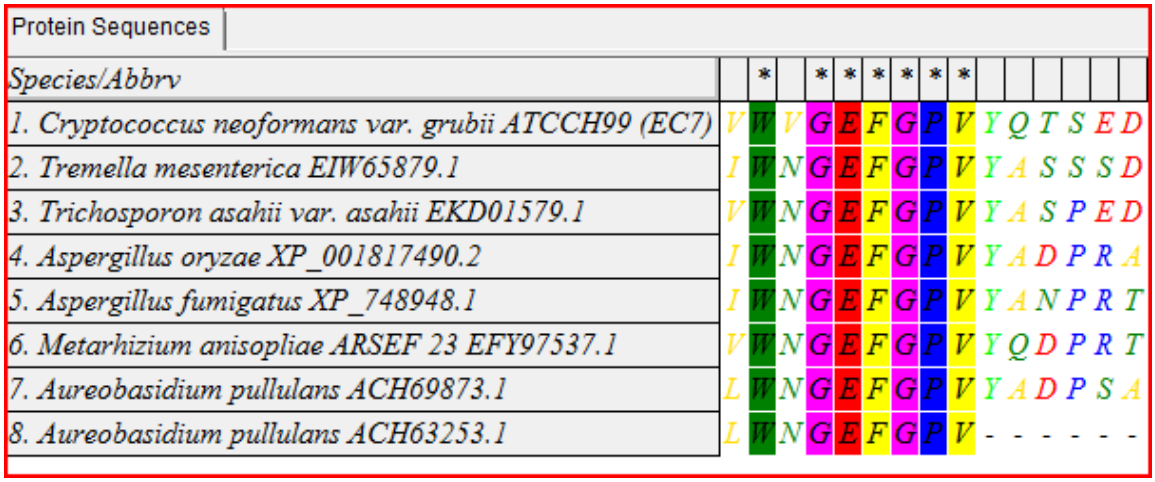

Figure 11 ClustalW alignment of amino acid sequences from Group 1 conducted using MEGA 5.2.1 (Tamura *et al.*, 2011) showing sequence conservation with regard to the nucleophile catalytic region. The conserved glutamic acid (E) residue is known to be the active residue (Hessrinat *et al.*, 1996; Jenkins *et al.*, 1995). All amino acids and their relative abbreviations are listed in Appendix B.

Phylogenetic analysis revealed that EC7 was closely related to cellulases isolated from the basidiomycetes *T. mesenterica* and *Trichosporon asahii* var. *asahii* (Fig 1) however, Group 1 appears to be dominated by cellulases originating from ascomycetes such as *Aspergillus fumigatus, A. oryzae, Aureobasidium pullulans* and *Metarhizium anisopliae*. Both *Aspergillus* and *Aureobasidium* are known as wood degraders and are found in soil and woody environments (Osono, 2008; de Hoog *et al.*, 2000; Kwon-Chung *et al.*, 1992; Federici, 1982; Stewart *et al.*, 1981). *Trichosporon* is an anamorphic genus known to occur in soil and form part of the normal skin microbiotia of humans (Kwon-Chung *et al.*, 1992). Interestingly, only the insect pathogen *M. anisopliae* and the fungal parasite *T. mesenterica* have not been associated with diseases in immune-comprised individuals (Freimoser *et al.*, 2003; de Hoog *et al.*, 2000; Kwon-Chung *et al.*, 1992). Both *A. fumigatus* and *A. pullulans* are associated with pulmonary complications (Tan *et al.*, 1997; de Hoog *et al.*, 2000; Kwon-Chung *et al.*, 1992); and are capable of dissemination throughout the human body (Mershon-Shier *et al.*, 2011; Ben-Ami *et al.*, 2010; Bolignano *et al.*, 2003; de Hoog *et al.*, 2000; Kwon-Chung *et al.*, 1992). Similar to *C. neoformans, T. asahii* var. *asahii* is one of only a few basidiomycetous species capable of causing systemic as well as subcutaneous infections in immune-compromised individuals (Walsh *et al.*, 1992; Watson *et al.*, 1970).

4. CONCLUSIONS

We have confirmed the presence of genes encoding for three putative glycoside hydrolases within the genome of *C. neoformans* var. *grubii* ATCC H99. Phylogenetic analysis of the amino acid sequences revealed that the putative enzymes, namely an endo-glucanase (EC7) and two cellulases (CC1 and CC6), grouped into separate clusters with other known GHF5 members. These clusters were mostly comprised of cellulases produced by known cellulolytic fungi frequently isolated from woody material. Not surprisingly, all three putative enzymes displayed a strong association with other GHF5 enzymes from the closely related jelly fungus *T. mesenterica* (Fig 1) (Fell *et al.,* 2000) and thereby strengthen the notion that *Filobasidiella* should indeed be grouped within the *Tremellales* (Scorzetti *et al.,* 2002).

As mentioned previously, GHF5 is the largest glycoside hydrolase family (Hessrinat *et al.,* 1989) and the catalytic regions, all containing glutamic acid (Glu – E), are conserved (Hessrinat *et al*., 1996; Jenkins *et al*., 1995). The amino acid sequence alignments allowed for the identification of these conserved catalytic sites for each of the three proteins (Table 1). This could help predict the function of the protein as the three-dimensional structure, mechanism employed, catalytic machinery as well as the molecular mechanisms involved is conserved in the majority of GHFs (Davies *et al.,* 1995; Henrissat *et al.,* 1995; Gebler *et al.,* 1992).

The putative enzyme CC1 appears to possess a possible carbohydrate binding module (CBM). The ricin-type B lectin identified within the protein sequence of CC1 is known to contain an R-type domain capable of binding galactose, mannose or xylose and is grouped in CBM family 13.

Interestingly, all three GHs identified in *C. neoformans* var. *grubii* ATCC H99 grouped with other GHs from human pathogens known to cause respiratory diseases (Fig 1). Most fungal infections are opportunistic and often pathogenic traits are required for survival within the environment (Moran *et al.,* 2011; Casadevall *et al.,* 2003). A variety of "dual-use" virulence factors have been identified in *C. neoformans* and include the enzymes laccase, phospholipase and urease (Casadevall *et al.,* 2003; Zhu *et al*., 2001; *Cox et al.,* 2000; Liu *et al*., 1999). It would therefore be interesting to determine if the identified cellulases of *C. neoformans* var. *grubii* would aid the yeast in terms of its pathogenicity and survival in the human body.

Although we have confirmed the presence of genes encoding for putative glycoside hydrolases in the genome of *C. neoformans* var. *grubii* ATCC H99, little is known about their chemical and physical properties, as well as transcriptional regulation. Knowledge of these aspects would greatly enhance our understanding of the interactions of this fungus in its natural environment and will be discussed in the next chapters of this dissertation.

5. REFERENCES

Bandoni R. J. (1965). Secondary control of conjugation in *Tremella mesenterica*. *Canadian Journal of Botany* **43 (6):** 627–30.

Bandoni R. J. (1995). Dimorphic heterobasidiomycetes: taxonomy and parasitism. *Studies in Mycology* **38:** 13-27.

Barras F., Bortoli-German I., Bauzan M., Rouvier J., Gey C., Heyraud A., Henrissat B. (1992). Stereochemistry of the hydrolysis reaction catalyzed by endoglucanase Z from *Erwinia chrysanthemi***.** *Federation of European Biochemical Societies Letters* **30(2):** 145-148.

Bauwens L., Swinne D., De Vroey C., De Meurichy W. (1986). Isolation of *Cryptococcus neoformans* var. neoformans in the aviaries of the Antwerp Zoological Gardens. *Mykosen* **29:** 291–294.

Ben-Ami R., Lewis R. E., Kontoyiannis D. P. (2010). Enemy of the immunosuppressed state: an update on the pathogenesis of *Aspergillus fumigatus* infection. *British Journal of Haematology* **150 (4):** 406-417.

Boekhout T, van Belkum A., Leenders A. C. A. P., Verbrugh H., Mukamurangwa P., Swinne D., Scheffers W. A. (1997). Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *International Journal of Systematic Bacteriology* **47(2):** 432-442.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans. Microbiology* **147:** 891–907.

Bolignano G., Criseo G. (2003). Disseminated nosocomial fungal infection by *Aureobasidium pullulans* var. *melanigenum*: a case report. *Journal of Clinical Microbiology* **41(9):** 4483-4485.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Boraston A. B., McLean B. W., Kormos J.M., Alam M., Gilkes N. R., Haynes C. A., Tomme P., Kilburn D. G., Warren R. J. A. (1999). Carbohydrate-binding modules: diversity of structure and function. In: Gilbert H. J., Davies G. J., Henrissat B., Svensson B. (eds.) *Recent advances in carbohydrate bioengineering*. Cambridge: The Royal Society of Chemistry, 201-211.

Boraston A. B., Bolam D. N., Gilbert H. J., Davies G. J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochemical Journal* **382:** 769-781.

Burchard A. C., Burchard R. P. and Kloetzel J. A. (1977). Intracellular, periodic structures in the gliding bacterium *Myxococcus xanthus*. *Journal of Bacteriology* **132:** 666-672.

Candy L., Peumans W. J., Menu-Bouaouiche L., Astoul C. H., Van Damme J., Van Damme E. J., Erard M., Rouqé P. (2001). The gal/GalNAc-specific lectin from the plant pathogen basidiomycete *Rhizoctonia solani* is a member of the ricin-B family. *Biochemical and Biophysical Research Communications* **282:** 655–61.

Cantarel B. L., Coutinho P. M., Rancurel C., Bernard T., Lombard V., Henrissat B (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research* **37:** 233-238.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready-made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Cooke W. B. (1961). The genus *Schizophyllum*. *Mycologia* **53(6):** 575-599.

Cox G. M., Mukherjee J., Cole G. T., Casadevall A., Perfect J. R. (2000). Urease as a virulence factor in experimental cryptococcosis. *Infection and Immunity* **68:** 443–448.

Cummings R. D., Etzler M. E. (2009). R-type Lectins. In: Varki A., Cummings R. D., Esko J. D. (eds.*) Essentials of Glycobiology (2nd edn).* New York: Cold Spring Harbor Laboratory Press, Chapter 28.

Davies G., Henrissat B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure* **3(9):** 853-859.

de Hoog G. S., Guarro J. (2000). Atlas of clinical fungi, 2nd edn. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

de Souza W. R. (2013). Microbial degradation of lignocellulosic biomass In: Chandel A. K., da Silva S. S. (eds.) *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization.* New York: InTech, Chapter 9.

Dunning Hotopp J. C., Clark M. E., Oliveira D. C..S. G., Foster J. M., Fischer P., Muñoz Torres M. C., Giebel J. D., Kumar N., Ishmael N., Wang S., Ingram J., Nene R. V., Shepard J., Tomkins J., Richards S., Spiro D. J., Ghedin E., Slatko B. E., Tettelin H., Werren J. H. (2007). Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science* **317:** 1753-1756.

Dworkin M. (1985). The Myxobacteria. In: Dworkin M. (ed.) *Development biology of the bacteria.* California: The Benjamin/Cummings Publishing Company, Inc., 123-127.

Dworkin M. (1996). Recent advances in the social and developmental biology of the myxobacteria. *Microbiological Reviews* **60:** 70–102.

Emmons C. W. (1951). Isolation of *Cryptococcus neoformans* from soil. *Journal of Bacteriology* **62:** 685–690.

Emmons C. W. (1955). Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (Columba livia). *American Journal of Hygiene* **62:** 227–232.

Ergin C., Ilkit M., Kaftanoglu O. (2004). Detection of *Cryptococcus neoformans* var. *grubii* in honeybee (*Apis mellifera*) colonies. *Mycoses* **47(9-10):** 431-434.

Eriksson K. E. L., Blanchette R. A., Ander P. (1990). Microbial and enzymatic degradation of wood components. Berlin: Springer.

Fell J. W., Boekhout T., Fonseca A., Scorzetti G., Statzell-Tallman A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domaim sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* **50:** 1351–1371.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783-791.

Floudas D., Binder M., Riley R., Barry K., Blanchette R. A., Henrissat B., Martínez A. T., Ortillar R., Spatafora J. W., Yadav J. S., Aerts A., Benoit I., Boyd A., Carlson A., Copeland A., Coutinho P. M., de Vries R. P., Ferreira P., Findley K., Foster B., Gaskell J., Glotzer D., Górecki P., Heitman J., Hesse C., Hori C., Igarashi K., Jurgens J. A., Kallen N., Kersten P., Khajamohiddin A., Kohler A., Kues U., Kumar T. K. A., Kuo A., LaButti K., Larrondo L. F., Lindquist E., Ling A., Lucas S., Lundell T., Martin R., McLaughlin D. J., Morgenstern I., Morin E., Murat C., Nolan M., Ohm R. A., Patyshakuliyeva A., Rokas A., Ruiz-Dueñas F. J., Sabat G., Salamov A., Samejima M., Schmutz J., Slot J. C., John F. S., Stenlid J., Sun H., Sun S., Tsang A., Wiebenga A., Young D., Pisabarro A., Eastwood D. C., Martin F., Cullen D., Grigoriev I. V.,

Hibbett D. S. (2012). The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science*. **336(6089):** 1715-1719.

Federici F. (1982). Extracellular enzymatic activities in *Aureobasidium pullulans*. *Mycologia* **74(5):** 738-743.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *Journal of Clinical Microbiology* **35(9):** 2243-2251.

Freimoser F. M., Screen S., Bagga, S., Hu G., St. Leger, R. J. (2003). EST analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* **149:** 239-247.

Fujimoto Z., Kuno A., Kaneko S., Kobayashi H., Kusakabe I., Mizuno H. (2002). Crystal structures of the sugar complexes of *Streptomyces olivaceoviridis* E-86 xylanase: sugar binding structure of the family 13 carbohydrate binding module. *Journal of Molecular Biology* **36(1):** 65-78.

Gebler J., Gilkes N. R., Claeyssens M., Wilson D. B., Béguin P., Wakarchuk W. W., Kilburn D. G., Miller R. C. Jr, Warren R. A., Withers S. G. (1992). Stereoselective hydrolysis catalyzed by related beta-1,4-glucanases and beta-1,4-xylanases. *Journal of Biological Chemistry* **267(18):** 12559-12561.

Gilkes N. R., Warren R. A., Miller R. J., Kilburn D. G. (1988). Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *Journal of Biological Chemistry* **263:** 10401–10407.

Gilkes N. R., Haynes C. A., Warren R. A, Kilburn D G. (1998). Characterization and affinity applications of cellulose-binding domains. *Journal of Chromatography B* **715:** 283–296.

Goldstein G., Scheid M., Hammerling U., Schlesinger D. H., Niall H. D., Boyse E. A. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proceedings of the National Academy of Science of the United States of America **72(1):** 11–5.

Graves M., Robin T., Chipman A. M., Wong J., Khashe S., Janda J. M. (1997). Four additional cases of *Burkholderia gladioli* infection with microbiological correlates and review. *Clinical Infectious Disease* **25(4):** 838-842.

Guarro J., Gené J; Stchigel A. M. (1999). Developments in fungal taxonomy. *Clinical Microbiology Reviews* **12(3):** 454–500.

Henrissat B., Claeyssens M., Tomme P., Lemesle L., Mornon J. P. (1989). Cellulase families revealed by hydrophobic cluster analysis. *Gene **81(1):** 83-95.*

Henrissat B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 309–316.

Henrissat B., Bairoch A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781–788.

Henrissat B., Callebaut I., Fabrega S., Lehn P., Mornon J. P., and Davies G. (1996). Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proceedings of the National Academy of Science of the United States of America* **93(11):** 5674.

Huntely S., Hamann N. Wegener-Feldbruegge S., Treuner-Lange A., Kube M., Reinhardt R., Klages S., Meuller R., Ronning C. M., Nierman W. C., Sogaard-Anderson L. (2011). Evolution of fruiting body formation in Myxococcales: a comparative genomic analysis. *Molecular Biology and Evolution* **28(2):** 1083-1097.

Jenkins J., Lo Leggio L., Harris G., and Pickersgill R. (1995). Beta-glucosidase, beta-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold beta/alpha architecture and with two conserved glutamates near the carboxy-terminal ends of beta-strands four and seven. *Federation of European Biochemical Societies Letters* **362(3):** 281-285.

Jones J. T., Furlanetto C., Kikuchi T. (2005). Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes. *Nematology* **7:** 641-646.

Kirk P. M., Cannon P. F., Minter D. W., Stalpers J. A. (2008). Dictionary of the Fungi (10th edn) Wallingford: CAB International.

Kony D. B., Damm W., Stoll S., van Gunsteren W. F., Hünenberger P. H. (2007). Explicit-solvent molecular dynamics simulations of the polysaccharide schizophyllan in water. *Biophysical Journal* **93:** 442–455.

Koshland D. E. (1953). Stereochemistry and the mechanism of enzymatic reactions. *Biological Reviews of the Cambridge Philosophical Society* **28:** 416–436.

Kwon-Chung K. J., Bennett J. E. (1992). *Medical mycology*. Philadelphia: Lea & Febiger.

Kwon-Chung K. J. (1998). *Filobasidiella* Kwon-Chung. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier Science BV, 656-662.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

Linder M., Margolles-Clark E., Reinikainen T., Teeri T. T. (1997). *Trichoderma reesei* cellobiohydrolase I with an endoglucanase cellulose binding domain: action on bacterial microcrystalline cellulose. *Journal of Biotechnology* **57:** 49–57.

Liu L., Tewari R. P., Williamson P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity* **67(11):** 6034-6039.

López-Martínez R., Castañón-Olivares L. R. (1995). Isolation of *Cryptococcus neoformans* var. *neoformans* from bird droppings, fruits and vegetables in Mexico City. *Mycopathologia* **129(1):** 25-28

Lord M. J., Roberts L. M. (2005). Ricin: structure, synthesis, and mode of action. In: Raffael S., Schmitt M. (eds.) *Microbial Protein Toxins. Topics in Current Genetics 11*. Berlin: Springer, 215–233.

Lum G., Min X. J. (2011). FunSecKB: the Fungal Sectretome KnowlegeBase. *Database - the Journal of Biological Databases and Curation.* **2011:** doi: 10.1093/database/bar001.

Mayer W. E., Schuster L. N., Bartelmes G., Dieterich C., Sommer R. J. (2011). Horizontal gene transfer of microbial cellulases into nematode genomes is associated with functional assimilation and gene turnover. *Biomedical Central Evolutionary Biology* **11:** 13-23.

Mershon-Shier K. L., Deville J. G., Delair S., Fothergill A. W., Wickes B., De Hoog G. S., Sutton D. A., Lewinski M.A. (2011). *Aureobasidium pullulans* var. *melanigenum* fungemia in a pediatric patient. *Medical Mycology* **49(1):** 80-3.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* **8(4):** 515-548.

Montagna M. T., Santacroce M. P., Caggiano G., Tatò D., Ajello L. (2003). Cavernicolous habitats harbouring *Cryptococcus neoformans*: results of a speleological survey in Apulia, Italy, 1999-2000. *Medical Mycology* **41(5):** 451-455.

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. dissertation, University of Delhi, Delhi, India.

Nei M. and Kumar S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Ohm R. A., de Jong J.F., Lugones L. G., Aerts A., Kothe E., Stajich J. E., de Vries R. P., Record E., Levasseur A., Baker S. E., Bartholomew K. A., Coutinho P. M., Erdmann S., Fowler T. J., Gathman A. C., Lombard V., Henrissat B., Knabe N., Kües U., Lilly W. W., Lindquist E., Lucas S., Magnuson J. K., Piumi F., Raudaskoski M., Salamov A., Schmutz J., Schwarze F. W., van Kuyk P. A., Horton J. S., Grigoriev I. V., Wösten H. A. (2010). Genome sequence of the model mushroom *Schizophyllum commune*. *Nature Biotechnology* **28(9):** 957–63.

Olsnes S., Pihl A. (1973). Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. *Biochemistry* **12(16):** 3121–6.

Osono T. (2008). Endophytic and epiphytic phyllosphere fungi of *Camellia japonica*: seasonal and leaf age-dependent variations. *Mycologia* **100(3):** 387-391.

Palfreyman J. W. (2001). The domestic dry rot fungus, *Serpula lacrymans*, its natural origins and biological control. *Ariadne workshop.*

Petrack B., Greengard P., Craston A., Sheppy F. (1965). Nicotinamide deamidase from mammalian liver. Journal of Biological Chemistry **240:** 1725–1730.

Pickart C.M., Eddins M. J. (2004). Ubiquitin: structures, functions, mechanisms. *ACTA Biochimica et Biophysica* **1695 (1–3):** 55–72.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Rauber A., Heard J. 1985). Castor bean toxicity re-examined: a new perspective. *Vet Human Toxicology* **27(6):** 498–502.

Reese E. T., Sui R. G. H., Levinson H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bacteriology* **59:** 485–497.

Reichenbach H., Dworkin M. (1992). The myxobacteria. In: Balows A., Tru H.G., Dworkin M., Harder W., Schleifer K. H. (eds.) *The Prokaryotes, (2nd edn).* New York: Springer, 3416-3487.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406-425.

Sanchez C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advances* **27:** 185-194.

Sarma D. S. R., Rajalakshmi S., Sarma S. (1964). Studies on the enzymes involved in nicotinamide adenine dinucleotide metabolism in Aspergillus niger. *Biochimica et Biophysica ACTA* **81:** 311–322.

Scorzetti G., Fell J. W., Fonseca A., Statzell-Tallman A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *Federation of European Microbiological Societies Yeast Research* **2:** 495–517.

Schmidt O. (2006). Wood and tree fungi. biology, damage, protection and use. New York: Springer.

Shmara A., Weinsteel N., Dery K. J., Chavideh R., Tolmasky M. E. (2001). Systematic analysis of a conserved region of the aminoglycoside 6′-*N*-acetyltransferase Type Ib. *Antimicrobial Agents and Chemotherapy*. **45(12):** 3287-3292.

Singh, B. N., Singh, N, B. (1971). Distribution of fruiting myxobacteria in Indian soils, bark of trees and dung of herbivorous animals. *Indian Journal of Microbiology* **11:** 47-92.

Stewart J. C., Parry J. B. (1981). Factors influencing the production of cellulase by *Aspergillus fumigatus* (Fresenius). *Journal of General Microbiology* **125(1):** 33-39.

Stoyanova, M., Pavlina, I., Moncheva, P., & Bogatzevska, N. (2007). Biodiversity and Incidence of *Burkholderia* Species. *Biotechnology and Biotechnological Equipment* **47:** 306-310.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28:** 2731-2739.

Tan H. P., Wahlstrom H. E., Zamora J. U., Hassanein T. (1997). *Aureobasidium* pneumonia in a post liver transplant recipient: a case report. *Hepatogastroenterology* **44(16):** 1215-1218.

Teeri T. T., Koivula A., Linder M., Wohlfahrt G., Divne C., Jones T. A. (1998). *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochemical Society Transactions* **26:** 173–178.

Thompson J. D., Higgins D. G., Gibson T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **11(22):** 4673-4680.

Tomme P., Van Tilbeurgh H., Pettersson G., Van Damme J., Vandekerckhove J., Knowles J., Teeri Claeyssens M. (1988). Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *European Journal of Biochemistry* **170:** 575–581.

Tomme P., Warren R. A., Miller R. C., Kilburn D. G., Gilkes, N. R. (1995). Cellulose-binding domains: classification and properties. In: Saddler J. N., Penner M. (eds.) *Enzymatic Degradation of Insoluble Polysaccharides.* Washington: American Chemical Society, 142-163.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil, *Medical Mycology* **41(5):** 383-390.

Vandamme P., Govan J., LiPuma J. (2007). Diversity and role of *Burkholderia* spp. In: Coenye T., Vandamme P. (eds.) Burkholderia*: Molecular Microbiology and Genomics*. Norfolk: Horizon Bioscience, Chapter 1.

Van Tilbeurgh H., Tomme P., Claeyssens M., Bhikhabhai R. (1986). Limited proteolysis of the cellubiohydrolase I from *Trichoderma reesei*. Separation of functional domains. *Federation of European Biochemical Societies Letters* **204:** 223–227.

Wales R., Richardson P. T., Roberts L. M., Woodland H. R., Lord J. M. (1991). Mutational analysis of the galactose binding ability of recombinant ricin B chain. *Journal of Biological Chemistry* **266(29):** 19172–19179.

Walsh T. J., Lee J. W., Melcher G. P., Navarro E., Bacher J., Callender D., Reed K. D., Wu T., Lopez-Berestein G., Pizzo P. A. (1992). Experimental *Trichosporon* infection in persistently granulocytopenic rabbits: implications for pathogenesis, diagnosis, and treatment of an emerging opportunistic mycosis. *Journal of Infectious Disease* **166(1):** 121-133.

Watson K. C., Kallichurum S. (1970). Brain abscess due to *Trichosporon cutaneum*. J. *Medical Microbiology* **3:** 191–193.

Weston S. A., Tucker A. D., Thatcher D. R., Derbyshire D. J., Pauptit R. A. (1994). X-ray structure of recombinant ricin A-chain at 1.8 Å resolution. *Journal of Molecular Biology* **244(4):** 410–22.

Wilkinson K. D. (2005). The discovery of ubiquitin-dependent proteolysis. *Proceedings of the National Academy of Science of the United States of America* **102(43):** 15280–15282.

Winsor G. L., Khaira B., Van Rossum T., Lo R., Whiteside M. D., Brinkman F. S. (2008). The *Burkholderia* Genome Database facilitating flexible queries and comparative analyses. *Bioinformatics* **24(23):** 2803-2804.

Wu S. L., Li C. C., Chen J. C., Chen Y. J., Lin C. T., Ho T. Y., Hsiang C. Y. (2009). Mutagenesis identifies the critical amino acid residues of human endonuclease G involved in catalysis, magnesium coordination, and substrate specificity. *Journal of Biomedical Science* **16:** 6-20.

Xhaard C., Fabre B., Andrieux A., Gladieux P., Barrés B., Frey P., Halkett F. (2011). The genetic structure of the plant pathogenic fungus *Melampsora larici-populina* on its wild host is extensively impacted by host domestication. *Molecular Ecology* **20(13):** 2739-55.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infection and Immunity* **69(9):** 5589-5596.

Zugmaier W., Bauer R., Oberwinkler F. (1994). Mycoparasitism of some *Tremella* species. *Mycologia* **86(1):** 49-56.

CHAPTER 4

Predicting the three-dimensional structure and cellular localization of three putative cellulases identified within the genome of the yeast pathogen *Cryptococcus neoformans* var. *grubii*

Predicting the three dimensional structure and cellular localization of three putative cellulases identified within the genome of the yeast pathogen *Cryptococcus neoformans* var. *grubii*

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Fungi play a pivotal role in the degradation of lignocellulosic material in natural environments. Hydrolysis is achieved via the secretion of various glycoside hydrolases that cleave the cellulose and hemi-cellulose within woody material into more soluble fractions, allowing their uptake across the fungal cell membrane. It is known that the opportunistic human pathogen, *Cryptococcus neoformans*, is able to grow and complete its life cycle on woody debris in the absence of additional nutrients. Completion of the genome sequence of this basidiomycetous yeast has allowed for the identification of three glycoside hydrolase family 5 (GHF5) enzymes involved in the catabolism of cellulose, however, little is known regarding the structural and chemical properties of these enzymes. Thus, the aim of this study was to evaluate the properties of two putative cellulases (CC1 and CC6) and an endo-glucanase (EC7), including their chemical and physical characteristics, tertiary structure and cellular location in *C. neoformans* var. *grubii*. The amino acid sequence of each protein was evaluated with regards to their chemical and physical properties. Automated homology modeling of the three-dimensional structures revealed that both CC1 and CC6 displayed the classical $(\alpha/\beta)_8$ triose-phosphate isomerase (TIM) barrel fold associated with GHF5 enzymes. Modeling of the EC7 protein did not produce the classic GHF5 associated structure, suggesting that this enzyme may be classed in a separate GH family. Green fluorescent protein (GFP tagging) confirmed that at least one of the identified putative proteins, cellulase C6, forms part of the cryptococcal secretome.

1. INTRODUCTION

Lignocellulose is widely regarded as the most prevalent renewable resource on the planet. Comprised of a combination of cellulose, hemicelluloses and lignin, these complex polymers represent a rich source of various mono and disaccharides. Fungi play a leading role in the biodegradation of lignocellulosic material by secreting ligninases, and a combination of glycoside hydrolases (GHs), including cellulases, mannanases, cellobiohydrolases and xylanases, into the surrounding medium (de Souza, 2013; Lum *et al.*, 2011; Sanchez, 2009).

Representatives of the well-known facultative opportunistic human pathogen, *Cryptococcus neoformans*, have been repeatedly isolated from tree hollows and woody debris (Randhawa *et al.*, 2011; 2008; 2006; 2000; Trilles *et al*., 2003; Fortes *et al*., 2001; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996) and it is known that this basidiomycetous yeast is able to grow and complete its life cycle on woody debris in the absence of additional nutrients (Botes *et al*., 2009). It was revealed that *C. neoformans* is one of many basidiomycetous species capable of utilizing woody material as carbon source (de Souza, 2013). Recently, we confirmed the presence of genes coding for three putative glycoside hydrolases within the genome of *C. neoformans* var. *grubii* ATCC H99 (Chapter 3). These putative enzymes, including an endo-glucanase (EC7) and two cellulases (CC1 and CC6), were all found to be members of glycoside hydrolase family 5. These findings support the contention that *C. neoformans* var. *grubii* does possess the enzymatic capability to degrade lignocellulose, a notion that was proposed a few years ago after the discovery of a cryptococcal laccase (Zhu *et al.*, 2001; Williamson, 1997).

Cryptococcus neoformans produces a cell wall bound laccase (Zhu *et al*., 2001) that is known to play a central role in the pathogenicity of this yeast (Min *et al*., 2001; Zhu *et al*., 2001; Liu *et al*., 1999). Laccase is essential in the degradation of lignin and is most prevalent within the white-rot basidiomycetes (Galliano *et al.*, 1991; Lobarzewski, 1990). Further research however, concluded that the cryptococcal laccase is not a true laccase, but rather a representative of a different family of multi-copper oxidases (Valderrama *et al*., 2003), suggesting that it would play no part in lignin degradation or modification.

Similar to the cryptococcal laccase, it remains unclear as to whether the above-mentioned putative glycoside hydrolases (CC1, CC6 and EC7) are truly involved in the degradation of woody material. Also, it is unknown whether these enzymes are

secreted by *C. neoformans*. To obtain further evidence for the cellulolytic nature of these enzymes and to determine whether they are part of the cryptococcal secretome, the enzymes were evaluated with regards to their chemical and physical properties, as well as their three-dimensional (3D) structures. In addition, recombinant technology was employed to obtain an indication of the cellular location of CC6 and EC7.

2. MATERIAL AND METHODS

2.1 Strains, Plasmids, Media and Culture Conditions

Cryptococcus neoformans var. *grubii* and *Escherichia coli* strains used in this study are listed in Table 1. Cloning vectors utilized and final plasmid constructs generated during this study are listed in Table 2 and Table 3 respectively. Yeast strains were maintained by periodic transfer to yeast peptone glucose (YPG; pH 5.5) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Gauteng, South Africa). Cultures of *E. coli* were grown in either Luria-Bertani broth (LB, pH 7.0) or on Luria-Bertani agar (LA, pH 7.0) plates (Sambrook *et al.*, 1989). Yeast transformants were selected and maintained by periodic transfer on agar plates with synthetic complete medium without uracil (SC^{-URA}, pH 6.0; du Plessis *et al.*, 2010) plates. Bacterial transformants were selected and maintained by periodic transfer to LA (pH 7.0) plates supplemented with 100 mg/L ampicillin (LA^{Ap+}). All media were prepared as per the manufacturer's instructions.

Table 1 Microbial strains used in this study.

Strain	Genotype	Reference or Source
Yeast and Bacterial strains		
Cryptococcus neoformans var. *grubii* ATCC H99 (CBS 10515)	*MATα*	Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands
Cryptococcus neoformans var. *grubii* CBS 10867	*MATα ura5 (FOA^r)*	CBS, Utrecht, The Netherlands
Escherichia coli DH5α	ϕ80Δ*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp., Wisconsin, USA

Table 2 Plasmids used in this study.

Plasmid	Description	Reference or Source
Plasmid vectors		
pGEM-T-Easy	Ap^r, T-tailed PCR product cloning vector	Promega Corp., Madison, Wisconsin, USA
pGFP	Ap^r, *lacZ*. ColE1 replicon, cloning vector	Clontech Laboratories Inc., California, USA
pJAF7	Ap^r, *URA5*	Fraser *et al*., 2003
pCIP3-GUST	Ap^r, *URA5*	Wickes *et al*., 1995

Table 3 Plasmids constructed during this study.

Plasmid	Description	Reference or Source
Plasmid constructs		
pGEM-CC6	Ap^r, 1293-bp *CC6* PCR product cloned into pGEM-T-Easy	This study
pGEM-CC6:GFP	Ap^r, *CC6*: 821-bp *KpnI-SpeI* fragment containing the GFP green fluorescent protein gene cloned into pGEM-*CC6*	This study
pJAF7-CC6:GFP	Ap^r, *URA5*, 2051-bp *EcoRI-EcoRI* fragment containing the fused *CC6*:*GFP* genes cloned into pJAF7	This study (Fig 1)
pCIP3-EC7	Ap^r, *URA5*, 1428-bp *NdeI-BglII* fragment containing the *EC7* gene cloned into pCIP3	This study
pCIP3-EC7:GFP	Ap^r, *URA5*, 800-bp *BglII-BglII* fragment containing the *GFP* gene cloned into pCIP3-*EC7*	This study (Fig 1)

2.2 Protein Evaluation

The deduced amino acid sequence of the *CC1*, *CC6* and *EC7* genes were obtained from the Broad Institute database (http://www.broadinstitute.org/) as described in Chapter 3. Various online tools were used to evaluate the putative proteins.

2.2.1 Physical and Chemical Parameters. The physical and chemical parameters of the three identified amino acid sequences were determined using the online ExPaSy-ProtParam tool (Gasteiger *et al.*, 2005; www.web.expasy.org/protparam). These include the molecular weight, theoretical iso-electric point (pI; Henderson, 1908), amino acid composition, atomic composition, aliphatic index (Ikai, 1980), instability index (Guruprasad *et al.*, 1990) and grand average of hydropathicity (GRAVY; Kyte *et al.*, 1982).

2.2.2 Prediction of Signal Peptides. The presence of signal peptides was determined using online severs SignalP 4.1 (Petersen *et al.*, 2011; www.cbs.dtu.dk/services/SignalP/) and TargetP 1.1 (Emanuelsson *et al.*, 2000; www.cbs.dtu.dk/services/TargetP/). The SignalP 4.1 server predicts the location and presence of signal peptides within an amino acid sequence based on a combination of several artificial neural networks. The results are validated by the value of Discrimination score (D-score) ranging from 0 to 1. Higher D-scores are indicative of secretory proteins. Similarly, TargetP 1.1 predicts the cellular location of eukaryotic proteins. The program is able to determine whether the protein is targeted towards the chloroplast, mitochondria or a secretory pathway. Reliability class (RC) scores are determined in order to estimate the reliability of the generated results. Values are given between 1 and 5, where 1 indicates the strongest prediction.

2.2.3 Three Dimensional (3D) Protein Model. The 3D structure of the three identified proteins (CC1, CC6 and EC7) were generated via an automated homology modeling program ESyPred3D (Lambert *et al.*, 2002; www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/). The program is able to incorporate alignment results from multiple alignment programs yielding better alignment of data. The final model is then constructed using MODELLER4 (Sali *et al.*, 1993). All generated protein models and Protein Database (PDB) structures were viewed in PyMOLWin version 0.99rc6 (DeLano Scientific LLC, California, USA).

2.2.4 3D Protein Model Quality Assessment. The quality of the 3D protein structures generated by ESyPred3D was assessed using ProQ (Wallner *et al.*, 2003; www.sbc.su.se/~bjornw/ProQ/ProQ.html). The ProQ program is a neural network-based predictor capable of assessing a protein model on the basis of various structural

features. The software generates two separate values indicating model quality, LG-score and MaxSub (Table 4).

Table 4 ProQ scores used to evaluate protein model integrity (Wallner *et al.*, 2003).

LG-score	MaxSub	Comment
>1.5	>0.1	Fairly good model
>2.5	>0.5	Very good model
> 4	>0.8	Extremely good model

2.3 Plasmid Construction

The identified proteins CC6 and EC7 were selected for further investigation with regards to their localization within the cryptococcal cell. Each gene was tagged with a green fluorescent protein and expressed in *C. neoformans* var. *grubii* CBS 10867 to visualize localization of the protein within the cell. Standard protocols were followed for DNA manipulations (Sambrook *et al.*, 1989). The enzymes used for restriction digests and ligations were sourced from Fermentas (Massachusetts, USA) and implemented as per the manufacturers' instructions. Digested DNA was eluted from agarose gels using the GeneJET Gel Extraction kit (Fermentas). The polymerase chain reaction (PCR) was performed in 20 μL reaction volumes using High Fidelity PCT Enzyme Mix (Fermentas) and the primers are listed in Table 5. Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at primer Tm (Table 5) for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products were separated in a Tris-EDTA agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory *et al.*, 1999). Detail regarding the final plasmid constructs are listed in Table 3 and shown in Fig 1.

Table 5 Primer sets used for cloning. All primers were produced by Inqaba Biotec, Gauteng, South Africa.

Primer	Primer sequence	Primer set Tm (°C)
CC6 F	5′-ATATCGCGAATGCGTTTGCTCATC-3′	62
CC6 R	5′-ATACTGGAGCTAGCCACATAACGAAG-3′	
EC7 F	5′-ATATCTGCAGATGAGCTCCGACGGT-3′	64
EC7 R	5′-ATATGGTACCTCACCAACTTGCCACA-3′	
GFP-BamHI-F	5′-ATAGGATCCATGAGTAAAGGAGAAGAAC-3′	61
GFP-BamHI-R	5′-ATAGGATCCCTATTTGTATAGTTCATCC-3′	

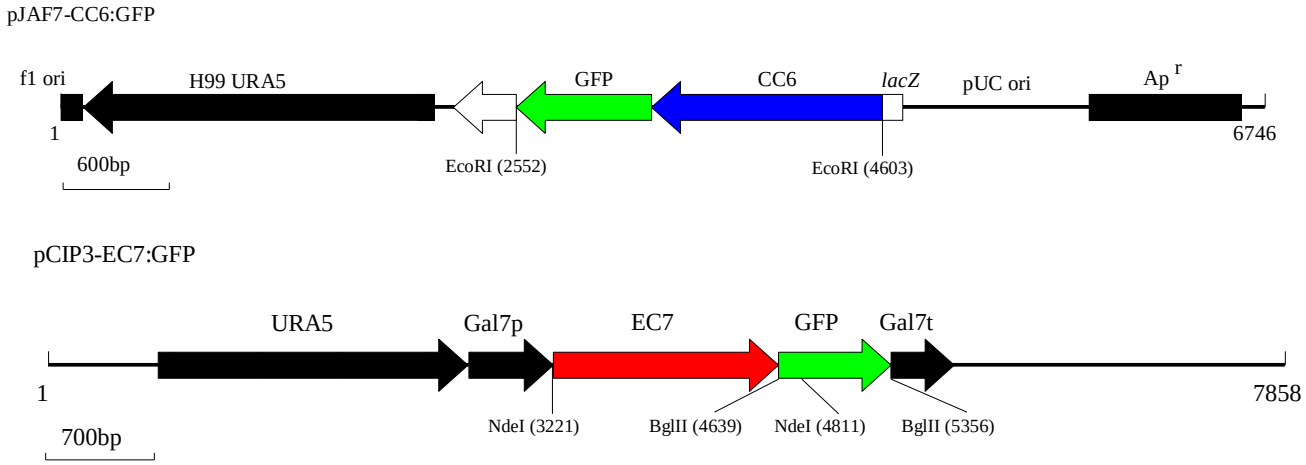

Figure 1 Plasmids to determine the cellular location of the putative cellulase CC6 and endo-glucanase EC7 with green fluorescent protein (GFP) tagging.

2.4 Yeast Transformation

Cryptococcus neoformans var. *grubii* CBS 10867 was transformed with the recombinant pJAF7-CC6:GFP or CIP3-EC7:GFP plasmids as previously described by Varma and co-workers (1992). Transformants were selected for growth on SC^{-URA} plates after incubation at 30 °C for five days.

2.5 Protein Localization using GFP Tagging

Cryptococcus neoformans var. *grubii* transformants harbouring the recombinant plasmids pJAF7-CC6:GFP or CIP3-EC7:GFP were cultured in 10 ml SC^{-URA} broth (pH 5.5), supplemented with 2 % (w/v) galactose and 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), for 18 hours at 30 °C on a tissue culture roll drum (7

rpm). A total of 2 mL of cell culture was harvested via centrifugation for 5 min at 13793 RCF (Biofuge fresco; Heraeus Instruments, Hanau, Germany). The supernatant was collected and filter-sterilized using a 0.22 µm pore filter (Merck-Millipore, South Africa). The cell pellets were re-suspended in 1 mL physiological saline solution. Both the filtered supernatant and washed cells were examined under ultraviolet light (365 nm) to localize the GFP-tagged recombinant proteins.

3. RESULTS AND DISCUSSION

3.1 Cellulase C1

3.1.1 Physical and Chemical Characteristics. The putative enzyme CC1 was the largest of the three putative proteins and had a total of 789 amino acids with a molecular weight of 85.302 kDa. Based on the size of CC1, one would expect that it is in fact a cellobiohydrolase (CBH) or exo-glucanase (Baldrian *et al.*, 2008; Bayer *et al.*, 1998). However large endo-glucanases have been isolated from the basidiomycetes *Sclerotium rolfsii* and *Gloeophyllum sepiarium* (Bhattacharjee *et al.*, 1993; Sadana *et al.*, 1984). Also, evaluation of the *C. neoformans* var. *grubii* genome shows that the yeast does not possess a CBH (Loftus *et al.*, 2005). The theoretical pI of CC1 was calculated to be 4.3 (Henderson, 1908). The high aliphatic index of 62.7 indicates that this protein may be stable over a wide range of temperatures (Ikai, 1980). Similarly, the calculated instability index of 33.84 and the negative GRAVY value (-0.359) indicate that the protein is regarded as being hydrophilic (Guruprasad *et al.*, 1990; Kyte *et al.*, 1982). The amino acid and atomic composition of CC1 are listed in Tables 6 and 7 respectively.

3.1.2 Prediction of Signal Peptides. Analysis revealed that CC1 does indeed have a short signal peptide (D-score = 0.891) comprising of 22 amino acid residues (Fig 2). The cleavage site is located between amino acid residues 22 (Ala - A) and 23 (Gly - G). Similarly, TargetP 1.1 predicted that CC1 would be targeted towards the secretory pathways (RC = 1). These results, coupled to the hydrophilic nature and overall stability of the protein, would strongly suggest that the putative cellulase enzyme (CC1) is secreted from the cell in order to act on an external substrate.

Table 6 Amino acid composition of CC1 as determined with ExPaSy-ProtParam (Gasteiger *et al.*, 2005).

Amino Acid	Total number	% mol/mol
Ala (A)	88	11.2
Arg (R)	22	2.8
Asn (N)	44	5.6
Asp (D)	55	7.0
Cys (C)	9	1.1
Gln (Q)	24	3.0
Glu (E)	38	4.8
Gly (G)	50	6.3
His (H)	15	1.9
Ile (I)	42	5.3
Leu (L)	37	4.7
Lys (K)	14	1.8
Met (M)	16	2.0
Phe (F)	22	2.8
Pro (P)	17	2.2
Ser (S)	116	14.7
Thr (T)	87	11.0
Trp (W)	35	4.4
Tyr (Y)	24	3.0
Val (V)	34	4.3

Table 7 Atomic composition of CC1 as determined with ExPaSy-ProtParam (Gasteiger *et al*., 2005).

Element	Number of atoms
Carbon (C)	3707
Hydrogen (H)	5562
Nitrogen (N)	1002
Oxygen (O)	1271
Sulphur (S)	25

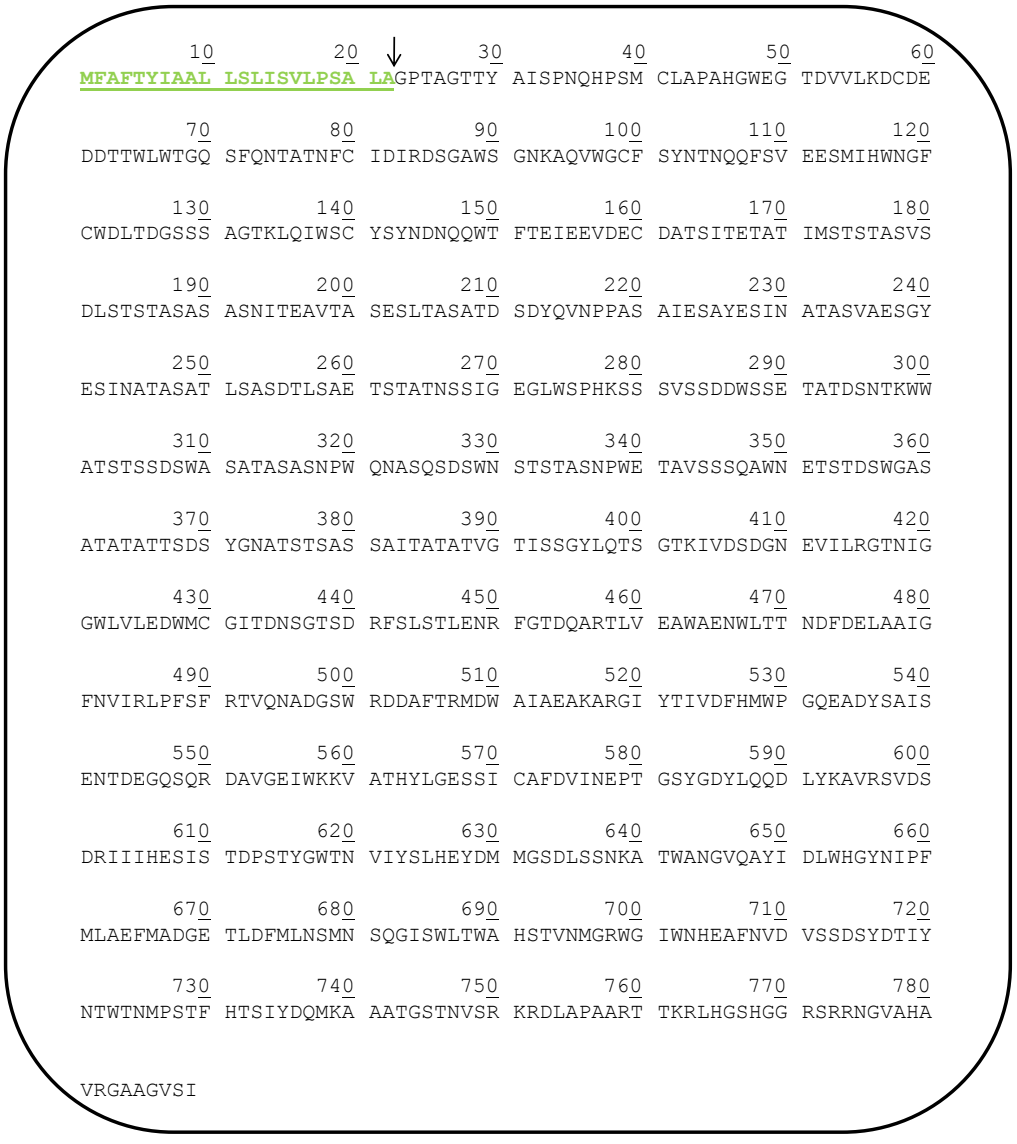

Figure 2 Signal peptide (marked in green) of CC1 as predicted by SignalP 4.1 (Petersen *et al*., 2011). The cleavage site is located between amino acid residues 22 (Ala - A) and 23 (Gly - G).

3.1.3 Protein Model (3D Structure). A 3D model (Fig 3) of CC1 was generated via an automated homology modeling program, ESyPred3D (Lambert *et al.*, 2002). The structure was validated using ProQ and received a LG-score of 2.710 and a MaxSub score of 0.365, indicating that the model ranged from very good to fairly good (Wallner *et al.*, 2003) depending on the selected criteria (Table 4). Phylogenetic analysis identified the protein as a glycoside hydrolase family 5 (GHF5) protein (Chapter 3, Fig 1). As discussed previously, GHF5 is one of the largest glycoside hydrolase families and comprises endo- and exo-glucanases, endo- and exo-mannanases, β-glucosidases, β-mansosidases, as well as xylo-glucanases. The 3D model of CC1 produced the classic $(\alpha/\beta)_8$ triose-phosphate isomerase (TIM) barrel fold associated with GHF5 proteins (Fig 3) and as such, the catalytic residues were identified as being the conserved glutamic acids (Figs 4 and 5) located at the C-terminal end of β-strands 4 (Glu-578) and 7 (Glu-664).

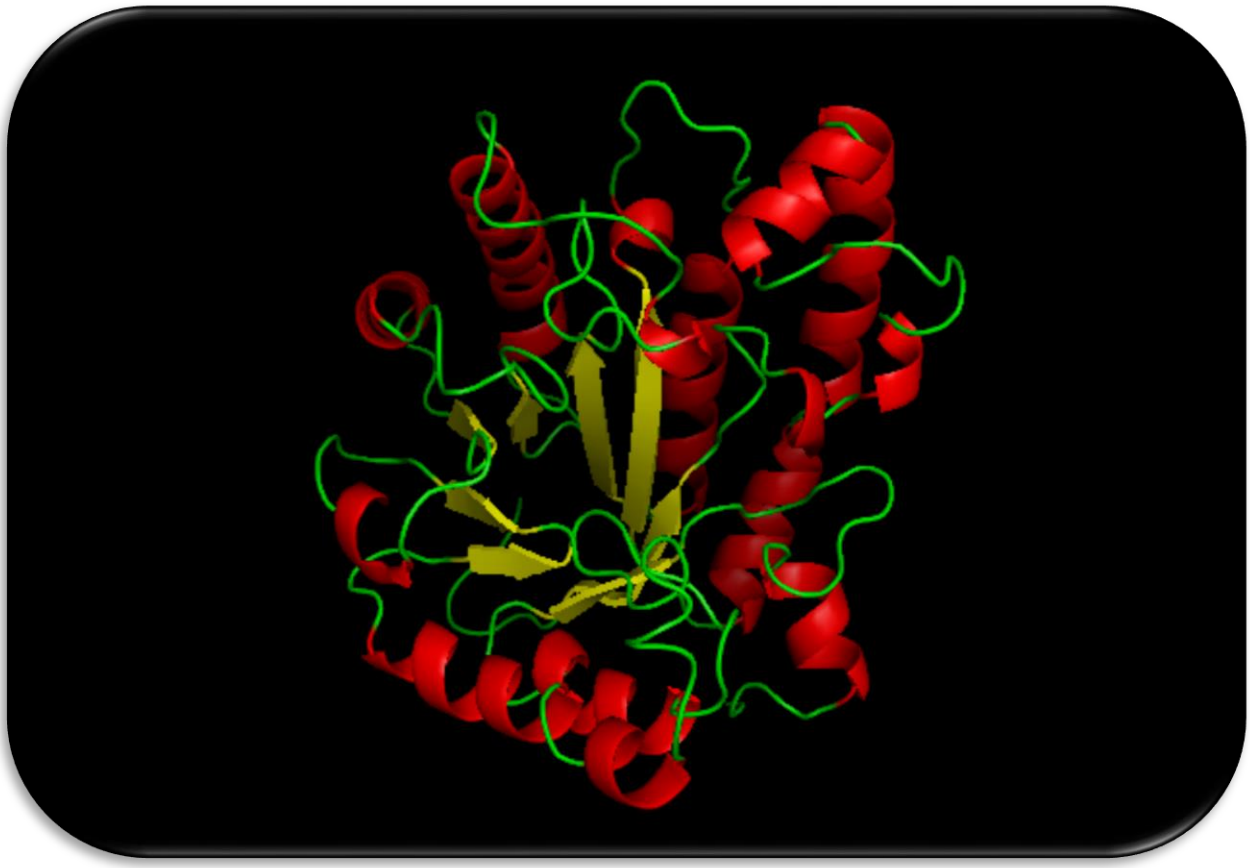

Figure 3 Three-dimensional model of the putative enzyme CC1 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). CC1 model LG score = 2.710 (Wallner *et al.*, 2003). An LG score > 2.5 indicates a very good model.

```
        10         20         30         40         50         60
MFAFTYIAAL LSLISVLPSA LAGPTAGTTY AISPNQHPSM CLAPAHGWEG TDVVLKDCDE

        70         80         90        100        110        120
DDTTWLWTGQ SFQNTATNFC IDIRDSGAWS GNKAQVWGCF SYNTNQQFSV EESMIHWNGF

       130        140        150        160        170        180
CWDLTDGSSS AGTKLQIWSC YSYNDNQQWT FTEIEEVDEC DATSITETAT IMSTSTASVS

       190        200        210        220        230        240
DLSTSTASAS ASNITEAVTA SESLTASATD SDYQVNPPAS AIESAYESIN ATASVAESGY

       250        260        270        280        290        300
ESINATASAT LSASDTLSAE TSTATNSSIG EGLWSPHKSS SVSSDDWSSE TATDSNTKWW

       310        320        330        340        350        360
ATSTSSDSWA SATASASNPW QNASQSDSWN STSTASNPWE TAVSSSQAWN ETSTDSWGAS

       370        380        390        400        410        420
ATATATTSDS YGNATSTSAS SAITATATVG TISSGYLQTS GTKIVDSDGN EVILRGTNIG

       430        440        450        460        470        480
GWLVLEDWMC GITDNSGTSD RFSLSTLENR FGTDQARTLV EAWAENWLTT NDFDELAAIG

       490        500        510        520        530        540
FNVIRLPFSF RTVQNADGSW RDDAFTRMDW AIAEAKARGI YTIVDFHMWP GQEADYSAIS

       550        560        570        580        590        600
ENTDEGQSQR DAVGEIWKKV ATHYLGESSI CAFDVINEPT GSYGDYLQQD LYKAVRSVDS
                                          *
       610        620        630        640        650        660
DRIIIHESIS TDPSTYGWTN VIYSLHEYDM MGSDLSSNKA TWANGVQAYI DLWHGYNIPF

       670        680        690        700        710        720
MLAEFMADGE TLDFMLNSMN SQGISWLTWA HSTVNMGRWG IWNHEAFNVD VSSDSYDTIY
  **
       730        740        750        760        770        780
NTWTNMPSTF HTSIYDQMKA AATGSTNVSR KRDLAPAART TKRLHGSHGG RSRRNGVAHA


VRGAAGVSI
```

Figure 4 Amino acid sequence of CC1 highlighting the catalytic residues in red and the C-terminal end of β-strands 4 and 7 in blue. *Predicted catalytic acid/base; **Predicted catalytic nucleophile.

Figure 5 Three-dimensional model of the putative enzyme CC1 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). The catalytic glutamic acid residues (Glu-578 and Glu-664) are shown in red. CC1 model LG score = 2.710 (Wallner *et al.*, 2003).
An LG score > 2.5 indicates a very good model.

3.2 Cellulase C6

3.2.1 Physical and Chemical Characteristics. The putative enzyme CC6 has a total of 431 amino acids with a molecular weight of 48.876 kDa. Similar to CC1, the theoretical pI was calculated to be 4.67 (Henderson., 1908); however, the aliphatic index of 73.81 was much higher indicating that this protein may be more stable than CC1 over a range of temperatures (Ikai, 1980). Similar to the properties of CC1, the calculated instability index (29.12) and the negative GRAVY value (-0.432) of CC6 indicated that the latter enzyme is very hydrophilic (Guruprasad *et al.,* 1990; Kyte *et al.*, 1982). The amino acid and atomic composition of CC6 are listed in Tables 8 and 9, respectively.

3.2.2 Prediction of Signal Peptides. Analysis revealed that CC6 possess a shorter signal peptide (D-score = 0.790) than CC1, comprising of 15 amino acid residues (Fig 6). The cleavage site is located between amino acid residues 15 (Ala - A) and 16 (Arg -R). TargetP 1.1 predicted that the CC6 would be targeted towards the secretory pathway (RC = 2). The predicted secretion of CC6, as well as its high hydrophilic nature and over all stability, strongly suggest that, similar to CC1, CC6 is also secreted from the cell in order to act on an external substrate.

Table 8 Amino acid composition of CC6 as determined with ExPaSy-ProtParam (Gasteiger *et al.*, 2005).

Amino Acid	Total number	% mol/mol
Ala (A)	36	8.4
Arg (R)	18	4.2
Asn (N)	30	7.0
Asp (D)	33	7.7
Cys (C)	5	1.2
Gln (Q)	15	3.5
Glu (E)	21	4.9
Gly (G)	35	8.1
His (H)	10	2.3
Ile (I)	21	4.9
Leu (L)	32	7.4
Lys (K)	12	2.8
Met (M)	4	0.9
Phe (F)	16	3.7
Pro (P)	18	4.2
Ser (S)	27	6.3
Thr (T)	25	5.8
Trp (W)	20	4.6
Tyr (Y)	27	6.3
Val (V)	26	6.0

Table 9 Atomic composition of CC6 as determined with ExPaSy-ProtParam (Gasteiger *et al*., 2005).

Element	Number of atoms
Carbon (C)	2211
Hydrogen (H)	3230
Nitrogen (N)	582
Oxygen (O)	664
Sulphur (S)	9

```
        10        ↓ 20        30        40        50        60
MRLLIPFFIV PLAIARALPA FPTVDVEKRS VNVGWPYGTD KIRGVNIGGW LVTEPFITPS

        70        80        90        100       110       120
LFEATGNNDI VDEWTFCQYQ DYDTARSALM NHWDTWFTED DFAKISAAGL NHVRIPIGFW

       130       140       150       160       170       180
AYDVQDGEPY IQGQADYLDR AIGWARKHNL AVIVDLHGAP GSQNGYDNSG RRGAADWATN

       190       200       210       220       230       240
NSNVDRTKNV ISLLSRKYSD SQYYGVVTAI ALLNEPATYL NELLLQTARQ YWYNAYGAAR

       250       260       270       280       290       300
YPFGNNDKSG LALVIHDGFQ PLNTFENYMT EPEYEDVLLD THNYQVFNDE YVAWNWDEHI

       310       320       330       340       350       360
SNICNKASTY STSPLWLVVG EWTLASTDCA KYLNGRGLGS RYDGSYPGSP YIGTCDDKSN

       370       380       390       400       410       420
DVDRFSEEYK AFMHRFWEVQ TQVYEQNGQG WIHWTWKTEN AADWSYEAGL DGGWIPWNAG

       430
SHDVSLSSLC G
```

Figure 6 Predicted signal peptide (marked in green) of CC6. The cleavage site is located between amino acid residues 15 Ala - A) and 16 (Arg - R).

3.2.3 Protein Model (3D Structure). A 3D model of CC6 was generated via an automated homology modeling program, ESyPred3D (Fig 7). The structure was validated using ProQ and received a LG-score of 5.499 and a MaxSub score of 0.566 indicating that the model ranged from extremely good to very good, depending on the selected criteria (Table 4). Phylogenetic analysis of the protein sequence identified the

protein as a glycoside hydrolase family 5 (GHF5) protein (Chapter 3, Fig 1). Similar to CC1, the 3D model of CC6 produced the classic $(\alpha/\beta)_8$ TIM barrel fold associated with GHF5 proteins (Fig 7) and as such, the catalytic residues were identified as being the conserved glutamic acids (Figs 8 and 9) located at the C-terminal end of β-strands 4 (Glu-215) and 7 (Glu-312). Further examination of the protein model also revealed the formation of two di-sulphide bonds between Cys304-Cys430 and Cys329-Cys355 (Fig 10 and 11). Such a di-sulphide bond, or SS-bond, is a covalent coupling of the thiol groups of the cysteine residues. These bonds play an integral role in the folding and stability of proteins, particularly those that are secreted (Betz, 1993; Thornton, 1981).

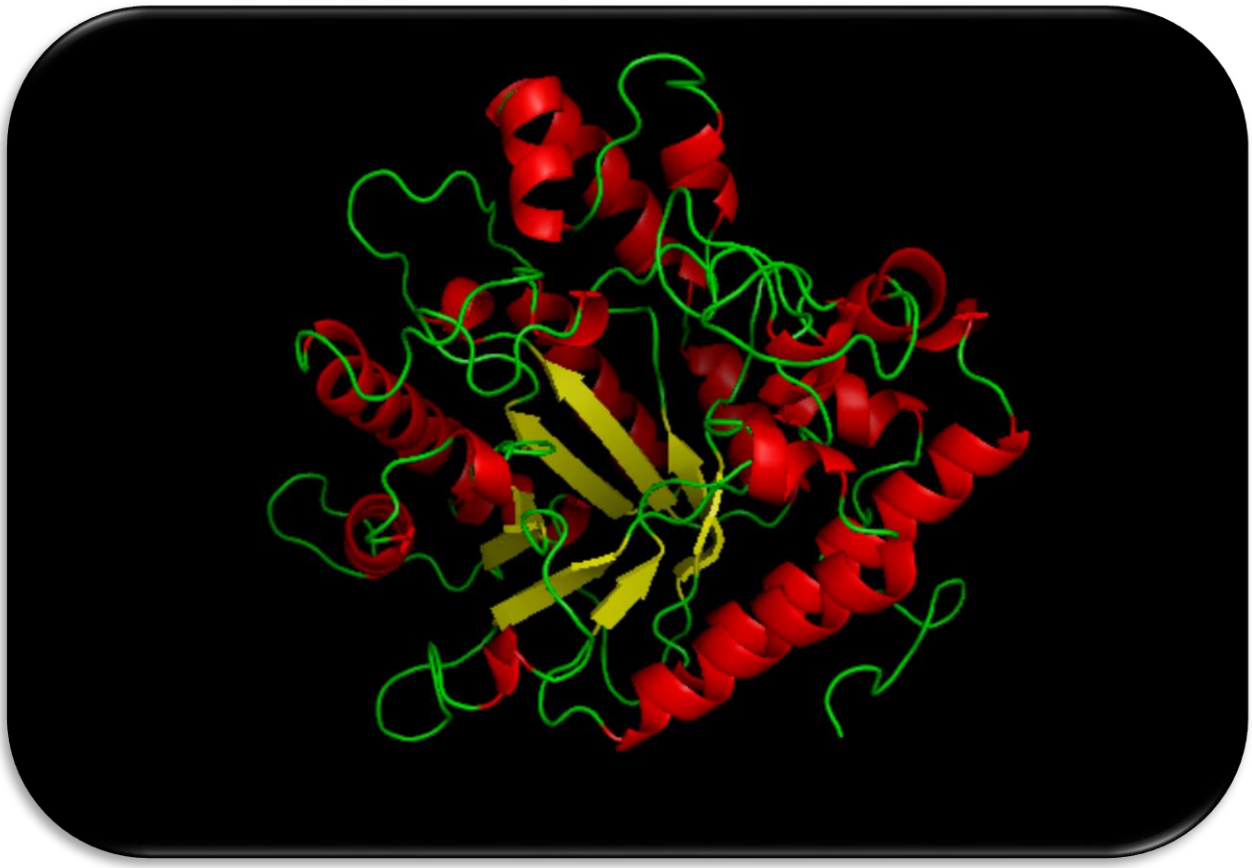

Figure 7 Three-dimensional model of the putative enzyme CC6 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). CC6 model LG score = 5.499 (Wallner *et al.*, 2003). An LG score > 4 indicates an extremely good model.

```
        10          20          30          40          50          60
MRLLIPFFIV PLAIARALPA FPTVDVEKRS VNVGWPYGTD KIRGVNIGGW LVTEPFITPS

        70          80          90         100         110         120
LFEATGNNDI VDEWTFCQYQ DYDTARSALM NHWDTWFTED DFAKISAAGL NHVRIPIGFW

       130         140         150         160         170         180
AYDVQDGEPY IQGQADYLDR AIGWARKHNL AVIVDLHGAP GSQNGYDNSG RRGAADWATN

       190         200         210         220         230         240
NSNVDRTKNV ISLLSRKYSD SQYYGVVTAI ALLNEPATYL NELLLQTARQ YWYNAYGAAR

       250         260         270         280         290         300
YPFGNNDKSG LALVIHDGFQ PLNTFENYMT EPEYEDVLLD THNYQVFNDE YVAWNWDEHI

       310         320         330         340         350         360
SNICNKASTY STSPLWLVVG EWTLASTDCA KYLNGRGLGS RYDGSYPGSP YIGTCDDKSN

       370         380         390         400         410         420
DVDRFSEEYK AFMHRFWEVQ TQVYEQNGQG WIHWTWKTEN AADWSYEAGL DGGWIPWNAG

       430
SHDVSLSSLC G
```

Figure 8 Amino acid sequence of CC6 highlighting the catalytic residues in red and the C-terminal end of β-strands 4 and 7 in blue. *Predicted catalytic acid/base; **Predicted catalytic nucleophile.

Figure 9 Three dimensional model of the putative enzyme CC6 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). The catalytic glutamic acid residues (Glu-215 and Glu-321) are shown in red. CC6 model LG score = 5.499 (Wallner *et al.*, 2003).
An LG score > 4 indicates an extremely good model.

```
        10         20         30         40         50         60
MRLLIPFFIV PLAIARALPA FPTVDVEKRS VNVGWPYGTD KIRGVNIGGW LVTEPFITPS

        70         80         90        100        110        120
LFEATGNNDI VDEWTFCQYQ DYDTARSALM NHWDTWFTED DFAKISAAGL NHVRIPIGFW

       130        140        150        160        170        180
AYDVQDGEPY IQGQADYLDR AIGWARKHNL AVIVDLHGAP GSQNGYDNSG RRGAADWATN

       190        200        210        220        230        240
NSNVDRTKNV ISLLSRKYSD SQYYGVVTAI ALLNEPATYL NELLLQTARQ YWYNAYGAAR

       250        260        270        280        290        300
YPFGNNDKSG LALVIHDGFQ PLNTFENYMT EPEYEDVLLD THNYQVFNDE YVAWNWDEHI

       310        320        330        340        350        360
SNICNKASTY STSPLWLVVG EWTLASTDCA KYLNGRGLGS RYDGSYPGSP YIGTCDDKSN
    *                     #                           #

       370        380        390        400        410        420
DVDRFSEEYK AFMHRFWEVQ TQVYEQNGQG WIHWTWKTEN AADWSYEAGL DGGWIPWNAG

       430
SHDVSLSSLC G
         *
```

Figure 10 Amino acid sequence of CC6 highlighting the cysteine residues predicted to form di-sulphide bonds. *Cys304-Cys430; #Cys329-Cys355.

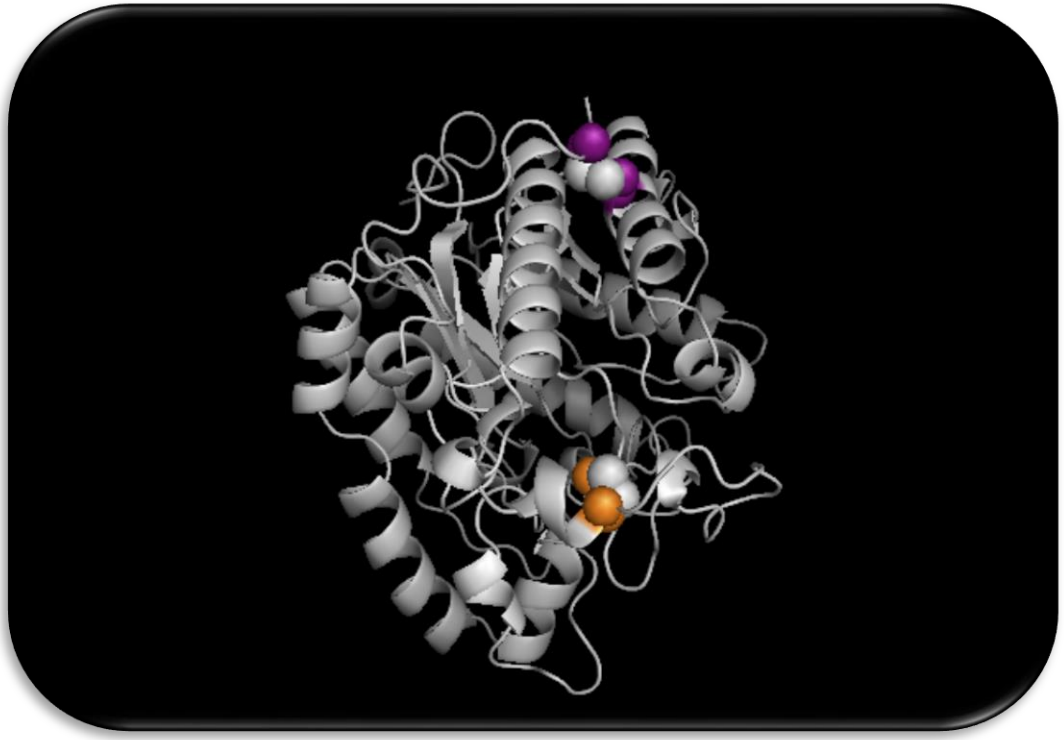

Figure 11 Three-dimensional model of the putative enzyme CC6 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). Two di-sulphide bonds occur in the model, i.e. Cys304-Cys430 (purple) and Cys329-Cys355 (orange). CC6 model LG score = 5.49 (Wallner *et al.*, 2003). LG score > 4 indicates an extremely good model.

3.2.4 Protein Localization. Due to the large size of the lignocellulosic constituents, cellulose and hemi-cellulose, they are unable to be transported across the cell membrane and be hydrolyzed within the cell. As a result, their respective hydrolytic enzymes generally form part of the secretome (de Souza, 2013; Lum *et al.*, 2011; Sanchez, 2009). Given the prediction of a signal peptide within the CC6 amino acid sequence, it was believed that this enzyme would indeed be targeted for secretion from the cryptococcal cell. To confirm the secretion of CC6, a green fluorescent protein (GFP) was cloned in frame at the C-terminal end of the recombinant CC6 to ensure the correct functioning of the native signal peptide (Table 3 and Fig 1) and transformed into *C. neoformans* var. *grubii* CBS 10867. Expression of the GFP was clearly visible in transformants, while minimal auto-fluorescence was noted in the parent strain (Fig 12). Subsequent filtration (0.22 µm) of the transformant strain confirmed that the fluorescence was located in the supernatant and not within the cell wall or membrane of *C. neoformans* var. *grubii* (Fig 13).

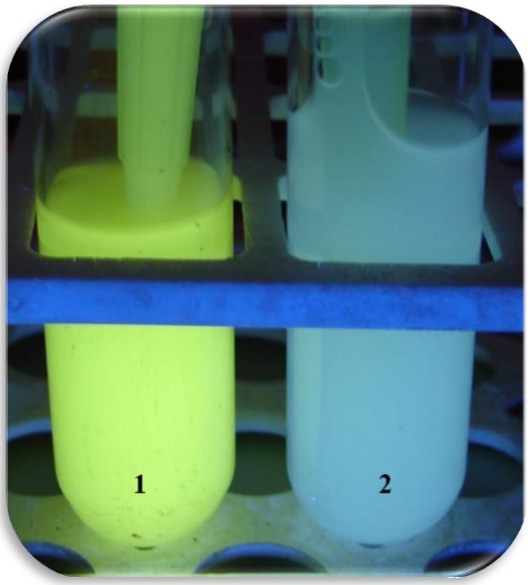

Figure 12 GFP visualization under ultraviolet light (365 nm). 1) *C. neoformans* var. *grubii* CBS 10867 pJAF7-*CC6*::*GFP*. 2) *C. neoformans* var. *grubii* CBS 10867 pJAF7.

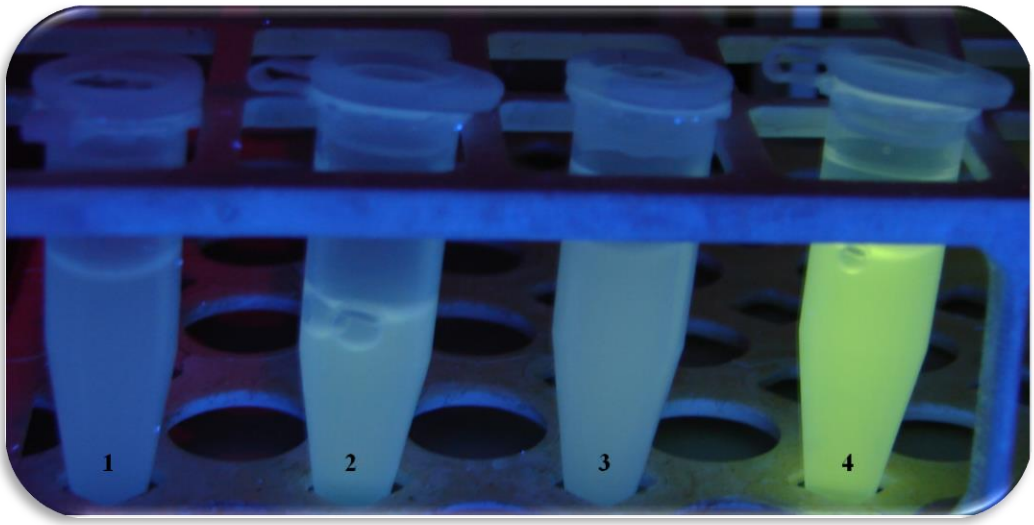

Figure 13 GFP visualization under ultra violet light (365 nm). 1) *C. neoformans* var. *grubii* CBS 10867 pJAF7 filtered supernatant. 2) *C. neoformans* var. *grubii* CBS 10867 pJAF7 whole cells re-suspended in physiological saline solution. 3) *C. neoformans* var. *grubii* CBS 10867 pJAF7-*CC6*::*GFP* whole cells re-suspended in physiological saline solution. 4) *C. neoformans* var. *grubii* CBS 10867 pJAF7-*CC6*::*GFP* filtered supernatant.

3. 3 Endo-glucanase C7

3.3.1 Physical and Chemical Characteristics. The putative enzyme EC7 has a total of 476 amino acids and a molecular weight of 55.130 kDa. Generally, the iso-electric points of fungal endo-glucanases are acidic ranging between 2.6 and 4.9 (Baldrian *et al.*, 2008; Ding *et al.*, 2001), but the theoretical pI of EC7 was calculated to be 6.56 (Henderson, 1908). Almost neutral pI values have also been noted for endo-glucanases isolated from the edible fungus *Volvariella volvacea* (Ding *et al.*, 2001) and the soil yeast *Rhodotorula glutinis* (Oikawa *et al.*, 1998). Of all three putative proteins, EC7 displayed the highest aliphatic index of 77.65 indicating that this protein would prove to be the most stable over a wide range of temperatures (Ikai, 1980). Similarly, EC7 was predicted to be the most stable in water and the most hydrophilic with a calculated instability index of 28.94 and the negative GRAVY value of -0.525 (Guruprasad *et al.*, 1990; Kyte *et al.*, 1982). The amino acid and atomic composition of EC7 are listed in Tables 10 and 11, respectively.

3.3.2 Prediction of Signal Peptides. Both servers predicted that EC7 lacked a signal peptide. Given that the components of lignocellulose, cellulose and hemi-cellulose, are largely insoluble and have a high molecular weight, it is unlikely that EC7 is not

secreted. The extreme hydrophilic nature and overall stability of the protein as indicated by the GRAVY value, as well as the instability and aliphatic indices strongly support the theory that EC7 is secreted from the cell. It would be tempting to suggest that similar to the cryptococcal laccase enzyme, EC7, is bound to the cell wall or embedded in the cell membrane (Zhu *et al*., 2001), however, comparison of the characteristics of the two proteins suggests that these two enzymes do not share a similar cellular location. The instability index of laccase was determined as 41.76 and suggests, that unlike EC7, laccase would be unstable when placed in water. Furthermore, the GRAVY value for laccase (-0.283) indicates that this protein is slightly hydrophobic and would be considered an integral protein embedded within the cell membrane or cell wall.

The lack of a signal peptide for EC7 may be due to the presence of alternative secretion pathways such as "leaderless" protein secretion. The secretion of proteins was thought to occur solely due to the presence of the N-terminal signal peptide that directs the protein towards the endoplasmic reticulum (ER) and subsequently the Golgi apparatus (Rodrigues *et al.,* 2012). An alternative pathway was suggested by Auron and co-workers (1987) when the human cytokine, interleukin 1, was secreted from human cells without a signal peptide in the presence of *Staphylococcus*. To date, leaderless protein secretion has been identified in numerous organisms, including the fungal species *Aspergillus niger* (Tsang *et al.,* 2009), *Saccharomyces cerevisiae* (Chen *et al.,* 1997) and *Coprinus cinereus* (Boulianne *et al.,* 2000), although it is still considered to be rare in fungi (Lum *et al.,* 2011: Tsang *et al.,* 2009). Research conducted on the virulence factors of *C. neoformans* have determined that the urease and certain elements of the polysaccharide capsule are secreted via alternative secretion pathways (Rodrigues *et al.,* 2012), suggesting that perhaps the putative enzyme EC7 is secreted in a similar manner.

Table 10 Amino acid composition of EC7 as determined with ExPaSy-ProtParam (Gasteiger *et al*., 2005).

Amino Acid	Total number	% mol/mol
Ala (A)	32	6.7
Arg (R)	26	5.5
Asn (N)	22	4.6
Asp (D)	35	7.4
Cys (C)	5	1.1
Gln (Q)	17	3.6
Glu (E)	31	6.5
Gly (G)	30	6.3
His (H)	19	4.0
Ile (I)	25	5.3
Leu (L)	40	8.4
Lys (K)	36	7.6
Met (M)	7	1.5
Phe (F)	28	5.9
Pro (P)	18	3.8
Ser (S)	28	5.9
Thr (T)	15	3.2
Trp (W)	11	2.3
Tyr (Y)	22	4.6
Val (V)	29	6.1

Table 11 Atomic composition of CC6 as determined with ExPaSy-ProtParam (Gasteiger *et al.*, 2005).

Element	Number of atoms
Carbon (C)	2500
Hydrogen (H)	3784
Nitrogen (N)	678
Oxygen (O)	713
Sulphur (S)	12

3.3.3 Protein Model (3D Structure). A 3D model of EC7 was generated via an automated homology modeling program, ESyPred3D (Fig 14). The structure was validated using ProQ and received a LG-score of 2.509 and a MaxSub score of 0.260 indicating that the model ranged from very good to fairly good, depending on the selected criteria (Table 4). Similar to CC1 and CC6, phylogenetic analysis of the protein sequence identified the protein as a glycoside hydrolase family 5 (GHF5) protein (Chapter 3, Fig 1). The 3D model of EC7 did not produce the classic $(\alpha/\beta)_8$ TIM barrel fold associated with GHF5 proteins (Fig 14), but rather displayed the characteristic $(\alpha)_8$ TIM barrel fold with only two parallel β-strands instead of eight. Alignment of the amino acid sequence identified the glutamic acid residues associated with GHF5 enzymes as Glu-203 and Glu-284 (Fig 15). However, given the altered 3D model of EC7, the previously identified catalytic glutamic acids were not located at the conserved C-terminal end of β-strands 4 and 7 (Fig 16). Such conformational changes could dramatically alter both the specificity and efficiency of the enzyme (Whiteman *et al.*, 2003; Romanos *et al.*, 1992).

It should be noted that the classification of GHs into GHFs is an on-going and dynamic process. In 2010, numerous GHF5 glucuronoxylan xylanohydrolases were found to be more closely related to GHF30 members and were subsequently re-classified accordingly (St John *et al.*, 2010). Even more recently, members of GHF5 were further classified into 51 sub-families on the basis of catalytic specificity (Aspeborg *et al.*, 2012). However, 20 sub-families were not linked to any confirmed enzymatic activity raising questions regarding their GHF5 status. As a result, the re-classification of EC7 within a new or alternative GFH would not be seen as unlikely.

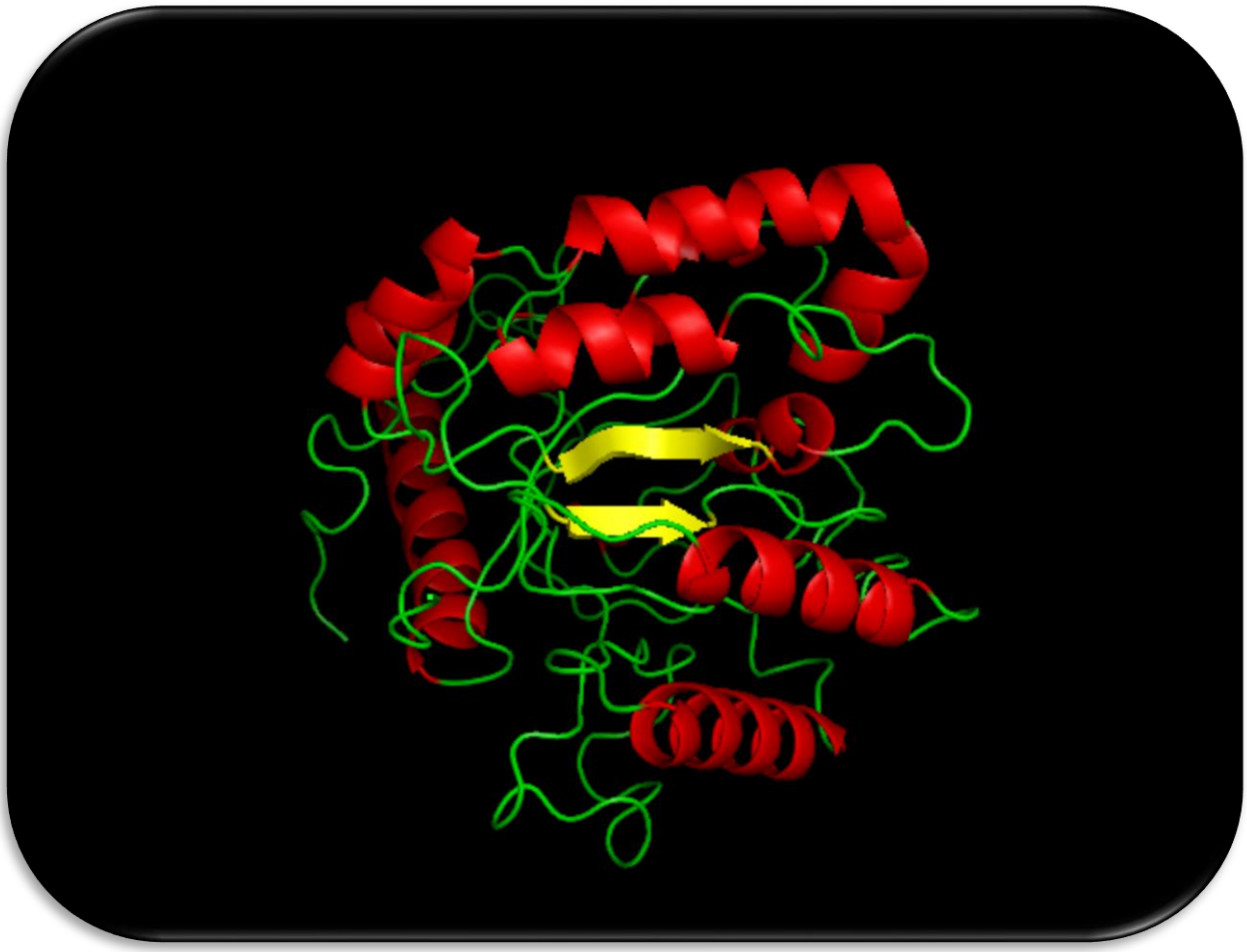

Figure 14 Three-dimensional model of the putative enzyme EC7 generated by the automated homology modeling program ESyPred3D (Lambert *et al.*, 2002). EC7 model LG score = 2.509 (Wallner *et al.*, 2003). An LG score > 2.5 indicates a very good model.

```
        10         20         30         40         50         60
MSSDGPDRGF LKVSGKDITL DGKPIILRVN MENFITGYAG HEHQARQALK QVLGTEKYNY

        70         80         90        100        110        120
FFEKFLEYFF AEDDANFFAS LGLNCIRIPV NYHHFEDDMN PRVFKRDGLK HLDRVIQIVC

       130        140        150        160        170        180
RSVHRTVTKP LSLAFIQCAK YGIYTVIDLH AAPGGQNFDW HSDNPTHKAL FYEHKDFQDR

       190        200        210        220        230        240
TVFIWENIAR DNAWVAGYNP LNEPSDEQHV RLVAFYNRIE KAIRAIDSNH ILFLDGKLTA

       250        260        270        280        290        300
NCYGFPNPPS LYEGSKEQIQ FHVDSYNGKT EYMRKHGSPV WVGEFGPVYQ TSEDGYPDWK

       310        320        330        340        350        360
HINDTRFDVL QLQLDIYAKA RASWSIWLYK DIGFQGMIYA GEDTAYVKLL KEFLHKKKVA

       370        380        390        400        410        420
AADKWGADDR AVRAMFAPLE SWLLETVPSI SDRYPQDWSV GEHLSRLVRN MLLSEELVKE

       430        440        450        460        470
YAEHFRGKSH KELDELAKSF KFSNCTQRKR LNDVLKSGSE RGIDEKKSLW QVGEKV
```

Figure 15 Amino acid sequence of EC7 highlighting the catalytic residues in red. *Predicted catalytic acid/base; **Predicted catalytic nucleophile.

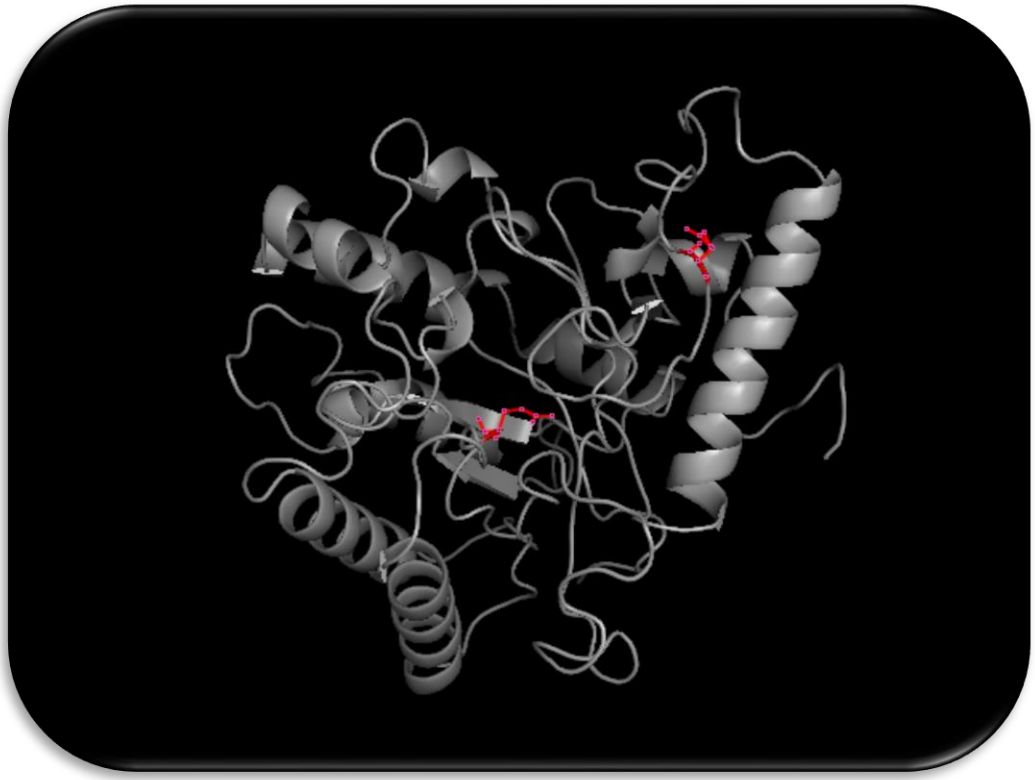

Figure 16 Three-dimensional model of the putative enzyme EC7 generated by the automated homology modeling program, ESyPred3D (Lambert *et al.*, 2002). The catalytic glutamic acid residues (Glu-203 and Glu-284) are shown in red. CC6 model LG score = 2.509 (Wallner *et al.*, 2003).
LG score > 2 indicates a very good model.

3.3.4 Protein Localization. As mentioned previously, the large size of lignocellulosic constituents precludes their entry into the eukaryotic cell. To determine the cellular location of EC7 a green fluorescent protein (GFP) was cloned in frame at the C-terminal end of the recombinant EC7, and the resulting recombinant plasmid, CIP3-EC7:GFP (Table 3), was transformed into *C. neoformans* var. *grubii* CBS 10867. No fluorescence was observed within any of the transformants or their filtered supernatant and is suspected to be as a result of protein mis-folding. Similar to poly-histidine affinity tags (His•Tag), the optimal placement of GFPs at either the C- or N-terminus of the recombinant protein is protein specific (Terpe, 2003; Halliwell *et al*., 2001). Incorrect placement of the GFP can alter the conformation, solubility and activity of the protein (Wu *et al.*, 1999). Insoluble or mis-folded protein would be exported to the cytosol, linked to ubiquitin, and targeted for protein degradation (Gasser *et al.*, 2008).

4. CONCLUSIONS

The physical and chemical characteristics of two putative cellulases (CC1 and CC6) and one endo-glucanase (EC7) identified in the genome *of C. neoformans* var. *grubii* ATCC H99, were evaluated. Using various online tools, including automated homology modeling, the protein sequences of each glycoside hydrolase were analyzed, and it was concluded that both CC1 and CC6 are putative cellulases. Both proteins displayed the classical $(\alpha/\beta)_8$ TIM barrel fold associated with GHF5 enzymes as well as the conserved glutamic acid catalytic residues located at the C-terminal end of β-strands 4 and 7. Green fluorescent protein tagging confirmed that the predicted signal peptide of CC6 was functional and that the cellulase CC6 does form part of the secretome of *C. neoformans* var. *grubii* ATCC H99.

Characterization of EC7 revealed marked differences with regards to its physical, chemical and structural properties when compared to CC1 and CC6. The putative endo-glucanase was found to have a higher than average pI compared to most fungal endo-glucanases, and despite the absence of a signal peptide, the protein is predicted to be extremely hydrophobic, an essential characteristic of secreted proteins. Futhermore, the protein structure did not produce the expected $(\alpha/\beta)_8$ TIM barrel fold associated with

GHF5 enzymes. Further characterization of CC6 and EC7, as recorded in the following chapter, may reveal both the functionality and specificity of these two enzymes.

5. REFERENCES

Aspeborg H., Coutinho P. M., Wang Y., Brumer H. 3rd, Henrissat B. (2012). Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5). *BMC Evolutionary Biology* **20(12):** 186-202.

Auron P. E., Warner S. J., Webb A. C., Cannon J. G., Bernheim H. A., McAdam K. J., Rosenwasser L. J., LoPreste G., Mucci S. F., Dinarello C. A. (1987). Studies on the molecular nature of human interleukin 1. *Journal of Immunology* **138:** 1447-56.

Baldrian P., Valaskova V (2008). Degradation of cellulose by basidiomycetous fungi. *Federation of European Microbiological Society Microbiology Reviews* **32:** 501-521.

Bayer E. A., Chanzy H., Lamed R., Shoham Y. (1998). Cellulose, cellulases and cellulosomes. *Current Opinions in Structural Biology* **8:** 548-557.

Betz S. F. (1993). Disulfide bonds and the stability of globular proteins. *Protein Science* **2:** 1551-1558.

Bhattacharjee B., Roy A., Majumder A. L. (1993). Carboxymethylcellulase from *Lenzites saepiaria*, a brown rotter. *Biochemistry and Molecular Biology International* **30:** 1143–1152.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Boulianne R. P., Liu Y., Aebi M., Lu B. C., Kües U. (2000). Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway. *Microbiology* **146:** 1841–1853.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Chen P., Sapperstein S. K., Choi J.D., Michaelis S. (1997). Biogenesis of the *Saccharomyces cerevisiae* mating pheromone a-factor. *Journal of Cell Biology* **136:** 251–269.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (eds.) *Current Protocols in Molecular Biology.* New York: Wiley Online Library, Chapter 2.

de Souza W. R. (2013). Microbial degradation of lignocellulosic biomass In: Chandel A. K., da Silva S. S. (eds.) *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization*. New York: InTech, Chapter 9.

Ding S. J., Buswell J. A. Ge W. (2001). Endoglucanase I from the edible straw mushroom, *Volvariella volvacae*: purification, characterization, cloning and expression. *European Journal of Biochemistry* **268:** 5687-5695.

du Plessis L., Rose S. H., Van Zyl W. H. (2010). Exploring improved endo-glucanase expression in *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology* **86:** 1503–1511.

Emanuelsson O., Nielsen H., Brunak S., von Heijne G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300:** 1005-1016.

Fraser J. A., Subaran R. L., Nichols C. B., Heitman, J. (2003). Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. *Eukaryotic Cell* **2:** 1036–1045.

Fortes S. T., Lazéra M. S., Nishikawa M. M., Macedo R. C., Wanke B. (2001). First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. *Mycoses.* **44(5):** 137-40

Galliano H., Gas G., Sevis J. L., Boudet A. M. (1991). Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. *Enzyme* and *Microbial Technol*ogy **13:** 478-82.

Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M. R., Appel R. D., Bairoch A. (2005). *Protein identification and analysis tools on the ExPASy server*. In: John M. Walker (ed.) *The Proteomics Protocols Handbook*. United Kingdom: Humana Press, 571-607.

Gasser B., Saloheimo M., Rinas U., Dragosits M., Rodríguez-Carmona E., Baumann K., Giuliani M., Parrilli E., Branduardi P., Lang C., Porro D., Ferrer P., Tutino M. L., Mattanovich D., Villaverde A. (2008). Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microbial Cell Factories* **7(11):** 1-18.

Guruprasad K, Reddy BV, Pandit MW (1990). Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence *Protein Engineering* **4(2):** 155–61.

Halliwell C. M., Morgan G., Ou C. P., Cass A. E. (2001). Introduction of a (poly)histidine tag in L-lactate dehydrogenase produces a mixture of active and inactive molecules. *Analytical Biochemistry* **295:** 257–261.

Henderson L. J. (1908). Concerning the relationship between the strength of acids and their capacity to preserve neutrality. *American Journal of Physiology* **21(4):** 173-179.

Ikai A. (1980). Thermostability and aliphatic index of globular proteins. *Journal of Biochemistry* **88(6):** 1895-1898.

Kyte J., Doolittle R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157:** 105-132.

Lambert C., Leonard N., De Bolle X., Depiereux E. (2002). ESyPred3D: Prediction of proteins 3D structures. *Bioinformatics* **18(9):** 1250-1256.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

Liu L., Tewari R. P., Williamson P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity* **67(11):** 6034-6039.

Lobarzewski J. (1990). The characteristics and functions of the peroxidases from *Trametes versicolor* in lignin biotransformation. *Journal of Biotechnol*ogy **13(2-3):** 111-117.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T. R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M.,

Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science* **307:** 1321-1324.

Lum G., Min X. J. (2011). FunSecKB: the Fungal Sectretome KnowlegeBase. *Database - the Journal of Biological Databases and Curation.* doi: 10.1093/database/bar001.

Min K. L., Kim Y. H., Kim Y. W., Jung H. S., Hah Y. C. (2001). Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis. Archives of Biochemistry and Biophysics* **392(2):** 279-286.

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. dissertation, University of Delhi, Delhi, India.

Oikawa T., Tsukagawa Y., Soda K. (1998). Endo-beta-glucanase secreted by a psychrotrophic yeast: purification and characterization. *Bioscience Biotechnology and Biochemistry* **62:** 1751–1756.

Petersen T. N., Brunak S., von Heijne G., Nielsen H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* **8:** 785-786.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Rodrigues M. L., Djordjevic J. T. (2012). Unraveling secretion in *Cryptococcus neoformans*: More than one way to skin a cat. *Mycopathologia* **173:** 407–418.

Romanos M. A., Scorer C. A., Clare J. J. (1992). Foreign gene expression in yeast: A review. *Yeast* **8:** 423-488.

Sadana J. C., Lachke A. H., Patil R. V. (1984). Endo-(1-4)-beta-D-glucanases from *Sclerotium rolfsii* – purification, substrate specificity, and mode of action. *Carbohydrate Research* **133:** 297–312.

Sali A., **Blundell T. L. (1993).** Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology* **234:** 779-815.

Sambrook J., Fritsch E. F., Maniatis T. (1989). Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Sanchez C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advances* **27:** 185-194.

St John F. J., González J. M., Pozharski E. (2010). Consolidation of glycosyl hydrolase family 30: a dual domain 4/7 hydrolase family consisting of two structurally distinct groups. *Federation of European Biochemical Societies Letters* **584(21)**: 4435-41.

Terpe K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology* **60:** 523–533.

Thornton J. M. (1981). Disulphide bridges in globular proteins. *Journal of Molecular Biology* **151:** 261-268.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil, *Medical Mycology* **41(5):** 383-390.

Tsang A., Butler G., Powlowski J., Panosko E. A., Baker S. E. (2009). Analytical and computational approaches to define the *Aspergillus niger* secretome. *Fungal Genetics and Biol*ogy **4:** 153–160.

Valderrama B., Oliver P., Medrano-Soto A., Vazquez-Duhalt R. (2003). Evolutionary and structural diversity of fungal laccases. *Antonie van Leeuwenhoek.* **84:** 289–299.

Varma A., Edman J. C., Kwon-Chung K. J. (1992). Molecular and genetic analysis of URA5 transformants of *Cryptococcus neoformans. Infection and Immunity* **60(3):** 1101-1108.

Wallner B., Elofsson A. (2003). Can correct protein models be identified? *Protein Science* **12(5):** 1073-1086.

Williamson P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* **2:** 99-107.

Whiteman P., Handford P. A. (2003). Defective secretion of recombinant fragments of fibrillin-1: implications of protein misfolding for the pathogenesis of Marfan syndrome and related disorders. *Human Molecular Genetics* **12(7):** 727–737.

Wickes B. L., Edman J. C. (1995). The *Cryptococcus neoformans* GAL7 gene and its use as an inducible promoter. *Molecular Microbiology* **16:** 1099–1109.

Wu J., Filutowicz M. (1999). Hexahistidine (His6)-tag dependent protein dimerization: a cautionary tale. *Acta Biochimica Polonica* **46:** 591–599.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*, Amsterdam: Elsevier., 77-98.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infection and Immunity* **69(9):** 5589-5596.

CHAPTER 5

Heterologous expression of glycoside hydrolases from *Cryptococcus neoformans* var. *grubii* in recombinant hosts.

Heterologous expression of glycoside hydrolases from *Cryptococcus neoformans* var. *grubii* in recombinant hosts.

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Lignocellulose is comprised of a three predominant polymers, namely cellulose, hemi-cellulose and lignin. The degradation of cellulose is achieved by a combination of enzymes, including β-1,4-endo-glucanases, β-1,4-exo-glucanases and β-glucosidases. Completion of the cryptococcal genome sequence allowed for the identification of two putative cellulases (CC1 and CC6) and an endo-glucanase (EC7), however, the activity of each has not yet been determined. The aim of this study was to express recombinant copies of the cryptococcal cellulase CC6 and endo-glucanase EC7 and confirm their activity. The recombinant EC7 protein was successfully expressed in *Escherichia coli* BL21 (DE3) ROS 2 and was able to hydrolyze carboxymethyl cellulose in plate assays. Both the recombinant CC6 and EC7 proteins failed to express correctly in *Saccharomyces cerevisiae*, which may be as a result of incorrect or hyper-glycosylation. This is the first known report of the isolation of an endo-glucanase from the human pathogen *Cryptococcus neoformans* var. *grubii*.

1. INTRODUCTION

Lignocellulose is comprised of three predominant polymers, namely cellulose, hemi-cellulose and lignin (Higuchi, 1997; Fengel *et al*., 1989). Cellulose is a linear polymer of β-1,4 glucosyl units that self-assemble to form a crystalline structure (Klemm *et al.*, 1998). Similarly, hemi-cellulose is a linear homo- or hetero-polymer with various side chains and is usually comprised of five different sugars, namely L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose (Fengel *et al.*, 1989). The amorphous nature of hemi-cellulose ensures that the polymer is more readily hydrolyzed than cellulose.

The degradation of cellulosic compounds is achieved by a combination of various enzymes including β-1,4-endo-glucanases, β-1,4-exo-glucanases (cellobiohydrolases) and β-glucosidases (Withers, 2001; Birsan *et al.*, 1998; Teeri, 1997). Endo-glucanases attack amorphous regions, cleaving the cellulose fibres internally to increase the availability of free chain ends. Cellobiose units are removed from these chain ends by exo-glucanase and are subsequently hydrolyzed by β-glucosidase to yield two glucose residues.

Completion of the cryptococcal genome sequence allowed for the identification of two putative cellulase enzymes, CC1 and CC6, and an endo-glucanase, EC7 (Chapter 3). All were grouped within the glycoside hydrolase family 5 (GHF5), which include numerous fungal cellulases. Both the putative enzymes CC1 and CC6 possess the classical $(\alpha/\beta)_8$ TIM barrel fold associated with GHF5 proteins and display chemical characteristics common amongst other fungal cellulases (Chapter 4). However, the endo-glucanase EC7 lacked the classic protein structure of GHF5 and has an almost neutral pI (6.56) as opposed to the usual acidic values reported for other fungal cellulases.

To further characterize CC6 and EC7, the aim of this study was to determine the activity of these enzymes by evaluating them in a recombinant host grown on a medium containing a cellulosic compound. This chapter therefore describes attempts to express the cryptococcal cellulase, CC6 and the endo-glucanase, EC7 in *Saccharomyces cerevisiae* and/or *Escherichia coli* to confirm the activity of these enzymes on a specific cellulosic substrate.

2. MATERIALS AND METHODS

2.1 Strains, Plasmids, Media and Culture Conditions

Saccharomyces cerevisiae, *Bacillus cereus* and *Escherichia coli* strains used in this study are listed in Table 1. Cloning vectors and final plasmid constructs generated during this study are listed in Table 2 and Table 3, respectively. Yeast strains were maintained by periodic transfer to yeast peptone glucose (YPG; pH 5.5) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol. Cultures of *E. coli* were grown in either Luria-Bertani broth (LB, pH 7.0) or on Luria-Bertani agar (LA, pH 7.0) plates (Sambrook *et al.*, 1989). Yeast transformants were selected and maintained by periodic transfer to synthetic complete medium without uracil (SC^{-URA}, pH 6.0; du Plessis *et al*., 2010) plates. Bacterial transformants were selected and maintained by periodic transfer to LA (pH 7.0) plates supplemented either with 100 mg/L ampicillin (LA^{Ap+}) or 50 mg/L kanamycin (LA^{Km+}). All media were prepared as per the manufacturer's instructions.

2.2 Plasmid Construction

Polymerase chain reactions (PCR) were performed in 20 µL reaction volumes using High Fidelity PCR Enzyme Mix (Fermentas). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at primer Tm (Table 4) for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products were separated in a Tris-EDTA agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining (Chory *et al.*, 1999).

The previously identified CC6 and EC7 proteins (Chapter 3) were selected for further investigation with regards to their enzyme activity. Each gene was cloned into the yeast expression vector yXYNSEC (Table 2, Fig 1) and expressed in *S. cerevisiae* strains Y294 and NI-C-D4. In addition, the gene coding for EC7 was cloned into the expression vectors pET21a(+) and pET28a(+), which contain and C- and N-terminal poly-histidine affinity tags (His•Tag), respectively, for expression in *E. coli* BL21 (DE3) Rosetta 2. Standard protocols were followed for DNA manipulations (Sambrook *et al.*, 1989). Enzymes for restriction digests and ligations were sourced from Fermentas

(Massachusetts, USA) and applied as per the manufacturers' instructions. Digested DNA was eluted from agarose gels using the GeneJET Gel Extraction kit (Fermentas). Details regarding the various plasmids constructs are listed and Table 3 in shown in Fig 1.

Table 1 Microbial strains used during this study.

Strain	Genotype	Reference or Source
Yeast and Bacterial strains		
Saccharomyces cerevisiae Y294	*MATα leu2-3,112 ura3-52 his3 trp1-289*	ATCC 201160
Saccharomyces cerevisiae NI-C-D4	*MATα, trp1, ura3, pep4*	Wang *et al*., 2001
Bacillus cereus CAB1112	Wild type	Culture collection Alfred Botha, Stellenbosch University, South Africa
Escherichia coli DH5α	ϕ80Δ*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp., Wisconsin, USA
Escherichia coli BL21(DE3) Rosetta 2	F^-*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	Novagen, Darmstadt, Germany

Table 2 Plasmids used during this study.

Plasmid	Description	Reference or Source
Plasmid vectors		
pGEM-T-Easy	Ap^r, T-tailed PCR product cloning vector	Promega Corp., Wisconsin, USA
yXYNSEC	Ap^r, *URA3 PGK1*$_P$-XYNSEC-*PGK1*$_T$	van Rooyen *et al*., 2005
pET21a(+)	Ap^r, T7 expression plasmid	Novagen, Darmstadt, Germany
pET28a(+)	Km^r, T7 expression plasmid	Novagen, Darmstadt, Germany

Table 3 Plasmids constructed during this study.

Plasmid	Description	Reference or Source
yXYNSEC-CC6	Ap^r, *URA5*, 1293-bp *CC6* PCR *EcoRI-XhoI* product cloned into yXYNSEC	This study (Fig 1)
yXYNSEC-EC7	Ap^r, *URA3*, 1428-bp *EC7* PCR *EcoRI-XhoI* product cloned into yXYNSEC	This study (Fig 1)
pET21a(+)-EC7	Ap^r, 1428-bp *NdeI-NotI* fragment containing the *EC7* gene cloned into pET21a	This study (Fig 1)
pET28a(+)-EC7	Km^r, 1428-bp-*NdeI-NotI* fragment containing the *EC7* gene cloned into pET28a	This study (Fig 1)

Table 4 Primer sets for cloning. All primers were produced by Inqaba Biotec, Gauteng, South Africa.

Primer	Primer sequence	Primer set Tm (°C)
CC6 xyn F	5′-ATATCGCGAATGCGTTTGCTCATC-3′	62
CC6 xyn R	5′-ATACTGGAGCTAGCCACATAACGAAG-3′	
EC7 xyn F	5′-ATATCAATTGATGAGCTCCGACGGT-3′	62
EC7 xyn R	5′-ATATGTCGACTCACACCTTCTCACCAAC-3′	
EC7 pET28 F	5′- ATATCATATGAGCTCCGACGG-3′	60
EC7 pET28 R	5′- ATATGCGGCCGCCACCTTCTCACCAAC-3′	
EC7 pET21 F	5′- ATATCATATGAGCTCCGACGG -3′	60
EC7 pET21 R	5′-ATATGCGGCCGCCACCTTCTCACCAAC -3′	

2.3 Yeast Transformation

Saccharomyces cerevisiae strains Y294 and NI-C-D4 were transformed with the recombinant plasmid yXYNSEC-CC6 or yXYNSEC-EC7 using the lithium acetate/DMSO (Hill *et al.*, 1991). Transformants were selected for growth on SC^{-URA} plates after incubation at 30 °C for five days. Successful transformation was confirmed by means of colony PCR using the primers sets CC6 xyn F/R and EC7 xyn F/R (Table 4).

Polymerase chain reactions (PCR) were performed in 20 µL reaction volumes using 2X PCR Master Mix (Fermentas). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 8 min, denaturation at 95 °C for 45 s, annealing 62 °C for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products were separated and visualized as previously described (Chory *et al.*, 1999).

2.4 Bacterial Transformation

Escherichia coli BL21(DE3) ROS2 was transformed with the recombinant plasmids pET21a-EC7 and pET28a-EC7 using the heat shock method (Sambrook *et al.*, 1989) and selected on LA^{Ap+} or LA^{Km+} after 24 hours incubation at 37 °C. The successful transformation of *Escherichia coli* BL21(DE3) ROS2 with the recombinant plasmids pET21a-EC7 and pET28a-EC7 was confirmed by means of colony PCR using the primers sets EC7 pET21 F/R and EC7 pET28 F/R (Table 4). Polymerase chain reactions (PCR) were performed in 20 µL reaction volumes using 2X PCR Master Mix (Fermentas). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 8 min, denaturation at 95 °C for 45 s, annealing 60 °C for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products were separated and visualized as previously described (Chory *et al.*, 1999).

2.5 Protein Isolation from *S. cerevisiae*

Total cellular proteins were isolated from *S. cerevisiae* Y294 and NI-C-D4 transformant strains using the Yeast Protein Kit (Zymo Research, California, USA) and subjected to SDS-PAGE analysis. To 15 µL of each sample, 5 µL of reducing sample buffer [12 % SDS (w/v); 6 % β-mercaptoetanol (v/v); 30 % glycerol (w/v); 0.05 % Coomassie blue G-250 (w/v) and 150 mM Tris-HCl (pH 7.0)] was added and the samples boiled (5 min, 95 °C). The protein fractions were separated on a 12 % (v/v) SDS-PAGE (Laemmli, 1970) together with the PageRuler Unstained Protein Ladder (Fermentas). Gel electrophoresis was conducted at 120 volts for approximately 2 hours, followed by visualization of protein bands with the rapid silver staining method (Bloom *et al.*, 1987).

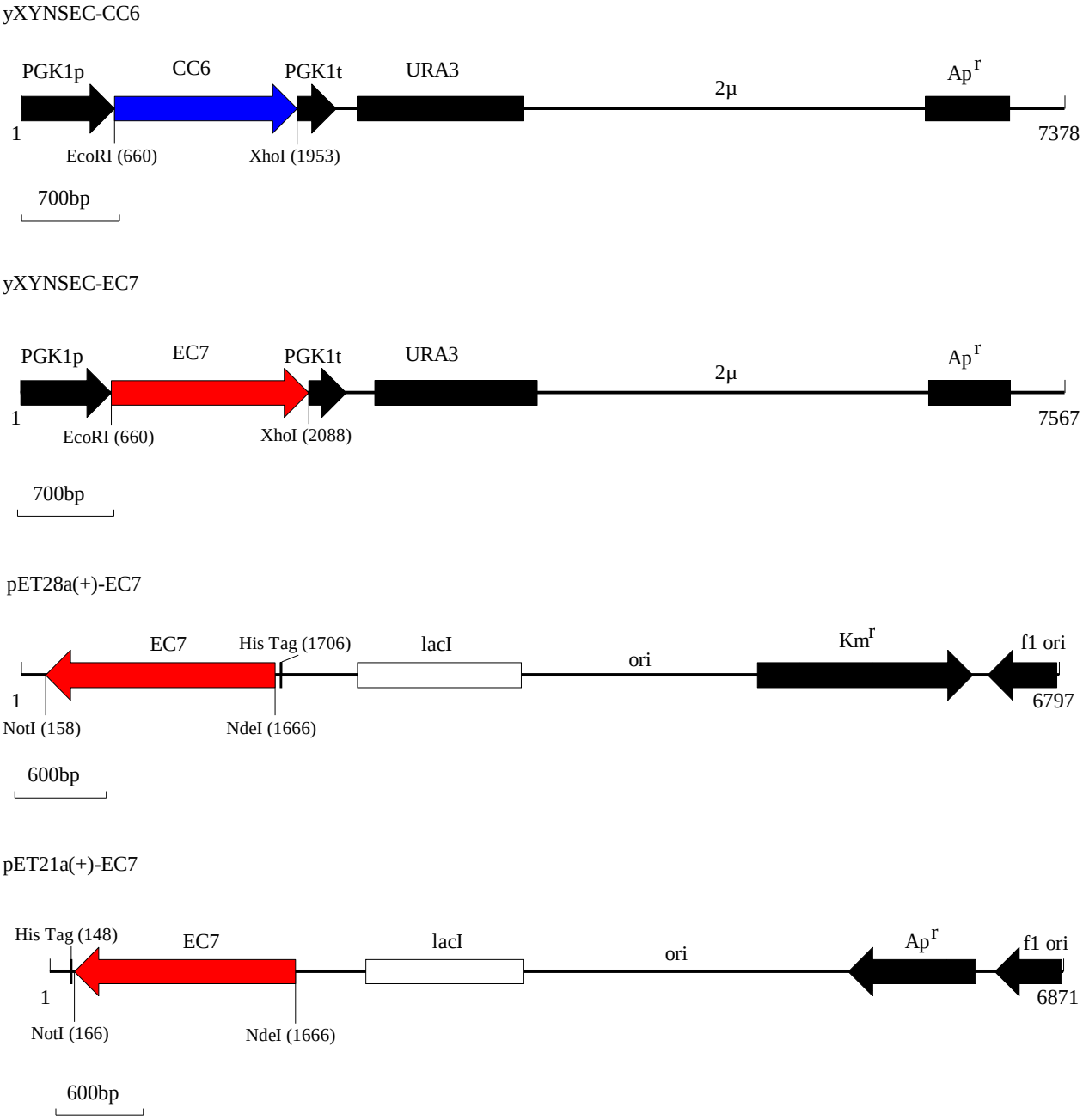

Figure 1 Plasmids constructed during this study to express recombinant copies of CC6 and EC7 in *Saccharomyces cerevisiae* and *Escherichia coli*

2.6 Induction of the Recombinant EC7 Protein in *E. coli*

Induction of the Novagen pET system (Darmstadt, Germany) in *E.coli* was modified method to ensure that the recombinant protein EC7 would occur predominantly within

the soluble fraction. Transformed strains were incubated in 5 ml LB (pH 7.0), supplemented with either 100 mg/L ampicillin or 50 mg/L kanamycin, overnight at 30 °C on a tissue culture roll drum (7 rpm). Overnight cultures were added to 200 ml LB (pH 7.0), supplemented with either 100 mg/L ampicillin or 50 mg/L kanamycin, in 1 L conical flasks. Cultures were re-incubated at 26 °C until an absorbance at 600 nm reached 0.6 units. A final concentration of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and cultures were incubated at 26 °C for approximately 18 hours. The cultures were collected via centrifugation for 5 min at 7740 RCF (Beckman Coulter, Avanti J-E; California, USA) and the pellets subjected to protein isolation.

2.7 Recombinant Protein Isolation from *E. coli*

Soluble recombinant protein was isolated from transformed *Escherichia coli* BL21 (DE3) ROS2 strains using the BugBuster Protein Extraction reagent (Novagen, Darmstadt, Germany). The recombinant His•tagged protein was purified using Novagen His•Bind Resin Ni-charged with His•Bind Quick Buffer Set (Novagen) and subjected to SDS-PAGE analysis. To 15 µL of each sample, 5 µL of reducing sample buffer [12 % SDS (w/v); 6 % β-mercaptoetanol (v/v); 30 % glycerol (w/v); 0.05 % Coomassie blue G-250 (w/v) and 150 mM Tris-HCl (pH 7.0)] was added and the samples boiled (5 min., 95 °C The protein fractions were separated on a 12 % (v/v) SDS-PAGE (Laemmli, 1970) together with the PageRuler Unstained Protein Ladder (Fermentas). Gel electrophoresis was conducted at 120 volts for approximately 2 hours, followed by visualization of protein bands with the rapid silver staining method (Bloom *et al.*, 1987). The purified recombinant EC7 His•Tag protein was dialyzed overnight using either 50 mM sodium citrate buffer (pH 5.0; 6.0) or 50 mM citrate phosphate buffer (pH 7.0). Dialyzed protein was stored at – 80 °C and evaluated for enzyme activity.

2.8 Enzyme Activity Assays

Liquid endo-β-1,4-glucanase activity was assayed according to the method previously described (Bailey *et al.*, 1992) using 1 % (w/v) carboxymethyl-cellulose (CMC, Sigma, Gauteng, South Africa) as the substrate at 50 °C. Serial dilutions of purified recombinant EC7 His•Tag protein in 50 mM sodium citrate buffer (pH 5.0; 6.0) and 50 mM citrate

phosphate buffer (pH 7.0) served as enzyme solution. The amount of released sugar was determined in triplicate by the di-nitro-salicylic (DNS) acid method (Miller *et al*., 1960).

For plate assays *E. coli* pET21a-EC7 transformants were inoculated onto LA (pH 7.0) plates, supplemented with 0.1 % (w/v) CMC and 0.2 mM IPTG, and incubated at 22 °C for one week (De Koker *et al.*, 2000). Plates were stained with 0.1 % congo red solution (B&M Scientific, Western Cape, South Africa) for 15 min and de-stained with a 1 M sodium chloride (NaCl) solution for 15 min. Plates were examined for the production of clear zones indicative of endo-glucanase activity. *Bacillus cereus* CAB 1112 served as positive control, while *E. coli* pET21a transformants served as negative control. All plate assays were conducted in triplicate

3. RESULTS AND DISCUSSION

3.1 Protein Expression and Characterization

The CC6 and EC7 genes were cloned into the yeast expression vector yXYNSEC under control of the phosphoglycerate kinase (PGK) promoter and terminator (Table 3, Fig 1) and transformed into *S. cerevisiae* Y294 and confirmed by colony PCR (data not shown). Unfortunately, neither of the recombinant proteins could be detected within the soluble fraction when the isolated proteins were visualized on a 12 % SDS-PAGE gel (results not shown).

Recombinant proteins that are expressed in *S. cerevisiae* are often hyper-glycosylated as a result of the addition of various side chains (So-Young *et al.*, 2007; Kang *et al.*, 1998; Romanos *et al.*, 1992). Incorrect or hyper-glycosylation of proteins can severely impact the overall heterogeneity of the protein in terms of its structure, solubility and activity (Whiteman *et al.*, 2003; Romanos *et al.*, 1992). Insoluble or mis-folded proteins would be exported to the cytosol linked to ubiquitin and targeted for protein degradation (Gasser *et a*l., 2008). Cellulases generally contain low amounts of glycosylation, ranging from 1 – 12 %, while some are not glycosylated at all (Cantarel *et al.*, 2009; Henriksson *et al.*, 1999; Kanda *et al.*, 1976; Kanda *et al.*, 1980; Eriksson *et al.*, 1975a; Eriksson *et al.*, 1975b). Hyper-glycosylation of the CC6 and EC7 proteins could

result in mis-folding and would therefore not be present in the soluble protein fraction isolated from the yeast cells.

The recombinant plasmids, yXYNSEC-CC6 and yXYNSEC-EC7, were subsequently transformed into *S. cerevisiae* NI-C-D4, a strain known to produce large amounts of un-glycosylated recombinant protein (Wang *et al.*, 2001), and confirmed by colony PCR (data not shown). Unfortunately, once again the recombinant proteins could still not be detected within the soluble fraction (Fig 2).

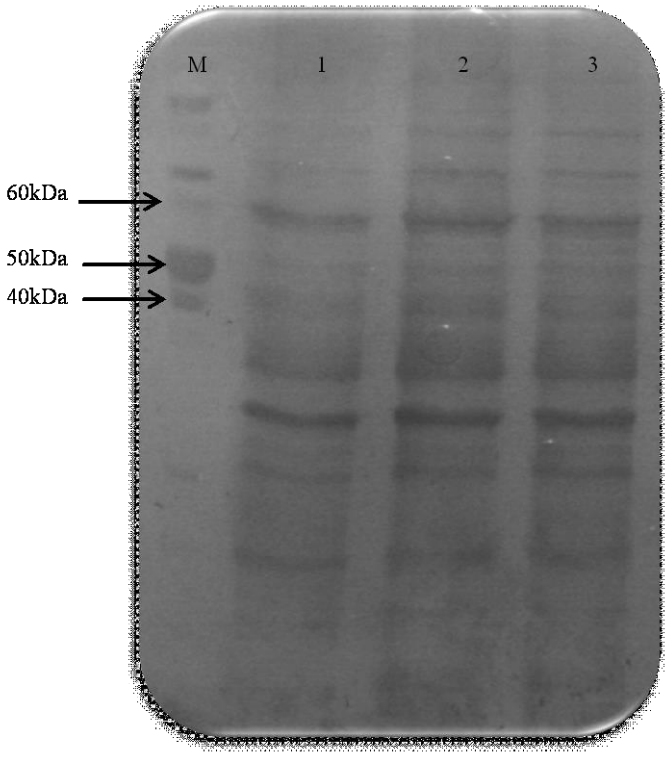

Figure 2 SDS-PAGE gel analysis of proteins extracted from recombinant *S. cerevisiae* strains: M: PageRuler Unstained Protein Ladder (Fermentas); Lane 1: *S. cerevisiae* NI-C-D4 yXYNSEC; Lane 2: *S. cerevisiae* NI-C-D4 yXYNSEC-CC6; *S. cerevisiae* NI-C-D4 yXYNSEC-EC7.

The EC7 gene was subsequently cloned into the *E. coli* expression vector pET28a (+) (Table 3 and Fig 1), that carries an N-terminal poly-histidine affinity tag (His•Tag) and expressed in a Rosetta 2 host strain, *E. coli* BL21 (DE3) ROS 2 (Table 1). A Rosetta 2 host strain was selected as it is designed to enhance the expression of eukaryotic proteins by providing transfer ribonucleic acids (tRNAs) for seven codons regarded as

rare in *E. coli*, thereby aiding in the translation of the recombinant protein (Baca *et al.*, 2000; Kurland *et al.*, 1996; Kane, 1995; Brinkmann *et al.*, 1989). Although the recombinant protein was expressed in *E.coli*, it was only present within the insoluble fraction. This could be ascribed to a number of factors, including high protein expression levels, the location of the His•Tag and the temperature used during expression.

The over-expression of recombinant proteins in *E. coli* commonly results in protein aggregation and the formation of inclusion bodies (Palomares *et al.*, 2004; Carrió *et al.*, 2002; Kopito, 2000). This is generally due to an increase in missense substitutions and errors during translation, which overloads the chaperone system (Kurland *et al.*, 1996). In many cases, the formation of inclusion bodies is preferred and be induced by culturing *E. coli* transformants at high temperatures and by employing protease deficient strains of *E. coli*. However, the subsequent re-solubilization of the recombinant protein can be inefficient and yield low volumes of active enzyme (De Bernadez., 2001).

Poly-histidine affinity tags (His•Tag) are commonly placed on either the C- or N-terminus of the recombinant protein, but optimal placement has been shown to be protein specific (Terpe, 2003; Halliwell *et al.*, 2001). Incorrect placement of the His•Tag can alter the enzymes conformation, solubility and activity (Wu *et al.*, 1999). The enzyme EC7 was therefore cloned into the *E. coli* expression vector pET21a (+) (Table 4 and Fig 1), which carries a C-terminal His•Tag, and once again expressed in *E. coli* BL21 (DE3) ROS 2 at a lower temperature. The relocation of the His•Tag, coupled to lower induction temperatures, resulted in increased solubility of the recombinant protein and subsequent purification (Fig 3). Based on SDS-PAGE analysis, the estimated molecular weight of EC7 was determined to be approximately 50 kDa. This is slightly lower than the previously calculated molecular weight of 55.130 kDa based on the number of amino acid residues (Chapter 4). This small discrepancy may be as a result of the incomplete disruption of the protein secondary structure. The protein can therefore not unfold correctly and it may migrate further than expected during SDS-PAGE analysis (Sallantin *et al.*, 1990; Hjelmeland *et al.*, 1981).

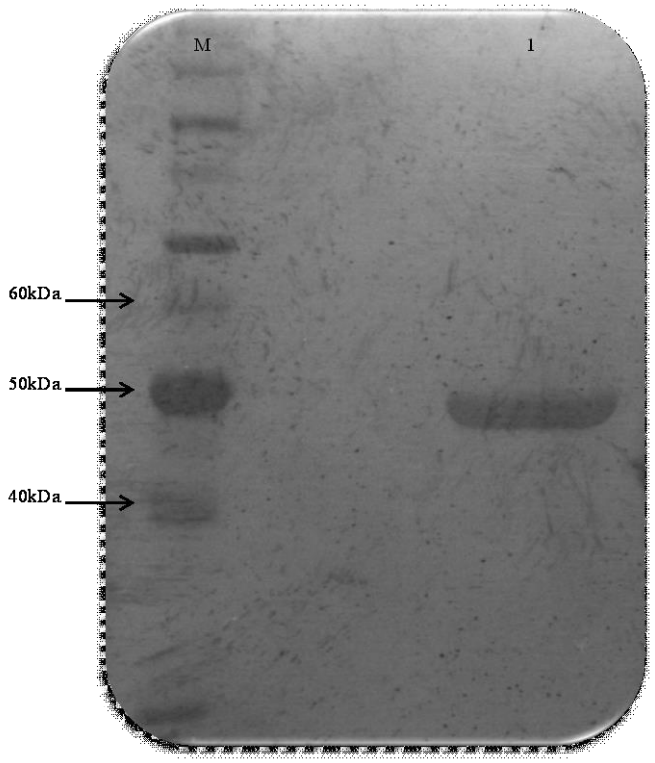

Figure 3 SDS-PAGE gel analysis of recombinant His•Tag EC7 protein. M: PageRuler Unstained Protein Ladder (Fermentas). Lane 1: EC7 His•Tag protein of approximately 50 kDa.

3.2 Enzyme Activity Assays

No endo-glucanase activity was observed for the recombinant EC7 His•Tag in liquid assays. However, the recombinant EC7 His•Tag tested positive for endo-glucanase activity in plate assays with LA^{Ap+} (pH 7.0) supplemented with 1 % (w/v) CMC and incubation at 22 °C (Fig 4).

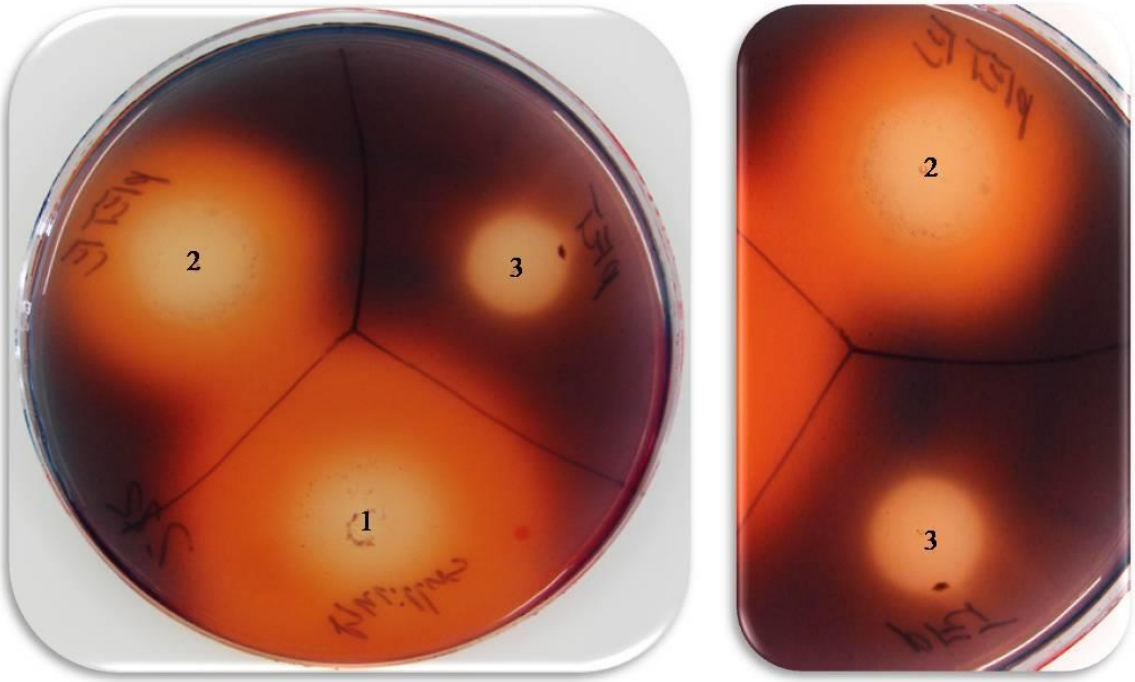

Figure 4 Plate assays for endo-glucanase activity. 1) *Bacillus cereus* CAB 1112. 2) After staining with congo red, *Escherichia coli* BL21 (DE3) ROS2 pET21a(+)-EC7 produced a halo around its colony on Luria-Bertini agar (LA, pH 7.0) supplemented with 100 mg/L ampicillin and 1 % (w/v) carboxymethyl cellulose (CMC). This is indicative of endo-glucanase activity. 3) *E. coli* BL21 (DE3) ROS2 pET21a(+) produced no halo around the colony after the plates were stained with congo red.

4. CONCLUSIONS

The cryptococcal endo-glucanase (EC7) was successfully expressed in *E. coli* BL21 (DE3) Rosetta 2 and was able to hydrolyze carboxymethyl cellulose. The tendency of *S. cerevisiae* to hyper-glycosylate recombinant proteins is believed to have inhibited the successful expression and isolation of CC6 and EC7 within a eukaryotic system. To our knowledge, this is the first report that the human pathogen *C. neoformans* var. *grubii* does possess a functional β-1,4-endo-glucanase. The functionality of this enzyme supports the contention that woody debris is indeed the true ecological niche of this yeast.

5. REFERENCES

Baca A. M., Hol W. G. (2000). Overcoming codon bias: a method for high-level overexpression of Plasmodium and other AT-rich parasite genes *in Escherichia coli*. *Internal Journal of Parasitology* **30(2):** 113-118.

Bailey M. J., Biely P., Poutanen K. (1992). Inter-laboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* **23:** 257-270.

Birsan C., Johnson P., Joshi M., MacLeod A., McIntosh L., Monem V., Nitz M., Rose D. R., Tull D., Wakarchuck W. W., Wang Q., Warren R. A. J., White A., Withers S. G. (1998). Mechanisms of cellulases and xylanases. *Biochemical Society of Transactions* **26:** 156-160.

Bloom H., Beier H., Gross H. S. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:** 93-99.

Brinkmann U., Mattes R. E., Buckel P. (1989). High-level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the dnaY gene product. *Gene* **85(1):** 109-114.

Cantarel B. L., Coutinho P. M., Rancurel C., Bernard T., Lombard V., Henrissat B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research* **37:** 233-238.

Carrió M. M., Villaverde, A. (2002). Construction and deconstruction of bacterial inclusion bodies. *Journal of Biotechnololgy* **96:** 3-12.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (eds.) *Current Protocols in Molecular Biology*. New York: Wiley Online Library, Chapter 2.

De Bernadez C. E. (2001). Protein refolding for industrial processes. *Current Opinion in Biotechnol*ogy **12:** 202-207.

De Koker T. H., Zhao J., Allsop S. F., Janse B. J. H. (2000). Isolation and enzymatic characterization of South African white rot fungi. *Mycological Research* **104:** 820-824.

du Plessis L., Rose S. H., Van Zyl W. H. (2010). Exploring improved endo-glucanase expression in *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology* **86:** 1503-1511.

Fengel D., Wegener G. (1989). Wood: Chemistry, ultrastructure, reaction. New York: Walter de Gruyter.

Eriksson K. E., Pettersson B. (1975a). Extracellular enzyme system utilized by fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for breakdown of cellulose. 1. Separation, purification and physicochemical characterization of 5 endo-1,4-beta-glucanases. *European Journal of Biochemistry* **51:** 193-206.

Eriksson K. E., Pettersson B. (1975b). Extracellular enzyme system utilized by fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for breakdown of cellulose. 3. Purification and physico-chemical characterization of an exo- 1,4-beta-glucanase. *European Journal of Biochemistry* **51:** 213-218.

Gasser B., Saloheimo M., Rinas U., Dragosits M., Rodríguez-Carmona E., Baumann K., Giuliani M., Parrilli E., Branduardi P., Lang C., Porro D., Ferrer P., Tutino M. L., Mattanovich D., Villaverde A. (2008). Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microbial Cell Factories* **7(11):** 1-18.

Halliwell C. M., Morgan G., Ou C. P., Cass A. E. (2001). Introduction of a (poly) histidine tag in L-lactate dehydrogenase produces a mixture of active and inactive molecules. *Analytical Biochemistry* **295:** 257-261.

Henriksson G., Nutt A., Henriksson H., Pettersson B., Stahlberg J., Johansson G., Pettersson G. (1999). Endoglucanase 28 (cel12A), a new *Phanerochaete chrysosporium* cellulase. *European Journal of Biochemistry* **259:** 88-95.

Higuchi T. (1997). Biochemistry and molecular biology of wood. London: Springer Verlag.

Hill J., Ian K. A., Donald G., Griffiths D. E. (1991). DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Research* **19:** 5791.

Hjelmeland L.M., Chrambach A. (1981). Electrophoresis and electrofocusing in detergent containing media: a discussion of basic concepts. *Electrophoresis* **2:** 1-11.

Kanda T., Wakabayashi K., Nisizawa K. (1976). Purification and properties of an endocellulase of avicelase type from *Irpex lacteus* (*Polyporus tulipiferae*). *Journal of Biochemistry* **79:** 977-988.

Kanda T., Wakabayashi K., Nisizawa K. (1980). Purification and properties of a lower molecular weight endo-cellulase from *Irpex lacteus* (*Polyporus tulipiferae*). *Journal of Biochemistry* **87:** 1625-1634.

Kane J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology* **6:** 494-500.

Kang H. A., Sohn J. H., Choi E. S., Chung B. H., Yu M. H., Rhee S. K. (1998). Glycosylation of human alpha 1-antitrypsin in *Saccharomyces cerevisiae* and methylotrophic yeasts. *Yeast* **14:** 371-381.

Klemm D., Philipp B., Heinze T., Heinze U., Wagenknecht W. (1998). Comprehensive cellulose chemistry. Chichester: Wiley VCH.

Kopito R. R. (2000). Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* **10:** 524-530.

Kurland C., Gallant J. (1996). Errors of heterologous protein expression. *Current Opinion in Biotechnology* **7(5):** 489-493.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Miller G. L., Blum R., Glennon W. E., Burton A. L. (1960). Measurement of carboxymethylcellulase activity. *Analytical Biochemistry* **2:** 127-132.

Palomares A. L., Estrada-Mondaca S., Ramírez O. T. (2004). Production of Recombinant Proteins: Challenges and Solutions. In: Balbas P., Lorence A. (eds.) *Recombinant Gene Expression: Reviews and Protocols. Volume 267 of Methods in Molecular Biology*. United Kingdom: Humana Press, Chapter 2.

Romanos M. A., Scorer C. A., Clare J. J. (1992). Foreign gene expression in yeast: A review. *Yeast* **8:** 423-488.

Sallantin M., Huet J. C., Demarteau C., Pernollet J. C. (1990). Reassessment of commercially available molecular weight standards for peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis using electroblotting and micro-sequencing. *Electrophoresis* **11(1):** 34-36.

Sambrook J., Fritsch E. F., Maniatis T. (1989). Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

So-Young K., Sohn J. H., Pyun Y. R., Choi E. S. (2007). Variations in Protein Glycosylation in *Hansenula polymorpha* Depending on Cell Culture Stage. *Journal of Microbiology and Biotechnology* **17(12):** 1949-1954.

Teeri T. T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in Biotechnology* **15:** 160-167.

Terpe K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology* **60:** 523-533.

Wang B. D., Chen D. C., Kuo T. T. (2001). Characterization of a *Saccharomyces cerevisiae* mutant with over secretion phenotype. *Applied Microbiology and Biotechnology* **55(6):** 712-720.

Whiteman P., Handford P. A. (2003). Defective secretion of recombinant fragments of fibrillin-1: implications of protein misfolding for the pathogenesis of Marfan syndrome and related disorders. *Human Molecular Genetics* **12(7):** 727-737.

Withers S. G. (2001). Mechanisms of glycosyl transferases and hydrolyses. *Carbohydrate Polymers* **44:** 325-337.

Wu J., Filutowicz M. (1999). Hexahistidine (His6)-tag dependent protein dimerization: a cautionary tale. *ACTA Biochimoca Polonica* **46:** 591-599.

Van Rooyen R., Hahn-Hägerdal B., La Grange D. C., Van Zyl W. H. (2005). Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. *Journal of Biotechnology* **120:** 284-295.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study.* Amsterdam: Elsevier., 77-98.

CHAPTER 6

Carbon regulation of two putative cellulases and an endo-glucanase identified in the genome of *Cryptococcus neoformans* var. *grubii*

Carbon regulation of two putative cellulases and an endo-glucanase identified in the genome of *Cryptococcus neoformans* var. *grubii*

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Cryptococcus neoformans (sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis and cryptococcal meningitis in immuno-suppressed individuals. Despite the repeated isolation of *C. neoformans* from a variety of different habitats rich in lignocellulosic material, as well as recent research indicating that this yeast is capable of using woody debris as a sole carbon and nitrogen source the lignocellulosic metabolism of this pathogen has never been identified or studied. Recently, three cellulases have been identified in *C. neoformans* var. *grubii* by means of phylogenetic screening, namely CC1, CC6 and EC7. The latter enzyme, EC7, has demonstrated endo-β-1,4-glucanase activity on carboxymethyl cellulose plate assays when expressed in *Escherichia coli*. The aim of this study was to gain insight into the transcriptional regulation of these genes in a woody environment. By using quantitative real-time polymerase chain reactions (qR-T PCR), we were able to determine that all three proteins showed varied expression profiles when the yeast was exposed to environments that differed in their carbohydrate composition. These environments ranged from media containing simple degradation products of lignocellulose, to media containing complex wood polymers. The putative cellulase (CC6) displayed the most dynamic expression profile, indicating up-regulation when *C. neoformans* var. *grubii* was exposed to mannose, galactose and cellobiose. Both the putative cellulase (CC1) and endo-glucanase (EC7) were also up-regulated in the presence of mannose. All three genes were induced in the presence of either *Acacia mearnsii* debris or an aqueous extract thereof. Further understanding of this complex regulatory system would greatly increase our understanding of the pathogen's survival in both environmental and clinical settings.

1. INTRODUCTION

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1997; Mitchell *et al.*, 1995), particularly those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). Strains of *C. neoformans* are responsible for approximately 6-10 % of all AIDS-related infections and 5 % of infections seen in organ transplant recipients (Park *et al.*, 2009; Vilchez *et al*., 2003; Husain *et al*., 2001; Liu *et al*., 1999; Williamson, 1997; Wang *et al*., 1996; Chuck *et al.*, 1989). Mortality rates are generally high, up to 50 % in organ transplant patients, despite the use of anti-fungal therapies to combat the yeast.

Transcriptional regulation studies to date have primarily focused on known virulence factors associated with the pathogenesis of *C. neoformans*, including mating, capsule production, melanin and urease synthesis, as well as the ability to withstand human physiological temperatures (Steen *et al*., 2002; Zhu *et al*., 2001; Liu *et al*., 1999). Research has determined that low levels of glucose result in an increase in melanin production, while low nitrogen levels trigger a mating response (Alspaugh *et al.*, 1997). Similarly, limiting amounts of the trace element iron have been linked to an increase in the transcription of genes associated with capsule and melanin synthesis, as well as numerous genes associated with growth at higher temperatures (Jung *et al.*, 2006; Kraus *et al.*, 2005; Janbon, 2004; Odom *et al.*, 1997; Vartivarian *et al.*, 1993).

Currently, little is known about the transcriptional regulation of *C. neoformans* within an environmental setting. Recent research demonstrated that similar to other fungi, *C. neoformans* exhibits nitrogen metabolite repression (Lee *et al.*, 2011to ensure that the yeast utilizes easily assimilated nitrogen sources before less preferred options (Marzluf *et al.*, 1997; Wiame *et al.*, 1985). The identification of two putative cellulases (CC1 and CC6) and an endo-glucanase (EC7) coded by genes within the genome of *C. neoformans* var. *grubii* (Chapter 3) raised questions as to whether these genes are regulated in a similar manner as other known cellulases.

It is generally accepted that lignocellulose itself does not induce transcription of cellulases, hemi-cellulases and ligninases as the substrate is relatively insoluble and too large to traverse the cell membrane (Suto *et al.*, 2001). It is therefore believed that a

number of lignocellulosic mono- and disaccharides are responsible for the induction of gene expression. To date, a number of carbohydrates have been shown to induce cellulase and hemi-cellulase transcription in a variety of fungi, and include cellobiose, cellotriose, D-galactose, gentiobiose, lactose, sophorose, L-sorbose and thiocellobiose (Fekete *et al.*, 2008; Seiboth *et al.*, 2007; Ding *et al.*, 2001; Chikamatsu *et al.*, 1999; Ilmèn *et al.*, 1997; Mernitz *et al.*, 1996; Morikawa *et al.*, 1995; Fowler *et al.*, 1992; Hrmova *et al.*, 1991; Bisaria *et al.*, 1989; Gritzali MaB *et al.*, 1979; Vaheri *et al.*, 1979). Similar to nitrogen metabolite repression, the presence of readily assimilated carbon sources, such as glucose, glycerol and fructose, has been shown to repress the expression of cellulases in the model organism *Trichoderma reesei* (Sidik *et al.*, 2001; Suto *et al.*, 2001; Sesták *et al.*, 1993).

With the above as background, the aim of this study was to gain insight into the regulation of the genes coding for CC1, CC6 and EC7 when *C. neoformans* var. *grubii* ATCC H99 is exposed to various lignocellulosic associated monosaccharides, disaccharides and complex wood polymers.

2. MATERIALS AND METHODS

2.1 Strain and Culture Conditions

Cryptococcus neoformans var. *grubii* ATCC H99 was obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands and maintained by periodic transfer to yeast peptone glucose (YPG, pH 5.5) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Gauteng, RSA) and incubated at 22°C . All media were prepared as per the manufacturer's instructions.

2.2 Gene Induction / Repression

A pre-inoculum of *C. neoformans* var. *grubii* ATCC H99 was cultured at 30 °C for 48 hours in 1 L conical flasks, containing 100 mL YPG (pH 5.5) on a rotary shaker (150 rpm; New Brunswick Scientific G53, Canada). Cells were collected via centrifugation for 5 min at 16278 RCF (Beckman Coulter, Avanti J-E, California, USA), washed twice in

distilled water, re-suspended in 100 mL yeast peptone broth (Merck Chemicals, South Africa; pH 5.5) and incubated at 30 °C on a rotary shaker (150 rpm; New Brunswick Scientific G53) for a further four hours. Cells were once again collected via centrifugation for 5 min at 16278 RCF (Beckman Coulter, Avanti J-E), washed twice in distilled water and re-suspended in 75 mL yeast nitrogen base (YNB, Becton Dickinson, Gauteng, South Africa; pH 6.0) supplemented with one of three simple sugars (5 % w/v), namely D-galactose, D-glucose and D-mannose or the disaccharide cellobiose (2.5 % w/v). This suspension was divided between three 250 mL conical flasks and re-incubated at 30 °C for 24 hours on a rotary shaker (150 rpm; New Brunswick Scientific G53). Biomass was subsequently harvested via centrifugation for 5 min at 13793 RCF (Biofuge fresco; Heraeus Instruments, Hanau, Germany) and subjected to ribonucleic acid (RNA) isolation as described below.

For gene induction in the presence of more complex carbohydrates, a pre-inoculum was prepared as described above, however, the 75 mL YNB (pH 6.0) suspension of *C. neoformans* var. *grubii* ATCC H99 was supplemented with either *Acacia* extract (5 % v/v) or divided and spotted onto 10 *A. mearnsii* agar plates. *Acacia mearnsii* extract was obtained by autoclaving (121 °C, 15 min) *A. mearnsii* debris suspended in water (10 % w/v) and decanting the supernatant (extract). The aqueous *A. mearnsii* debris extract was evaluated for the presence of reducing sugars (Benedict, 1909). *Acacia mearnsii* agar was prepared as described previously (Botes *et al*., 2009) and were incubated at 30 °C for one week after which yeast colonies were scraped from the plates and subjected to RNA isolation.

2.3 RNA Isolation and Real-Time PCR

Ribonucleic acid (RNA) was isolated using the RiboPure™ Yeast Kit (Ambion, California, USA) from 24 hour-induced cultures described above. The quality of the RNA was assayed in a Tris-EDTA agarose gel (1 % w/v) and quantified using a BioDrop-µLITE spectrophotometer. The RNA samples were assayed for the presence of contaminating DNA by amplifying the internal transcribed spacer (ITS) region of the ribosomal gene cluster using universal primers ITS1/4 (White *et al*., 1990, Table 1). Polymerase chain reactions (PCR) were performed in 20 µL reaction volumes with final

concentrations of ~400 ng RNA, 2.5 mM magnesium chloride ($MgCl_2$), 0.4 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer and 0.5 U Taq DNA polymerase recombinant (Fermentas, Massachusetts, USA). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products, indicative of DNA contamination, were separated in a Tris-EDTA agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999). A total of 1 µg RNA was subsequently converted to complementary deoxyribonucleic acid (cDNA) using the RevertAid Premium First Strand cDNA synthesis kit (Fermentas).

Relative gene expression of CC1, CC6 and EC7 was evaluated using quantitative real-time polymerase chain reactions (qR-T PCR) in a Roche Lightcycler (version 2.0) using specific primers (Table 1) and HOT FIREPol® EvaGreen® qPCR Mix Plus Capillary (Solis BioDyne, Tartu, Estonia). The cDNA was diluted and approximately 50 ng was used in each qR-T PCR reaction with the parameters set as follows; 95 °C for 15 min; 23 cycles of 95 °C for 15 s, at primer Tm (Table 1) for 20 s and 70 °C for 20 s with a final extension at 72 °C for 7 min. Relative gene expression was determined using the REST analysis tool (2009) (Pfaffl *et al.*, 2002) and the actin primer set (Table 1) was used as a calibrator. The amplification efficiency was determined for each set of real-time primers (Table 1) by means of standard curves. Real-time PCR was performed, as described above, using serial 10 fold dilutions of target cDNA. The amplification efficiency was subsequently calculated using the REST analysis tool (2009).

Table 1. Primer sets used for semi-quantitative real time gene expression during experimentation. All primers were produced by Inqaba Biotec, Gauteng, South Africa

Primer	Primer sequence	Primer set Tm (°C)
General primers		
ITS1	5′- TCCGTAGGTGAACCTGCGG-3′	53
ITS4	5′- TCCTCCGCTTATTGATATGC-3′	
Real time primers		
Actin-F	5′-GCCCTTGCTCCTTCTTCTAT-3′	60
Actin-R	5′-GACGATTGAGGGACCAGACT-3′	
CC1-F	5′-TTGACAGATGGAAGCTCT-3′	55
CC1-R	5′-AGAAGCATGAGAAGTCGA-3′	
CC6-F	5′-CTTATGGAAGCGACAAGATC-3′	58
CC6-R	5′-GCAGCTTGAGCAGTATCATA-3′	
EC7-F	5′-CAGACAATCCGACTCACA-3′	55
EC7-R	5′-TGTAGAATGCAACAAGGC-3′	

3. RESULTS AND DISCUSSION

3.1 Transcriptional Gene Regulation

Transcriptional regulation of CC1, CC6 and EC7 within *C. neoformans* var. *grubii* ATCC H99 was evaluated with regards to the presence of lignocellulosic constituents and substrates in the surrounding medium. Cellulose is a linear polymer comprised of β-1,4 glucosyl units (Klemm *et al.,* 1998), while hemi-cellulose is a linear homo- or hetero-polymer with various side chains and is usually comprised of five different sugars, namely L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose (Fengel *et al.*, 1989). The yeast's metabolism was therefore presented with various lignaceous components including the monosaccharides glucose and mannose, and the disaccharides cellobiose and galactose. More complex substrates were also selected namely woody debris from the tree species *A. mearnsii* and the aqueous extract thereof. *Acacia mearnsii* is classified as a hardwood species possessing higher cellulose to hemi-cellulose ratios, as well as a glucuronoxylan dominated hemi-cellulose fraction (Laine, 2005). The aqueous

extract was evaluated for the presence of reducing sugars, particularly glucose, with Benedict's solution (Benedict, 1909). All the aqueous extracts yielded a green to orange colour with a red precipitate after heating, indicating that low amounts of reducing sugars were present.

The CC1 protein displayed increased transcription levels in the presence of *A. mearnsii* debris, glucose and mannose (Table 2), but no notable transcriptional regulation was observed when galactose or the aqueous extract from the woody debris was presented to the yeast (results not shown). Mannose resulted in a substantial increase; (mean factor of 30.72), in the transcriptional level of CC1 and may be attributed to the ricin B chain (RTB) lectin located at the N-terminal of the protein (Chapter 3) that is known to bind the sugar moieties galactose or mannose similar to various carbohydrate binding modules (CBMs) (Wales *et al.*, 1991). Recently, a bound CBM found in *Clostridium thermocellum* has been shown to trigger a conformational change within an intracellular protein, the anti-sigma domain (Rsg1) in the case of *C. thermocellum*, thereby inducing the transcription of the cellulase gene (Yaniv *et al.*, 2012). Similar results have been observed in the pathogenic white rot fungus *Heterobasidion irregulare* where the transcription of genes encoding CBMs is up-regulated in the presence of cellulose (Yakovlev *et al.*, 2012). Given the lack of transcriptional up or down regulation of CC1 in the presence of galactose, it can be speculated that the RTB of CC1 is capable of binding only mannose.

The increased level of transcription, (9.22), observed for CC1 (Table 2) on woody debris was in stark contrast to the indifference of CC1 transcriptional levels towards the aqueous extract of this debris and may be due to low inducer concentrations of the reducing sugars as indicted by the Benedict's test. Many responses to external stimuli require a threshold concentration before induction or repression can occur (Alberts *et al.*, 2002; Meijer *et al.*, 1998). Also, the presence of inducers and repressors other than carbohydrates within the woody debris and aqueous extract cannot be excluded. *Acacia mearnsii* is known to produce large quantities of water-soluble polyphenolic compounds known as tannins (Nathan, 2006; Smith *et al.*, 2003). The cellulases of the human pathogens *Schizophyllum commune* and *Chaetomium globosum* have been shown to be repressed by various phenols (Varadi, 1972). In contrast, the cellulase of the brown rot

fungi *Postia placenta* and *Gloeophyllum trabeum* are induced by low concentrations of phenolics (Highley *et al.*, 1989).

Table 2. Relative gene expression levels of enzymes, CC1, CC6 and EC7 when *C. neoformans* var. *grubii* ATCC H99 was exposed to various carbon sources. Relative gene expression was determined using the REST analysis tool (2009) (Pfaffl *et al.*, 2002). Values represent a mean factor of up or down regulation when the sample group is compared with the control group. Only those carbon sources that resulted in a significant ($p < 0.05$) response are shown (n = 3).

Carbon source	**Relative gene expression**		
	Cellulase C1	**Cellulase C6**	**Endo-glucanase C7**
Acacia debris	9.22	-	-
Acacia extract	-	5.58	1.75
Cellobiose	-	81.65	-
D-galactose	-	7.50	-
D-glucose	1.91	-	-0.70
D-mannose	30.72	2.31	2.24

The cellulase CC6 displayed increased transcription levels in the presence of the aqueous debris extract, cellobiose, galactose and mannose (Table 2), but in contrast with CC1, no notable up or down regulation was observed when woody debris or D-glucose was presented to the yeast (results not shown). Cellobiose was the strongest inducer (81.65) it is known to be a common cellulase inducer in a number of fungal species including, *Aspergillus nidulans*, *Penicillium janthinellum*, *Volvariella volvaceae*, and *Trichoderma reesei* (Ding *et al.*, 2001; Chikamatsu *et al.*, 1999; Ilmèn *et al.*, 1997; Mernitz *et al.*, 1996).

The increased levels of transcription (5.58) observed for CC6 (Table 2) in the presence of the aqueous extract of *A. mearnsii* debris, was in stark contrast to the indifference of CC6 transcriptional levels towards the debris itself. This may be due to the presence of other water insoluble phenolic compounds within the woody debris that could inhibit the transcription of CC6 (Karetnikova, 2006; Varadi, 1972).

The endo-glucanase EC7 displayed increased transcription levels in the presence of the aqueous debris extract, cellobiose, and D-mannose, but transcription was repressed in the presence of glucose (-0.70) (Table 2). No notable up or down regulation was observed when woody debris or D-galactose was presented to the yeast (results not shown).

The endo-glucanase EC7 was the only evaluated protein to display carbon catabolite repression in the presence of glucose (Table 2). Carbon catabolite repression prevents the expression of certain genes required to metabolize complex carbon sources, thereby allowing the organism to fully utilize the preferred simple carbon sources (Gancedo *et al.*, 1998; Ronne, 1995). Catabolite repression of endo-glucanases by glucose is a common occurrence amongst other fungal endo-glucanases and has been observed in *Aspergillus terreus*, *Daldinia eschscholzii*, *Penicillium purpurogenum*, *T. reesei* and *V. volvacea* (Karnchanatat *et al.*, 2008; Ding *et al.*, 2001; Sidik *et al.*, 2001; Suto *et al.*, 2001; Sesták *et al.*, 1993).

Similar to CC6, the transcription levels of EC7 increased in the presence of the aqueous extract of *A. mearnsii* debris and appeared indifferent towards the debris itself. As mentioned previously, the presence of unknown repressors or low concentrations of required inducers cannot be excluded (Karetnikova, 2006; Alberts *et al.*, 2002; Meijer *et al.*, 1998; Varadi, 1972).

4. CONCLUSIONS

We examined the carbon regulation of three previously identified genes (Chapter 3) involved in the lignocellulosic metabolism of *C. neoformans* var. *grubii* ATCC H99, specifically two cellulases (CC1 and CC6) and an endo-glucanase (EC7). The cellulase CC6 displayed the most dynamic expression profile indicating up-regulation when *C. neoformans* var. *grubii* ATCC H99 was exposed to the monosaccharides mannose and galactose, and the disaccharide cellobiose. Both cellulase CC1 and endo-glucanase EC7 were up-regulated in the presence of mannose. The transcription of EC7 was inhibited by glucose, a common carbon catabolite repressor amongst other fungal endo-glucanases.

In contrast to CC1, induction of CC6 and EC7 occurred in the presence of the *A. mearnsii* aqueous extract and not the solid woody debris. It is speculated that various-

water soluble and insoluble phenolic compounds produced by *A. mearnsii* may impact on the regulation of these specific genes. Nonetheless, the induction of the cellulases CC1, CC6 and the endo-glucanase EC7 by various lignocellulosic components further support the notion that the ecological niche of *C. neoformans* var. *grubii* is woody material.

5. REFERENCES

Alberts B., Johnson A., Lewis J, Raff M., Roberts K., Walter P. (2002). General principles of cell communication. In: Alberts B., Johnson A., Lewis J, Raff M., Roberts K., Walter P. (eds.). *Molecular Biology of the Cell 4th (edn).* New York: Garland Science.

Alspaugh J., Perfect J., Heitman J. (1997). *Cryptococcus neoformans* mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. *Genes and Development* **11:** 3206 –3217.

Benedict S. R. (1909). A reagent for the detection of reducing sugars. *Journal of Biological Chemistry* **5:** 485-487.

Bisaria V. S., Mishra S. (1989). Regulatory aspects of cellulase biosynthesis and secretion. *Critical Reviews in Biotechnology* **9(2):** 61-103.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready-made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chikamatsu G., Shirai K., Kato M., Kobayashi T., Tsukagoshi N. (1999). Structure and expression properties of the endo-β-1,4-glucanase A gene from the filamentous fungus *Aspergillus nidulans*. *Federation of European Microbiological Societies Microbiology Letters* **175:** 239-245.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (eds.) *Current Protocols in Molecular Biology*. New York: Wiley Online Library, Chapter 2.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **321:** 794–799.

Ding S. J., Ge W., Buswell J. A. (2001). Endoglucanase I from the edible straw mushroom, *Volvariella volvacea*. Purification, characterization, cloning and expression. *European Journal of Biochemistry* **268(22):** 5687-5695.

Fekete E., Padra J., Szentirmai A., Karaffa L. (2008). Lactose and D-galactose catabolism in the filamentous fungus *Aspergillus nidulans. ACTA Microbiologica et Immunologica Hungarica* **55(2):** 119-24.

Fengel D., Wegener G. (1989). Wood: Chemistry, ultrastructure, reaction. New York: Walter de Gruyter.

Fowler T., Brown R. D. Jr. (1992). The bgl1 gene encoding extracellular beta-glucosidase from *Trichoderma* reesei is required for rapid induction of the cellulase complex. *Molecular Microbiol*ogy **6(21):** 3225-35.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *Journal of Clinical Microbiology* **35(9):** 2243-2251.

Gancedo J. M. (1998). Yeast carbon catabolite repression. *Microbiology and Molecular Biology Reviews* **62:** 334–361.

Gritzali MaB R. D. J. (1979). The cellulase system of *Trichoderma*: relationship betweeen purified extracellular enzymes from induced or cellulose-grown cells. *Advances in Chemistry Series* **181:** 237–60.

Highley T. L., Micales J. A. (1989). Effect of aromatic monomers on production of carbohydrate degrading enzymes by white-rot and brown-rot fungi. *Federation of European Microbiological Societies Microbiology Letters* **66:** 15-22.

Hrmova M., Petrakova E., Biely P. (1991). Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillus terreus* by homo- and heterodisaccharides composed of glucose and xylose. *Journal of General Microbiology* **137(3):** 541-547.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerging Infectious Diseases* **7:** 375-381.

Ilmèn M., Saloheimo A., Onnela M. L., Penttilaë M. E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Applied Environmental Microbiology* **63:** 1298-1306.

Janbon G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *Federation of European Microbiological Societies Yeast Research* **4:** 765–771.

Jung W. H., Sham A., White R., Kronstad J. W. (2006). Iron regulation of the major virulence factors in the AIDS-associated pathogen *Cryptococcus neoformans*. *PLoS Biology* **4(12):** e410.

Karetnikova E. A. (2006). Consumption of water-insoluble phenolic products of lignin pyrolysis by the strain *Penicillium tardum* H-2. *Prikladnaia biokhimiia i mikrobiologiia* **42(1):** 55-8.

Karnchanatat A., Petsom A., Sangvanich P., Piapukiew J., Whalley A. J. S., Reynolds C. D, Gaddd G. M., Sihanonth P. (2008). A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. *Enzyme and Microbial Technology* **42:** 404–413.

Klemm D., Philipp B., Heinze T., Heinze U., Wagenknecht W. (1998). Comprehensive cellulose chemistry. Chichester: Wiley VCH.

Kraus P. R., Nichols C. B., Heitman J. (2005). Calcium- and calcineurinin dependent roles for calmodulin in *Cryptococcus neoformans* morphogenesis and high-temperature growth. *Eukaryotic Cell* **4:** 1079–1087.

Laine C. (2005). Structure of hemicelluloses and pectins in wood and pulp. Ph.D. dissertation, Helsinki University of Technology, Helsinki, Finland.

Lee I. R., Chow E. W. L., Morrow C. A., Djordjevic J. T., Fraser J. A. (2011). Nitrogen metabolite repression of metabolism and virulence in the human fungal pathogen *Cryptococcus neoformans*. *Genetics* **188(2):** 309-323.

Liu L., Tewari R. P., Williamson P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity* **67(11):** 6034-6039.

Marzluf G. A. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiology and Molecular Biology Reviews* **61:** 17–32.

Meijer M. M., Boonstra J., Verkleij A. J., Verrips C. T. (1998). Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *Journal of Biological Chemistry* **273(24):** 102–24 107.

Mernitz G., Koch A., Henrissat B., Schultz G. (1996). Endoglucanase II (EGII) of *Penicillium janthinellum*: cDNA sequence, heterologous expression and promotor analysis. *Current Genetics* **29:** 490-495.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews.* **8(4):** 515-548.

Morikawa Y., Ohashi T., Mantani O., Okada H. (1995). Cellulase induction by lactose in *Trichoderma reesei* PC-3-7. *Applied Microbiology and Biotechnology* **44:** 106–111.

Nathan S. S. (2006). The use of *Eucalyptus tereticornis* Sm. (Myrtaceae) oil (leaf extract) as a natural larvicidal agent against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). *Bioresource Technology* **98(9):** 1856-1860.

Odom A., Muir S., Lim E., Toffaletti D. L., Perfect J., Heitman J. (1997). Calcineurin is required for virulence of *Cryptococcus neoformans*. *The European Molecular Biology Organization Journal* **16:** 2576–2589.

Park B. J., Wannemuehler K. A., Marston B. J., Govender N., Pappas P. G., Chiller T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Pfaffl M. W., Horgan G. W., Dempfle L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30(9):** 1-10.

Ronne H. (1995). Glucose repression in fungi. *Trends in Genetics* **11:** 12–17.

Seiboth B., Gamauf C., Pail M., Hartl L., Kubicek C. P. (2007). The D-xylose reductase of *Hypocrea jecorina* is the major aldose reductase in pentose and D-galactose catabolism and necessary for beta-galactosidase and cellulase induction by lactose. *Molecular Microbiology* **66(4):** 890-900.

Sesták S., Farkas V. (1993). Metabolic regulation of endoglucanase synthesis in *Trichoderma reesei*: participation of cyclic AMP and glucose-6-phosphate. *Canadian Journal of Microbiology* **39(3):** 342-347.

Sidik N. M., Nasir S. N., Sharifuddin S. A. (2011). Expression of a new endoglucanase gene from *Aspergillus terreus* SUK-1 suppressed by glucose. *Australian Journal of Basic and Applied Sciences* **5(9):** 1956-1961.

Smith A. H., ImLay J. A., Mackie R. I. (2003). Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. *Applied and Environmental Microbiology* **69(6):** 3406-3411.

Steen B. R., Lian T., Zuyderuyn S., MacDonals W. K., Marra M., Jones S. J. M., Kronstad J. W. (2002). Temperature-regulated transcription in the pathogeneic fungus *Cryptococcus neoformans*. *Genome Research* **12:** 1386-1400.

Suto M., Tomita F. (2001). Induction and catabolite repression mechanisms of cellulase in fungi. *Journal of Bioscience and Bioengineering* **92:** 305-311.

Vaheri M., Leisola M., Kauppinen V. (1979). Transglycosylation products of cellulase system of *Trichoderma reesei*. *Biotechnology Techniques* **1:** 696–699.

Varadi J. (1972). The effect of aromatic compounds on cellulase and xylanase production of the fungi *Schizophyllum commune* and *Chaetomium globosum*. In: Walters A. H., Hueck-van der Plas E. H. (eds.) *Biodeterioration of materials, Vol. 2*. London: Applied Science, 129-135.

Vartivarian S. E., Anaissie E. J., Cowart R. E., Sprigg H. A., Tingler M. J., Jacobson E. S. (1993). Regulation of cryptococcal capsular polysaccharide by iron. *Journal of Infectious Disease* **167:** 186–190.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transplant International* **16:** 336-340.

Wales R., Richardson P. T., Roberts L. M., Woodland H. R., Lord J. M. (1991). Mutational analysis of the galactose binding ability of recombinant ricin B chain. *Journal of Biological Chemistry* **266 (29):** 19172–19179.

White T. J., Bruns T. D., Lee S. B., Taylor J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J., White T. J. (eds.) *PCR protocols. A guide to methods and applications.* San Diego: Academic Press, 315-322.

Williamson P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* **2:** 99-107.

Wiame J. M., Grenson M., Arst, Jr H. N., (1985). Nitrogen catabolite repression in yeasts and filamentous fungi. *Advances in Microbial Physiology* **26:** 1–88.

Yakovlev I., Vaaje-Kolstad G., Hietala A. M., Stefańczyk E., Solheim H., Fossdal C. G. (2012). Substrate-specific transcription of the enigmatic GH61 family of the pathogenic white-rot fungus *Heterobasidion irregular* during growth on lignocellulose. *Applied Microbiology and Biotechnology* **95:** 979–990.

Yaniv O., Frolow F., Levy-Assraf M., Lamed R., Bayer E. A. (2012). Interactions between family 3 carbohydrate binding modules (CBMs) and cellulosomal linker peptides. *Methods in Enzymology* **510:** 247-259.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier, 77-98.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infection and Immunity* **69(9):** 5589-5596.

CHAPTER 7

Growth of *Cryptococcus neoformans* var. *grubii* ATCC H99 on mucosal-associated glycoproteins

Growth of *Cryptococcus neoformans* var. *grubii* ATCC H99 on mucosal-associated glycoproteins

A Botes, A Botha

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Cryptococcus neoformans (sanfelice) Vuillemin, anamorph of *Filobasidiella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis and cryptococcal meningitis in immuno-suppressed individuals. It is widely accepted that the most common portal of entry are the lungs, however, little research has been conducted on how *C. neoformans* is able to survive within the lung tissue. The respiratory system contains numerous glycoproteins, called mucins, which are rich in carbohydrates, particularly galactose. The objectives of this study was first to determine if *C. neoformans* var. *grubii* ATCC H99 was capable of utilizing mucin as sole nutrient source, and secondly, to evaluate the transcriptional regulation of the *C. neoformans* var. *grubii* cellulases (CC1 and CC6) and endo-glucanase (EC7) in the presence of mucin. Growth curves confirmed that *C. neoformans* var. *grubii* ATCC H99 was capable of growth on mucin in the absence of other nutrients. Real-time quantitative PCR (qPCR) revealed that transcription of cellulase CC6 was induced in the presence of mucin. These results would suggest that cellulase CC6 may aid the survival and therefore the pathogenicity of *C. neoformans* var. *grubii* during the initial stages of infection.

1. INTRODUCTION

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1997; Mitchell *et al.*, 1995), particularly those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). The estimated global incidence of this infection, also known as cryptococcosis, among these individuals is approximately 30 % (Jarvis *et al.*, 2009; Park *et al*., 2009; Vilchez *et al*., 2003; Husain *et al*., 2001; Williamson, 1997; Chuck *et al.*, 1989) with a mortality rate of between 30 – 60 %.

Cryptococcus neoformans is known to have a worldwide distribution (Sorrell *et al*., 1997) and has been isolated from a number of environmental sources including soil, avian guano contaminated soil, avian guano, fir trees, almond trees, eucalyptus trees, woody debris and decaying wood (Randhawa *et al.*, 2011; Randhawa *et al.*, 2008; 2006; 2000; Baroni *et al.*, 2006; Ergin *et al.*, 2004; Montagna *et al.*, 2003; Trilles *et al*., 2003; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996; López *et al.*, 1995; Bauwens *et al*.,1986; Emmons, 1955; 1951). It is clear, however, that the variant *C. neoformans* var. *grubii* is more predominant with regards to environmental and clinical sources than *C. neoformans* var. *neoformans* with approximately 90 % of all clinical cases being attributed to the former variety (Mitchell *et al.*, 1995).

While the exact mechanisms of infection are not yet fully elucidated, it is widely accepted that the most common portal of entry are the lungs. Infectious propagules such as desiccated yeast cells or viable basidiospores of *C. neoformans*; are inhaled and may remain localized within the lungs, where they are thought to remain relatively dormant prior to dissemination (Feldmesser *et al*., 2001). The human airways are rich in glycoproteins, called mucins that aid in the defence against pathogens (Lamblin *et al.*, 2001). Mucins are can contain one to several hundred carbohydrate chains, typically consisting of fucose, galactose and small amounts of mannose (Lamblin *et al.*, 2001; 1991).

The mechanism of how *C. neoformans* is able to survive within the lung tissue is not fully understood. During the initial stages of infection, yeast cells are rapidly engulfed by alveolar macrophages, however, extracellular localization of yeast cells

becomes more prevalent after 24 hours of infection (Feldmesser *et al.*, 2000). Interestingly, research evaluating the *in vivo* regulation of *C. neoformans* cells isolated from lung tissue determined that processes fundamental to carbon and energy metabolism were greatly enhanced (Hu *et al.*, 2008). These included transcripts associated with the transport of mono-saccharides, and enzymes involved in glycolysis and gluconeogenesis.

Due to the relatively high carbohydrate content of the mucin-rich respiratory epithelium and the importance of the yeast's carbohydrate metabolism during infection (Hu *et al.*, 2008), we hypothesized that the glycoside hydrolases we previously characterised (Chapters 3, 4 and 5) and found to be implicated in wood degradation, i.e. putative cellulases (CC1 and CC6) and endo-glucanase (EC7), may play a role during lung colonization. To test this hypothesis, the first aim of this study was to determine whether a representative of *C. neoformans* var. *grubii* is able to grow on mucin, as sole nutrient source. The second aim of the study was test whether expression of the genes coding for the above-mentioned glycoside hydrolases is increased in the presence of mucin.

2. MATERIALS AND METHODS

2.1 Strain and Culture Conditions

Cryptococcus neoformans var. *grubii* ATCC H99 (CBS 10515) was obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and maintained by periodic transfer to yeast peptone glucose (YPG, pH 5.5) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Gauteng, South Africa) and incubated at 22°C . All media used were prepared as per the manufacturer's instructions.

2.2 Growth on Mucin

A pre-inoculum of *C. neoformans* var. *grubii* ATCC H99 was at 30 °C for 48 hours in 250 mL conical flasks, containing 25 mL YPG broth (pH 5.5) on a rotary shaker (150 rpm; New Brunswick Scientific G53, Canada). A total of 2 mL of culture was centrifuged (Biofuge fresco, Heraeus Instruments, Hanau, Germany) for 5 min at 13 793 RCF (Beckman Coulter, Avanti J-E, California, USA) and the pellet was re-suspended in 2 mL

distilled water. Total cell counts of the resulting suspensions that served as final inoculums were done using a Neubauer haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

Mucin substrate was prepared by pre-autoclaving (121 °C, 15 min) a series of 250 mL conical flasks containing 25 mL distilled water that were subsequently supplemented with 0.1 % mucin (w/v) (Sigma, Gauteng, South Africa)

Each substrate was inoculated with *C. neoformans* var. *grubii* ATCC H99 to a final concentration of 1 x 10^6 yeast cells / mL of substrate and incubated at 30 °C on a rotary shaker (150 rpm; New Brunswick Scientific G53, Canada). A total of 500 µL was sampled immediately after inoculation from each flask and yeasts were enumerated using dilution plates with malt extract agar (MEA). Plates were incubated at 30 °C for 48 hours before the colonies were counted. Enumeration of yeasts was repeated every four hours. All experiments were conducted in triplicate and the data were plotted on a log graph using Microsoft Office Excel 2010.

2.3 Gene Induction / Repression

A pre-inoculum of *C. neoformans* var. *grubii* ATCC H99 was cultured at 30 °C for 48 hours in 1 L conical flasks, containing 100 mL YPG (pH 5.5) on a rotary shaker (150 rpm; New Brunswick Scientific G53). Cells were collected via centrifugation for 5 min at 16278 RCF (Beckman Coulter, Avanti J-E), washed twice in distilled water and re-suspended in 100 mL yeast peptone broth (pH 5.5) and incubated at 30 °C on a rotary shaker (150 rpm; New Brunswick Scientific G53) for a further four hours. Cells were once again collected via centrifugation for 5 min at 16278 RCF (Beckman Coulter, Avanti J-E), washed twice in distilled water and re-suspended in 75 mL distilled water. The total suspension was divided and spotted onto 10 mucin agar plates. Mucin agar was prepared by supplementing pre-autoclaved (121 °C, 15 min) water agar (1.5 % w/v) with mucin (1 % w/v). Plates were poured using Petri dishes (90 mm diameter) and allowed to solidify. Mucin agar plates were incubated at 30 °C for one week after which yeast colonies were scraped from the plates and subjected to RNA isolation.

2.4 RNA Isolation and Real-Time PCR

Ribonucleic acid (RNA) was isolated using the RiboPure™ Yeast Kit (Ambion, California, USA) from 24 hour induced cultures described above. The quality of the RNA was assayed in a Tris-EDTA agarose gel (1 % w/v) and quantified using a BioDrop-µLITE spectrophotometer. The RNA samples were assayed for the presence of contaminating DNA by amplifying the internal transcribed spacer (ITS) region of the ribosomal gene cluster using universal primers ITS1/4 (White *et al.*, 1990, Table 1). Polymerase chain reactions (PCR) were performed in 20 µL reaction volumes with final concentrations of ~400 ng RNA, 2.5 mM magnesium chloride ($MgCl_2$), 0.4 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer and 0.5 U Taq DNA polymerase recombinant (Fermentas, Massachusetts, USA). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400, California, USA) for 30 cycles with: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplified products, indicative of DNA contamination, were separated in a Tris-EDTA agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory *et al.*, 1999). A total of 1 µg RNA was subsequently converted to complementary deoxyribonucleic acid (cDNA) using the RevertAid Premium First Strand cDNA synthesis kit (Fermentas).

Relative gene expression of CC1, CC6 and EC7 was evaluated using quantitative real-time polymerase chain reactions (R-T qPCR) performed in a Roche Lightcycler (version 2.0) using specific primers (Table 1) and HOT FIREPol® EvaGreen® qPCR Mix Plus Capillary (Solis BioDyne, Tartu, Estonia). The cDNA was diluted and approximately 50 ng was used in each R-T qPCR reaction with the parameters set as follows; 95 °C for 15 min; 23 cycles of 95 °C for 15 s, at primer Tm (Table 1) for 20 s and 70 °C for 20 s with a final extension at 72 °C for 7 min. Relative gene expression was determined using the REST analysis tool (2009) (Pfaffl *et al.*, 2002) with the actin primer set (Table 1) used as a calibrator. The amplification efficiency was determined for each set of real-time primers (Table 1) by means of standard curves. Real-time PCR was performed, as described above, using serial 10 fold dilutions of target cDNA. The

amplification efficiency was subsequently calculated using the REST analysis tool (2009).

Table 1 Primer sets used for semi-quantitative real time gene expression during experimentation. All primers were produced by Inqaba Biotec, Gauteng, South Africa.

Primer	Primer sequence	Primer set Tm (°C)
General primers		
ITS1	5′- TCCGTAGGTGAACCTGCGG-3′	53
ITS4	5′- TCCTCCGCTTATTGATATGC-3′	
Real time primers		
Actin-F	5′-GCCCTTGCTCCTTCTTCTAT-3′	60
Actin-R	5′-GACGATTGAGGGACCAGACT-3′	
CC1-F	5′-TTGACAGATGGAAGCTCT-3′	55
CC1-R	5′-AGAAGCATGAGAAGTCGA-3′	
CC6-F	5′-CTTATGGAAGCGACAAGATC-3′	58
CC6-R	5′-GCAGCTTGAGCAGTATCATA-3′	
EC7-F	5′-CAGACAATCCGACTCACA-3′	55
EC7-R	5′-TGTAGAATGCAACAAGGC-3′	

3. RESULTS AND DISCUSSION

3.1 Growth on Mucin

Respiratory track mucins could serve as a suitable nutrient source for lung pathogens, such as *C. neoformans*, as they are a rich source of both proteins and carbohydrates. Previously, we demonstrated that *C. neoformans* var. *grubii* ATCC H99 is capable of growth on both galactose and mannose (Chapter 2, Fig 1). Both sugars occur naturally within hemi-cellulose as well as the glycoprotein mucin (Lamblin *et al.*, 2001; 1991; Fengel *et al.*, 1989). Similar to hemi-cellulose, the great variation in carbohydrate side chains add to the complexity of mucin. Mucin is associated with mucosal membranes within the human body, including the respiratory system, and could thus serve as a nutrient source during the initial stages of lung infection by *C. neoformans*.

The ability of *C. neoformans* var. *grubii* ATCC H99 to utilize mucin as sole nutrient source was therefore evaluated. The yeast was found to be capable of growth on mucin (Fig 1) and displayed a similar growth pattern to the one observed when it was grown on glucomannan and bean gum locust (Fig 2). These results suggest that the previously identified cellulases (CC1 and CC6) and the endo-glucanase (EC7) may aid the survival of *C. neoformans* var. *grubii* in the lungs.

3.2 Transcriptional Gene Regulation

The transcriptional regulation of CC1, CC6 and EC7 within *C. neoformans* var. *grubii* ATCC H99 was evaluated with regards to the presence of the glycoprotein mucin in the surrounding medium. Previously, we found that expression of CC6 was up-regulated in the presence of both galactose and mannose, while an increase in expression of CC1 and EC7 was observed in the presence of mannose (Chapter 6). The transcription of neither CC1 nor EC7 was found to be up-regulated by galactose. Given that mucin is known to be comprised of both galactose and small amounts of mannose (Lamblin *et al.*, 2001; 1991), it was hypothesized that the three proteins may display increased expression levels in the presence of this glycoprotein. However, real-time PCR analyses indicated that only CC6 was up-regulated in the presence of mucin (Table 2).

As determined previously, transcription of CC6 was induced by the presence of *Acacia* extract, cellobiose, D-galactose and D-mannose (Chapter 6). Cellobiose remains the strongest inducer of CC6 transcription followed by D-galactose, while mucin resulted in slightly (though not significant) higher levels of transcription than D-mannose (Table 2). Neither CC1 nor EC7 displayed a notable difference with regards to their transcriptional regulation (results not shown), despite the presence of mannose in mucin (Lamblin *et al.*, 2001; 1991). As mentioned previously, mannose is only present in small amounts and may therefore not reach the minimum threshold required to induce an increase in transcription of CC1 and EC7.

The lack of transcriptional induction of these glycoside hydrolases in the presence of mucin may not only have been as a result of the absence of a suitable inducer. Mucin is known to contain proteins with anti-microbial activity, such as lactoferrin (Arnold *et al.*, 1980; Arnold *et al.*, 1977). Lactoferrin is classified as a transferrin protein and is

responsible for regulating the levels of free iron in the body (Farnaud *et al.*, 2003; Brock, 1980; Johansson *et al.*, 1960). Iron is an important metal required for the growth of both fungi and bacteria and is regularly associated with trefoil peptides such as mannose receptors, clostridium neurotoxins, fascin, as well as ricin (Xu *et al.*, 2005; Farnaud *et al.*, 2003; Brych *et al.*, 2001; Lamblin *et al.*, 2001; Brock, 1980).

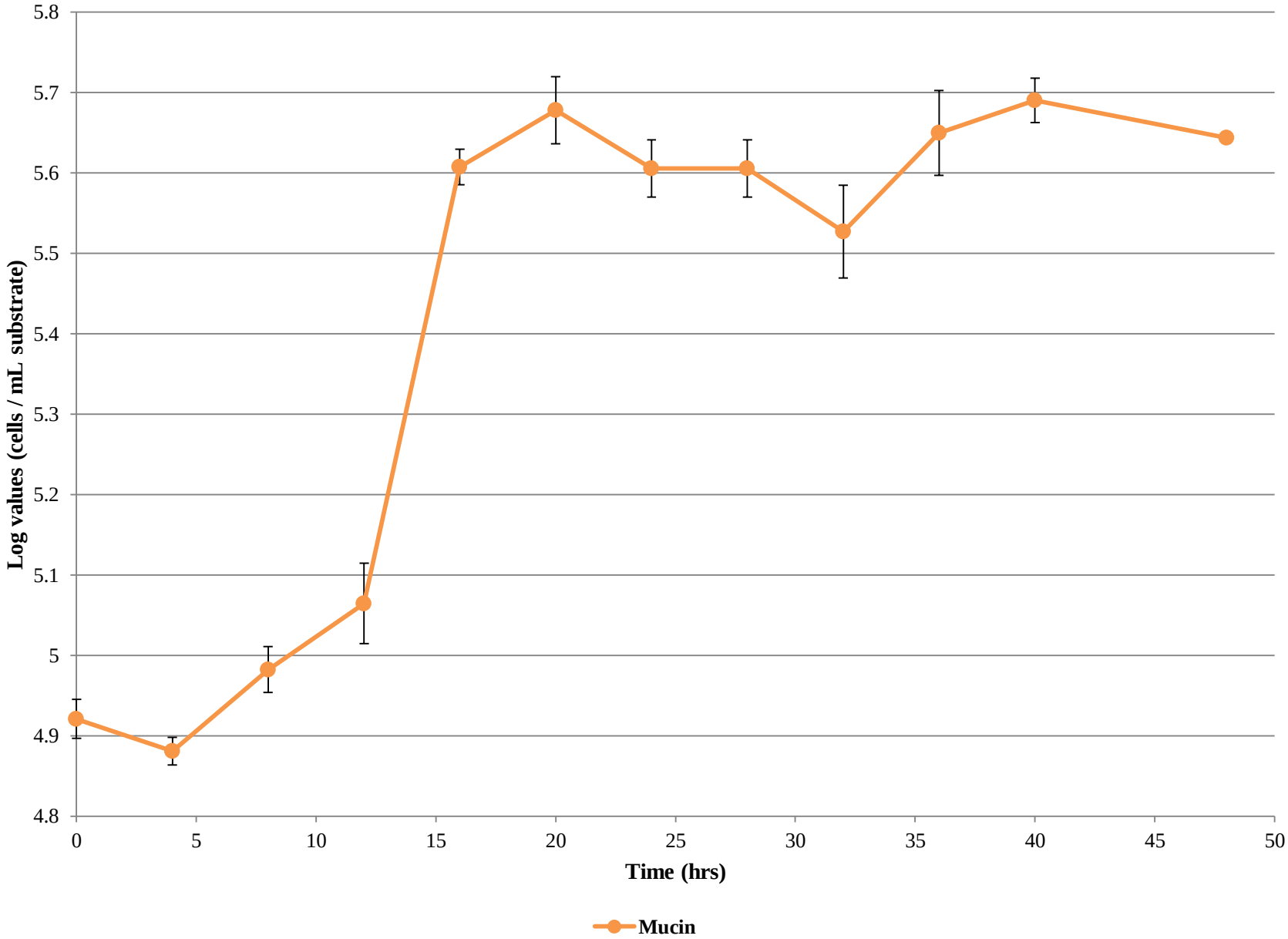

Figure 1 Growth curve of *C. neoformans* var. *grubii* ATCC H99 in liquid cultures containing 0.1% (w/v) mucin. Values represent the mean of three repetitions while bars denote standard errors.

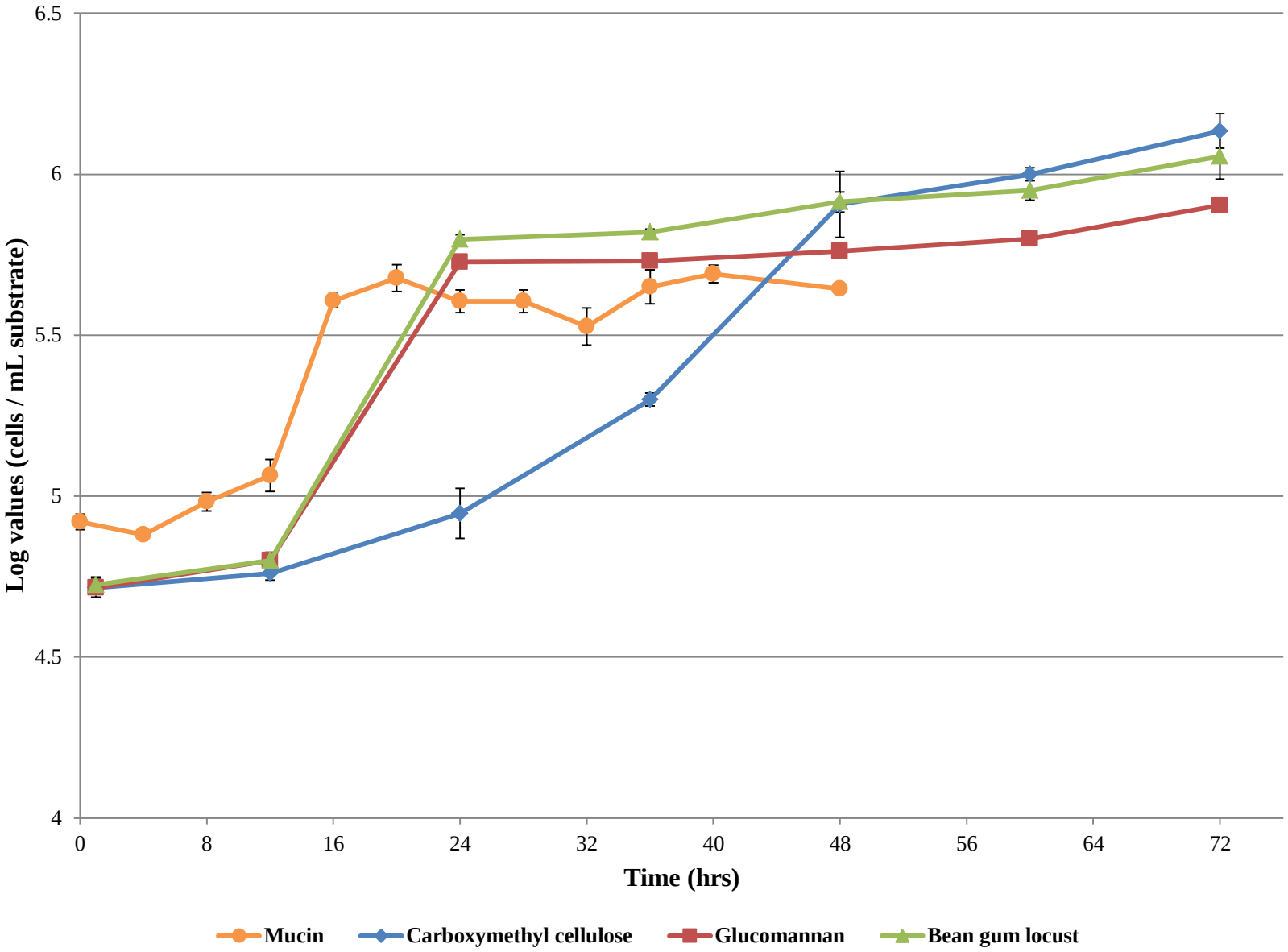

Figure 2 Growth curve of *C. neoformans* var. *grubii* ATCC H99 in liquid cultures containing either 0.1% (w/v) mucin; carboxymethyl cellulose (CMC); glucomannan (GM) or bean gum locust (LBG). Values represent the mean of three repetitions while bars denote standard errors. Data of growth on CMC, GM and LBG were obtained from the results recorded in Chapter 2.

Table 2. Relative gene expression levels of enzyme CC6 when *C. neoformans* var. *grubii* ATCC H99 was exposed to various carbon sources. Relative gene expression was determined using the REST analysis tool (2009) (Pfaffl *et al.*, 2002). Values represent a mean factor of up or down regulation when the sample group is compared with the control group. Only those carbon sources that resulted in a significant ($p < 0.05$) response are shown (n = 3). Data of relative gene expression in the presence of *Acacia extract*, cellobiose, D-galactose and D-mannose were obtained from the results recorded in Chapter 6.

Substrate	**Relative gene expression (95 % Confidence Interval)**
	Cellulase C6
Acacia extract	5.58
Cellobiose	81.65
D-galactose	7.50
D-mannose	2.31
Mucin	2.65

Research has shown that in the case of *C. neoformans*, the level of iron plays a critical role in the regulation of various virulence factors, including capsule production, melanin synthesis and the ability to grow at elevated temperatures (Jung *et al.*, 2006; Kraus *et al.*, 2005; Odom *et al.*, 1997; Jacobson *et al.*, 1996; Vartivarian *et al.*, 1993). Due to the action of lactoferrin, the iron dependent transcription factor would induce the expression of the previously mentioned virulence factors and possibly inhibited the expression of CC1. Previous analysis of CC1 revealed a ricin-type B lectin domain within the protein sequence (Chapter 3) and may therefore require iron in order to function correctly. However, further research will have to be conducted to confirm this.

4. CONCLUSIONS

We confirmed that *C. neoformans* var. *grubii* ATCC H99 is capable of growth on mucin in the absence of additional nutrients. The yeast displayed a similar growth pattern on mucin when compared to glucomannan and bean gum locust (galactomannan) suggesting similarities in terms of hydrolysis and metabolism. Real-time qPCR revealed that an increase in transcription of cellulase CC6 occurred in the presence of mucin. These

results suggest that similar to other "dual-use" virulence factors, such as laccase and urease, cellulase CC6 may aid in the survival of *C. neoformans* during the initial colonization of the lung tissue by ensuring that the pathogen has access to a nutrient source.

5. REFERENCES

Arnold R. R., Cole M. F., McGhee J. R. (1977). A bactericidal effect for human lactoferrin. *Science* **197:** 263–265.

Arnold R. R., Brewer M., Gauthier J. J (1980). Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infection and Immunity* **28:** 893–898.

Bauwens L., Swinne D., De Vroey C., De Meurichy W. (1986). Isolation of *Cryptococcus neoformans* var. neoformans in the aviaries of the Antwerp Zoological Gardens. *Mykosen* **29:** 291–294.

Brock J. H. (1980). Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in the newborn infant. *Archives of Disease in Childhood* **55:** 417–421.

Brych S. T., Blaber S. I., Logan T. M., Blaber M. (2001). Structure and stability effects of mutations designed to increase the primary sequence symmetry within the core region of a β-trefoil. *Protein Science* **10:** 2587–2599.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready-made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (eds.) *Current Protocols in Molecular Biology*. New York: Wiley Online Library, Chapter 2.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **321:** 794–799.

Emmons C. W. (1951). Isolation of *Cryptococcus neoformans* from soil. *Journal of Bacteriology* **62:** 685–690.

Emmons C. W. (1955). Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (Columba livia). *American Journal of Hygiene* **62:** 227–232.

Ergin C., Ilkit M., Kaftanoglu O. (2004). Detection of *Cryptococcus neoformans* var. *grubii* in honeybee (*Apis mellifera*) colonies. *Mycoses* **47(9-10):** 431-434.

Farnaud S., Evans R. W. (2003). Lactoferrin—a multifunctional protein with antimicrobial properties. *Molecular Immunology* **40(7):** 395–405.

Feldmesser M., Kress Y., Novikoff P., Casadevall A. (2000). *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infection and Immunity* **68:** 4225– 4237.

Fengel D., Wegener G. (1989). Wood: Chemistry, ultrastructure, reaction. New York: Walter de Gruyter.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *Journal of Clinical Microbiology* **35(9):** 2243-2251.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia *Journal of Clinical Microbiology* **37:** 2920-2926.

Hu G., Cheng P., Sham A., Perfect J. R, Kronstad J. W. (2008). Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. *Molecular Microbiology* **69(6):** 1456–1475.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerging Infectious Disease* **7:** 375-381.

Jacobson E. S., Compton G. M. (1996). Discordant regulation of phenol oxidase and capsular polysaccharide in *Cryptococcus neoformans*. *Journal of Medical and Veterinary Mycology* **34:** 289–291.

Jarvis J. N., Boulle A., Loyse A., Bicanic T., Rebel K., Williams A., Harrison T. S., Meintjes G. (2009). High ongoing burden of cryptococcal disease in Africa despite antiretroviral roll out. *AIDS* **23(9):** 1182–1183.

Johansson B., Virtanen A. I., Tweit R. C., Dodson R. M. (1960). Isolation of an iron-containing red protein from human milk. *ACTA Chemica Scandinavica* **14(2):** 510–512.

Jung W. H., Sham A., White R., Kronstad J. W. (2006). Iron regulation of the major virulence factors in the AIDS-associated pathogen *Cryptococcus neoformans*. *PLOS Biology* **4(12):** 2282-2295.

Kraus P. R., Nichols C. B., Heitman J. (2005). Calcium- and calcineurinin dependent roles for calmodulin in *Cryptococcus neoformans* morphogenesis and high-temperature growth. *Eukaryotic Cell* **4:** 1079–1087.

Lamblin G., Lhermitte M., Klein A., Houdret N., Scharfman A., Ramphal R., Roussel P. (1991). The carbohydrate diversity of human respiratory mucins: A protection of the underlying mucosa? *American Review of Respiratory Disease* **144:** 19–24.

Lamblin G., Degroote S., Perini J. M., Delmotte P., Scharfman A., Davril M., Lo-Guidice J. M., Houdret N., Dumur V., Klein A., Roussel P. (2001). Human airway mucin glycosylation: A combinatory of carbohydrate determinants which vary in cystic fibrosis. *Glycoconjugate Journal* **18:** 661–684.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

López-Martínez R., Castañón-Olivares L. R. (1995). Isolation of *Cryptococcus neoformans* var. *neoformans* from bird droppings, fruits and vegetables in Mexico City. *Mycopathologia* **129(1):** 25-28.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* **8(4):** 515-548.

Montagna M. T., Santacroce M. P., Caggiano G., Tatò D., Ajello L. (2003). Cavernicolous habitats harbouring *Cryptococcus neoformans*: results of a speleological survey in Apulia, Italy, 1999-2000. *Medical Mycology* **41(5):** 451-455.

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. thesis, University of Delhi, Delhi, India

Odom A., Muir S., Lim E., Toffaletti D. L., Perfect J., Heitman J. (1997). Calcineurin is required for virulence of *Cryptococcus neoformans*. *The European Molecular Biology Organization Journal* **16:** 2576–2589.

Park B. J., Wannemuehler K. A., Marston B. J., Govender N., Pappas P. G., Chiller T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Pfaffl M. W., Horgan G. W., Dempfle L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30(9):** 1-10.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Randhawa H. S., Kowshik T., Chowdhary A., Prakash A., Khan Z. U., Xu J. (2011). Seasonal variations in the prevalence of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* in decayed wood inside trunk hollows of diverse tree species in north-western India: a retrospective study. *Medical Mycology* **49(3):** 320-323.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Revista Iberoamericana de Micologia* **14:** 42-43.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Medical Mycology* **41(5):** 383-390.

Vartivarian S. E., Anaissie E. J., Cowart R. E., Sprigg H. A., Tingler M. J., Jacobson E. S. (1993). Regulation of cryptococcal capsular polysaccharide by iron. *Journal of Infectious Disease* **167:** 186–190.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transplant International* **16:** 336-340.

White T. J., Bruns T. D., Lee S. B., Taylor J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J., White T. J. (eds.) *PCR protocols. A guide to methods and applications.* San Diego: Academic Press, 315-322.

Williamson P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* **2:** 99-107.

Xu R., Xiao Y. (2005). A common sequence-associated physicochemical feature for proteins of beta-trefoil family. *Computational Biology and Chemistry* **29:** 79–8.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (eds.) *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier, 77-98.

CHAPTER 8

General Conclusions and Future Research

1. GENERAL CONCLUSIONS

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen that causes cryptococcal meningitis, also known as cryptococcosis (Casadevall *et al*., 2003; Franzot *et al*., 1997; Mitchell *et al.*, 1995), predominantly in immuno-compromised individuals, particularly in those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). This basidiomycetous yeast species is subdivided into two main varieties, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), as well as a rare hybrid variety, *C. neoformans* (serotype AD) (Boekhout *et al.*, 2001; Boekhout *et al.*, 1997). Currently, the global incidence of cryptococcosis, among individuals suffering from AIDS, is estimated at approximately 30 % with a mortality rate ranging between 30 – 60 % (Park *et al*., 2009; Vilchez *et al*., 2003; Husain *et al*., 2001; Boekhout *et al.*, 1997; Powderly, 1993; Chuck *et al.*, 1989).

From a global medical perspective, *C. neoformans* var. *grubii* (Serotype A, MATα) is responsible for approximately 90 % of all cryptococcosis infections (Halliday *et al.*, 1999; Boekhout *et al.*, 1997; Mitchell *et al.*, 1995). Although representatives of this variant can be grouped into two different genotypes, namely VNI and VNII, the genotype VNI appears to be dominant, having a worldwide distribution (Meyer *et al*., 1999; Boekhout *et al.*, 1997). This genotype has been isolated from 78 % of global AIDS related cryptococcal infections.

Representatives of *C. neoformans* have been isolated from a variety of environmental sources including avian and bat guano, soil, fruits, insects, vegetative debris and particularly decaying wood and tree hollows (Randhawa *et al.*, 2011; 2008; 2006; 2000; Baroni *et al.*, 2006; Ergin *et al.*, 2004; Montagna *et al.*, 2003; Trilles *et al*., 2003; Chakrabarti *et al.*, 1997; Mussa, 1997; Lazéra *et al*., 1996; López *et al.*, 1995; Bauwens *et al.*,1986; Emmons, 1955; 1951). It is believed that infection occurs when infectious particles such as desiccated yeast cells or basidiospores, which originate from these previously mentioned environments, are inhaled.

The ecological niche of *C. neoformans* was first believed to be avian guano (Sorrell *et al.*, 1997); indeed, *C. neoformans* has been shown to reproduce sexually when mated on pigeon guano under laboratory conditions (Neilsen *et al.*, 2007). Recent findings, however, suggest that the true ecological niche of *C. neoformans* may be woody

material (Botes *et al.*, 2009). Representatives of this species, particularly *C. neoformans* var. *grubii*, have been found to grow on agar plates containing carboxymethyl cellulose as carbon source. However, little is known about the ability of *C. neoformans* var. *grubii* to degrade the hemi-cellulosic components of wood.

Results obtained during this study indicate that *C. neoformans* var. *grubii* ATCC H99 interacts positively with cellulosic and hemi-cellulosic materials. Growth studies revealed that *C. neoformans* var. *grubii* ATCC H99 was capable of utilizing carboxymethyl cellulose, glucomannan as well as bean gum locust (galactomannan) as sole carbon source. In addition, the yeast was able to assimilate simple degradation products of woody materials, such as L-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose and D-xylose. D-Mannose and D-glucose resulted in the highest the maximum specific growth rates (μ_{max}), i.e. 0.414 h^{-1} (± 0.003) and 0.290 h^{-1} (± 0.07) respectively.

These results suggest that *C. neoformans* var. *grubii* is potentially producing glycoside hydrolases (GHs), such as cellulases and mannanases, which are able to hydrolyze lignaceous material (de Souza, 2013; Lum *et al.*, 2011; Sanchez, 2009).

Screening the genome of *C. neoformans* var. *grubii* ATCC H99 led to the identification of three putative enzymes that may play a role during the degradation of woody materials, specifically an endo-glucanase (EC7) and two cellulases (CC1 and CC6). Evaluation of the deduced amino acid sequences indicated that all three enzymes belong to the largest known glycoside hydrolase family (GHF), namely GHF5, which is noted for its inclusion of fungal endo-glucanases and endo-mannanases (Cantarel *et al.*, 2009; Hessrinat *et al.*, 1993; Hessrinat, 1991). Phylogenetic analyses revealed that the three cryptococcal enzymes grouped in distinct clades that could be distinguished on the basis of conserved amino acid regions associated with the known catalytic sites. Interestingly, all three putative proteins grouped with various well known plant, insect and human pathogens species such as *Burkholderia gladioli* (Vandamme *et al.*, 2007; Graves *et al.*, 1997); *Melampsora larici-populina (*Xhaard *et al.*, 2011*); Metarhizium anisopliae* (Freimoser *et al.*, 2003); *Schizophyllum commune* (Guarro *et al.*, 1999); *Trichosporon asahii* var. *asahii* (Walsh *et al.*, 1992; Watson *et al.*, 1970).

The amino acid sequence of each protein was evaluated to deduce its chemical and physical properties, which suggested that all three proteins could be regarded as being extra-cellular in nature. Automated homology modelling of the three dimensional structure revealed that both CC1 and CC6 displayed the classical $(\alpha/\beta)_8$ TIM barrel fold generally associated with GHF5. However, modelling of the EC7 protein did not produce the classic GHF5 structure, suggesting that this enzyme may be classed in a separate GH family. Green fluorescent protein (GFP tagging) confirmed that at least one of the identified putative proteins, CC6, forms part of the cryptococcal secretome. Plate assays also confirmed that EC7 is indeed an endo-glucanase. To our knowledge, this is the first report that the human pathogen *C. neoformans* var. *grubii* does possess a functional β-1,4-endo-glucanase.

By using real-time quantitative PCR (qPCR), we were able to determine that the proteins displayed varied expression profiles when the yeast was exposed to environments that differed in their carbohydrate composition. The putative cellulase CC6 displayed the most dynamic expression profile indicating up-regulation when *C. neoformans* var. *grubii* was exposed to mannose, galactose and cellobiose. Both the putative cellulase CC1 and endo-glucanase EC7 were up-regulated in the presence of mannose. The use of either *Acacia mearnsii* debris or the aqueous extract thereof as an inducer resulted in a significant up-regulation of all three enzymes. The inductions of CC1 (by *A. mearnsii* debris) as well as CC6 and EC7 (by the aqueous extract) implicate the presence of other naturally occurring inducers/repressors such as the various phenolic compounds that are known to be produced by *Acacia* species. These results confirm previous findings that the woody phyloplane is likely to be a natural habitat of *C. neoformans* var. *grubii*.

In the presence of mucin, real-time qPCR revealed that the transcription of CC6 was up-regulated. These results suggest that similar to other "dual-use" virulence factors, such as laccase and urease, cellulase CC6 may aid the survival of *C. neoformans*, both within the lungs and its natural habitat. Further understanding of the carbohydrate metabolic regulatory system, and its resulting impact on virulence, would greatly increase our understanding of the pathogen's survival in both environmental and clinical settings.

2. FUTURE RESEARCH

A number of unanswered questions remain concerning the lignocellulosic lytic abilities of *C. neoformans* var. *grubii*. Expression of the endo-glucose EC7 and the cellulase CC6 in the ascomycetous host, *Saccharomyces cerevisiae,* was unsuccessful and this experiment will have to be re-designed to characterize the proteins in terms of their substrate, temperature and pH ranges. Similarly, the cellular location of CC1 and EC7 can be determined using GFP-fusion technology.

The three-dimensional structure of EC7 as determined by automated homology modeling did not yield the classical $(\alpha/\beta)_8$ TIM barrel characteristic of GHF5 members. The use of X-ray crystallography (Kendrew *et al.,* 1958; Bragg, 1913; 1912) could be employed in order to elucidate the protein structure and evaluate protein-substrate interactions.

The regulation and impact of the identified cellulases on virulence needs to be studied in greater depth. Micro-array analyses (Lashkari *et al.,* 1997: Schena *et al.,* 1995; Maskos *et al.,* 1992; Augenlicht *et al.,* 1987) would prove useful in examining the expression levels of the entire cryptococcal genome when the yeast is exposed to a lignaceous environment. Knockout mutant strains (Liu *et al.,* 2008) could be generated to determine if these proteins are vital for the asexual and sexual reproduction of *C. neoformans* var. *grubii* on woody material. Similarly, animal model experiments would give an indication of the virulence of knockout strains compared to the wild-type yeast (Neilsen *et al.,* 2005a; 2005b; 2003; Kwon-Chung *et al.,* 1992).

Finally, it is surprising to note that a number of respiratory pathogens, both bacterial and fungal species, are confirmed cellulase producers. The tuberculosis causing prokaryote *Mycobacterium tuberculosis* is known to be a member of the cellulase producing family (Varrot *et al.,* 2005) and co-habit the lungs with *C. neoformans* in HIV and AIDS patients. The interaction of *C. neoformans* with *M. tuberculosis,* as well as with other respiratory pathogens, needs to be examined with regards to their cellulosic metabolism.

REFERENCES

Augenlicht L. H., Wahrman M. Z. Halsey H., Anderson L., Taylor J., Lipkin M. (1987). Expression of cloned sequences in biopsies of human colonic tissue and in colonic carcinoma cells induced to differentiate *in vitro*. *Cancer Research* **47(22):** 6017–6021.

Baroni F. A., Paula C. R., Silva E. G., Viani F. C., Rivera I. N. G., Oliveira M. T., Gambale W. (2006). *Cryptococcus neoformans* strains isolated from church towers in Rio de Janeiro City, RJ, Brazil. *Rev Inst Med Trop. São Paulo* **48:** 71-75.

Bauwens L., Swinne D., De Vroey C., De Meurichy W. (1986). Isolation of *Cryptococcus neoformans* var. neoformans in the aviaries of the Antwerp Zoological Gardens. *Mykosen,* **29:** 291–294.

Boekhout T., van Belkum A., Leenders A. C. A. P., Verbrugh H., Mukamurangwa P., Swinne D., Scheffers W. A. (1997). Molecular Typing of *Cryptococcus neoformans*: Taxonomic and epidemiological aspects. *International Journal of Systematic Bacteriology* **47(2):** 432-442.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* **147:** 891-907.

Botes A., Boekhout T., Hagen F., Vismer H., Swart J., Botha A. (2009). Growth and mating of *Cryptococcus neoformans* var. *grubii* on woody debris. *Microbial Ecology* **57(4):** 757-765.

Bragg W. L. (1912). The spectacular reflexion of X-rays. *Nature* **90(2250):** 410.

Bragg W. L. (1913). The diffraction of short electromagnetic waves by a crystal. *Proceedings of the Cambridge Philosophical Society* **17:** 43.

Cantarel B. L., Coutinho P. M., Rancurel C., Bernard T., Lombard V., Henrissat B (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research* **37:** 233-238.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment – the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology* **6:** 332-337.

Chakrabarti A., Jatana M., Kumar P., Chatha L., Kaushal A., Padhye A. A. (1997). Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *Journal of Clinical Microbiology* **35:** 3340–3342.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **321:** 794–799.

de Souza W. R. (2013). Microbial degradation of lignocellulosic biomass In: Chandel A. K., da Silva S. S. (eds.) *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization*. New York: InTech, Chapter 9.

Emmons C. W. (1951). Isolation of *Cryptococcus neoformans* from soil. *Journal of Bacteriology* **62:** 685–690.

Emmons C. W. (1955). Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (Columba livia). *Am J Hyg,* **62:** 227–232.

Ergin C., Ilkit M., Kaftanoglu O. (2004). Detection of *Cryptococcus neoformans* var. *grubii* in honeybee (*Apis mellifera*) colonies. *Mycoses* **47(9-10):** 431-434.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *Journal of Clinical Microbiology* **35(9):** 2243-2251.

Freimoser F. M., Screen S., Bagga, S., Hu G., St. Leger, R. J. (2003). EST analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* **149:** 239-247.

Graves M., Robin T., Chipman A. M., Wong J., Khashe S., Janda J. M. (1997). Four additional cases of Burkholderia gladioli infection with microbiological correlates and review. *Clinical Infectious Disease* **25(4):** 838-842.

Guarro J., Gené J; Stchigel A. M. (1999). Developments in fungal taxonomy. *Clinical Microbiology Reviews* **12(3):** 454–500.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and a mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gattii* strains from Australia. *Journal of Clinical Microbiol*ogy **37:** 2920-2926.

Henrissat B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 309–316.

Henrissat B., Bairoch A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781–788.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerging Infectious Disease* **7:** 375-381.

Kendrew J. J., Bodo G., DIntzis H. M., Parrish R. G., Wyckoff H., Phillips D. C. (1958). A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. *Nature* **181(4610):** 6662-6666.

Kwon-Chung K. J., Edman J. C., Wickes B. L. (1992). Genetic association of mating type**s** and virulence in *Cryptococcus neoformans. Infection and Immunity* **60:** 602-605.

Lashkari D. A., DeRisi J. L., McCusker J. H., Namath A. F., Gentile C., Hwang S. Y., Brown P. O., Davis R. W. (1997). Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proceedings of the National Academy of Sciences of the United States of America* **24(24):** 13057-13062.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *Journal of Medical and Veterinary Mycology* **34(2):** 127-31.

Liu O. W., Chun C. D., Chow E. D., Chen C., Madhani H. D., Noble S .M. (2008). Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell* **135(1):** 174–188.

López-Martínez R., Castañón-Olivares L. R. (1995). Isolation of *Cryptococcus neoformans* var. *neoformans* from bird droppings, fruits and vegetables in Mexico City. *Mycopathologia* **129(1):** 25-28.

Lum G., Min X. J. (2011). FunSecKB: the Fungal Sectretome KnowlegeBase. *Database - the Journal of Biological Databases and Curation.* 2011: doi: 10.1093/database/bar001.

Maskos U., Southern E. M. (1992). Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesized *in situ*. *Nucleic Acids Research* **20(7):** 1679–1684.

Meyer W., Marszewska K., Amimostofian M., Igreja R. P., Hardtke C., Methling K., Viviani M. A., Chindamporn A., Sukroongreung S., John M. A., Ellis D. H., Sorrell T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20:** 1790-1799.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 Years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* **8(4):** 515-548.

Montagna M. T., Santacroce M. P., Caggiano G., Tatò D., Ajello L. (2003). Cavernicolous habitats harbouring *Cryptococcus neoformans*: results of a speleological survey in Apulia, Italy, 1999-2000. *Medical Mycology* **41(5):** 451-455.

Mussa A. Y. (1997). Studies on respiratory mycotic disease agents with special reference to their natural habitats. Ph.D. dissertation, University of Delhi, Delhi, India.

Nielsen K., Cox G. M., Wang P., Toffaletti D. L., Perfect J. R., Heitman J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic *a* and α isolates. *Infection and Immunity* **71(9):** 4831-4841.

Nielsen K., Marra R. E., Hagen F., Boekhout T., Mitchell T. G., Cox G. M., Heitman J. (2005a). Interaction between genetic background and the mating-type locus in *Cryptococcus neoformans* virulence potential. *Genetics* **171(3):** 975-983.

Nielsen K., Cox G. M., Litvintseva A. P., Mylonakis E., Malliaris S. D., Benjamin D. K. Jr, Giles S. S., Mitchell T. G., Casadevall A., Perfect J. R., Heitman J. (2005b). *Cryptococcus neoformans* {alpha} strains preferentially disseminate to the central nervous system during coinfection. *Infection and Immunity* **73(8):** 4922-4933.

Nielsen K., De Obaldia A. L., Heitman J. (2007). *Cryptococcus neoformans* mates on pigeon guano: Implications for the realized ecological niche and globalization. *Eukaryotic Cell* **6(6):** 949–959.

Park B. J., Wannemuehler K. A., Marston B. J., Govender N., Pappas P. G., Chiller T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Powderly W. G. (1993). Cryptococcal meningitis and AIDS. *Clinical Infectious Disease* **17:** 837–842.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia* **151:** 63-69.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Preeti Sinha K., Chowdhary A., Khan Z. U., Yan Z., Xu J., Kumar A. (2006). Distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed trunk wood of *Syzygium cumini* trees in north-western India. *Medical Mycology* **44:** 623–630.

Randhawa H. S., Kowshik T., Chowdhary A., Preeti Sinha K., Khan Z. U., Sun S., Xu J. (2008). The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46(8):** 823-833.

Sanchez C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advances* **27:** 185-194.

Schena M., Shalon D., Davis R. W., Brown P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270(5235):** 467–470.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Revista Iberoamericana de Micologia* **14:** 42-43.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Medical Mycology* **41(5):** 383-390.

Vandamme P., Govan J., LiPuma J. (2007). Diversity and role of *Burkholderia* spp. In: Coenye T., Vandamme P. (eds.) Burkholderia*: Molecular Microbiology and Genomics*. Norfolk: Horizon Bioscience, Chapter 1.

Varrot A., Leydier S., Pell G., Macdonald J. M., Stick R. V., Henrissat B., Gilbert H. J., Davies G. J. (2005). *Mycobacterium tuberculosis* strains possess functional cellulases. *Journal of Biological Chemistry* **280(21):** 20181-20184.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transplant International* **16:** 336-340.

Walsh T. J., Lee J. W., Melcher G. P., Navarro E., Bacher J., Callender D., Reed K. D., Wu T., Lopez-Berestein G., Pizzo P. A. (1992). Experimental *Trichosporon* infection in persistently granulocytopenic rabbits: implications for pathogenesis, diagnosis, and treatment of an emerging opportunistic mycosis. *Journal of Infectious Disease* **166(1):** 121-133.

Watson K. C., Kallichurum S. (1970). Brain abscess due to *Trichosporon cutaneum*. J. *Medical Microbiology* **3:** 191–193.

Xhaard C., Fabre B., Andrieux A., Gladieux P., Barrés B., Frey P., Halkett F. (2011). The genetic structure of the plant pathogenic fungus *Melampsora larici-populina* on its wild host is extensively impacted by host domestication. *Molecular Ecology* **20(13):** 2739-55.

APPENDICES

Appendix A

Amino Acid Sequences used to Construct a Neighbour-Joining Phylogenetic Tree

Cellulase protein sequences used to construct a neighbour-joining phylogenetic tree in order to identify putative cellulases within the genome of *Cryptococcus neoformans* var. *grubii* ATCC H99. All sequences were obtained from the Broad Institute database (www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/) and/or the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov.) All amino acids and their relative abbreviations are listed in Appendix B.

***Aspergillus fumigatus* XP_748948.1**

MAVGILKVKGNKVVDNDGNEVLLRGAAIGGWMNMENFITGYPGHESQHRAAMRRVLGPEKYEFFF
DRWLEYFFTEADAKFFAGLGLNCIRIPFNYRHFEDDMNPRVLKESGFKHLDRVIELCAKEKIYTI
LDMHTAPGGQNGDWHSDNPTSYAAFWDFKDHQDRTVWLWEQIAARYKDNPWVAGYNPLNEPCDPE
HVRLPAFYERVEKAIRAIDPDHILWLDGNTFAMEWKGFDKVLPNCVYAMHDYSSMGFPTGERYKG
TPEQKEHLERQYLRKAEFMNKNGTVIWNGEFGPVYANPRTEAEAETINQERYSLLGEQLRIYDKY
KIHWSIWLYKDIGLQGMIHTSPDSKWNRTIQPLLEKKKKFWLDAWGREPFAEPEAALKPLVDWID
KVSPTAKETYPTPWNTERHLLRNVFQTFLAASFVNEFAELFRGMDEKELDELAHSFHFDECEQRG
GLNEILREHARATKH

***Aspergillus oryzae* XP_001817490.2**

MSINLLEKTSIMSSGILKVKGNKIVDNDGNEVLLRGAAIGGWMNMENFITGYPGHESQHRTAMKK
VLGPEKYEFFFDRWLEYFFTEADAKFFAGLGLNCIRIPFNYRHLEDDMNPRVLKESGFKHLDRVV
DLCSKQNIYTILDMHTAPGGQNGDWHSDNFTSYAAFWDYKDHQDRTVWLWEQIAARYRSNPWVAG
YNPLNEPCDPEHVRLPAFYERVEKAIRAVDPDHILWLDGNTFAMEWKGFDKVLPNCVYSIHDYAS
MGFPTGERYKGTAEQNAHLERTYLRKVQPLNEKGTAIWNGEFGPVYADPRADAEASTINQERYNL
LGEQLRIYDKYNIHWSIWLYKDIGLQGMIYTSPDSKWNKTIQPFLEKKNHFWLDCWGRRPSAEPE
AALKPLVEWIDKVSPQAKETYPTPWNTERHLLRNVFQTFLAASFADEFAELFRGMNEAELDSLAR
SFHFEECVQRDGLNEILREHAHARQA

***Aureobasidium pullulans* ACH63253.1**

MAPHAEPQSQTTEQTSSGQFTNPPCNMLHVDGADIVDASGSRIILKGAGLGGHMNFENFITGYPG
HEHEHRAAMAEVLGKEKAQFFFDRLIHYFFTDADAAYFQSLGLNCIRVPFNYRHFIDDDNPTVIK
DSGFKLLDNIVDICGRHNLYVILDLHAVPGGQNQDWHSDSGMGKALFWEFKVFQDQMINLWVALA
KHYVGNPVIAGYNPLNEPADPKHTRLINWYDRIEKAIRKVDPDHILWVDGNTYAMDFSHFTTIMP
NTVYACHDYANLGFPIPGQDPYTNTPEQNSKLRRQFNRKAEFSRTHNVPLWNGEFGPV

***Aureobasidium pullulans* ACH69873.1**

MAPHAEPQSQTTEQTSSGQFTNPPCNMLHVDGADIVDASGSRIILKGAGLGGHMNFENFITGYPG
HEHEHRAAMAEVLGKEKAQFFFDRLIHYFFTDADAAYFQSLGLNCIRVPFNYRHFIDDDNPTVIK
DSGFKLLDNIVDICGRHNLYVILDLHAVPGGQNQDWHSDSGMGKALFWEFKVFQDQMINLWVALA
KHYVGNPVIAGYNPLNEPADPKHTRLINWYDRIEKAIRKVDPDHILWVDGNTYAMDFSHFTTIMP
NTVYACHDYANLGFPIPGQDPYTNTPEQNSKLRRQFNRKAEFSRTHNVPLWNGEFGPVYADPSAD
PEADKTNISRFGVLKEQLKIYAESQIHWTIWLYKDIGYQGMVHVSRNSAYMQLIAAFVAKKQRLG
LDFWGVVDKSGVSETYDPFLRDLKAMVPEHLQGIKYPDHWSFDRQFERIIRECLLSEYLGMEFAE
LFKGKSEQELDELAQSFKFENCVTRDTLTEILQEDAAKSRAQK

***Burkholderia gladioli* YP_004348142.1**

MKTPRKSAIGRLRQAAILASAAFAMFAASGADAQAALSMLHASGRQIVDTSGKTVQLKGFNLGGW
FVMEGYMSPMDAGSLTDTYSVMKQLDAAYGVTEERALMKAYQDTWIQAQDFANIKAAGFNVVRVP
LWWGQFFDLDNPTIPGWRSDAFVELDQLVANAAANGIYVIFDMHGVIGGQGTDVDTGQGNRNAFW
SNTEFQSDTAWLWWQIANHFKGNTTVAGYDLINEPTGAPNNNAVWSAYSRFYNSIRSIDPDHMIF
IEGTWGNWNWDMLPPPSQYGWTNVVYEMHEYQWGANGNASVIKNGADNQARDFANHASWNVPGYV
GEFNAFSTDPSVWQYCITAYNNAGLSWTQWAYKAVNGTAPNYWGWYDPTFWPARPNVSTDSASEI
ERKWRLWSTSATFAKNTALNIKPSY

***Coniophora puteana* EIW86987.1**

MHSLAKLALFGLAAVRQACAISPGFPYGSEKVRGVNLGGWLVLEPWITPSLFDNTGNDAIVDEWT
FGEYQNYDTALGVLQNHWNTWITESDFAAIAAAGLNHVRVPIGYWAFEVGPGEPYIQGQLPYLQN
AVTWAGQNGLKVIVDLHGAPGSQNGYDNSGHRIPYPEWQSNQTNVQRTDAIIKQLEGMFESQTNV
VSIIAPLNEPAGYDGDQILSVVRQYWYDSYGNIRYPYGTSQQSNTIELIHDAFQPLSYWTGFMTP
PNYQGVAMDTHIYQVFSDADVAMTWPQHISAACAEQSALSGFDLWLIVGEWSTSPTDCATYLNGR
GVGSRYDGTYSGSTYVGSCTGLTGSASTFSAAYKTFLRQFWEAQVISYEAAADGWVMWAWKTESA
DEWSYSAGLQNGWIPQNPTDLEYPSICG

***Cryptococcus flavus* AAC60541.1**

MKTATLLAALSVLAGALAAPLAGDSALHRRSLPRLGGVNLAGCDFGIDIYGNSGTPACPGTEQVG
HFIADGANLFRLPAGWQYLVGNNQASTSLAPDFFAQYDALVQAVISKGAYAIIDVHNYARWNGAI
IGQGGPSNQDFANLWTLLATKVTSNDPNVIFGLMNEPHDLDVSTWAGSVQAAVNAIRAAGATSQY
ILIPGTGFTNANAWFQGQDNALLGVTDPVGGTDKLLLDVHRYNDVDFSGTHAECTTNSLDVLSSL
DSWLKGNGRKAIVSETGGGHTTSCETDLGEFLNGIKEDYPSVLGFAVWAAGSFDPSYVLSITPTN
GVDNQLFDIAVKPNLP

Cryptococcus flavus Q04469

MKTATLLAALSVLAGALAAPLAGDSALHRRSLPRLGGVNLAGCDFGIDIYGNSGTPACPGTEQVG
HFIADGANLFRLPAGWQYLVGNNQASTSLAPDFFAQYDALVQAVISKGAYAIIDVHNYARWNGAI
IGQGGPSNQDFANLWTLLATKVTSNDPNVIFGLMNEPHDLDVSTWAGSVQAAVNAIRAAGATSQY
ILIPGTGFTNANAWFQGQDNALLGVTDPVGGTDKLLLDVHRYNDVDFSGTHAECTTNSLDVLSSL
DSWLKGNGRKAIVSETGGGHTTSCETDLGEFLNGIKEDYPSVLGFAVWAAGSFDPSYVLSITPTN
GVDNQLFDIAVKPNLP

Cryptococcus neoformans var. *grubii* ATCCH99 (CC1)

MFAFTYIAALLSLISVLPSALAGPTAGTTYAISPNQHPSMCLAPAHGWEGTDVVLKDCDEDDTTW
LWTGQSFQNTATNFCIDIRDSGAWSGNKAQVWGCFSYNTNQQFSVEESMIHWNGFCWDLTDGSSS
AGTKLQIWSCYSYNDNQQWTFTEIEEVDECDATSITETATIMSTSTASVSDLSTSTASASASNIT
EAVTASESLTASATDSDYQVNPPASAIESAYESINATASVAESGYESINATASATLSASDTLSAE
TSTATNSSIGEGLWSPHKSSSVSSDDWSSETATDSNTKWWATSTSSDSWASATASASNPWQNASQ
SDSWNSTSTASNPWETAVSSSQAWNETSTDSWGASATATATTSDSYGNATSTSASSAITATATVG
TISSGYLQTSGTKIVDSDGNEVILRGTNIGGWLVLEDWMCGITDNSGTSDRFSLSTLENRFGTDQ
ARTLVEAWAENWLTTNDFDELAAIGFNVIRLPFSFRTVQNADGSWRDDAFTRMDWAIAEAKARGI
YTIVDFHMWPGQEADYSAISENTDEGQSQRDAVGEIWKKVATHYLGESSICAFDVINEPTGSYGD
YLQQDLYKAVRSVDSDRIIIHESISTDPSTYGWTNVIYSLHEYDMMGSDLSSNKATWANGVQAYI
DLWHGYNIPFMLAEFMADGETLDFMLNSMNSQGISWLTWAHSTVNMGRWGIWNHEAFNVDVSSDS
YDTIYNTWTNMPSTFHTSIYDQMKAAATGSTNVSRKRDLAPAARTTKRLHGSHGGRSRRNGVAHA
VRGAAGVSI

Cryptococcus neoformans var. *grubii* ATCCH99 (CC6)

MRLLIPFFIVPLAIARALPAFPTVDVEKRSVNVGWPYGTDKIRGVNIGGWLVTEPFITPSLFEAT
GNNDIVDEWTFCQYQDYDTARSALMNHWDTWFTEDDFAKISAAGLNHVRIPIGFWAYDVQDGEPY
IQGQADYLDRAIGWARKHNLAVIVDLHGAPGSQNGYDNSGRRGAADWATNNSNVDRTKNVISLLS
RKYSDSQYYGVVTAIALLNEPATYLNELLLQTARQYWYNAYGAARYPFGNNDKSGLALVIHDGFQ
PLNTFENYMTEPEYEDVLLDTHNYQVFNDEYVAWNWDEHISNICNKASTYSTSPLWLVVGEWTLA
STDCAKYLNGRGLGSRYDGSYPGSPYIGTCDDKSNDVDRFSEEYKAFMHRFWEVQTQVYEQNGQG
WIHWTWKTENAADWSYEAGLDGGWIPWNAGSHDVSLSSLCG

***Cryptococcus neoformans* var. *grubii* ATCCH99 (EC7)**

MSSDGPDRGFLKVSGKDITLDGKPIILRVNMENFITGYAGHEHQARQALKQVLGTEKYNYFFEKF
LEYFFAEDDANFFASLGLNCIRIPVNYHHFEDDMNPRVFKRDGLKHLDRVIQIVCRSVHRTVTKP
LSLAFIQCAKYGIYTVIDLHAAPGGQNFDWHSDNPTHKALFYEHKDFQDRTVFIWENIARDNAWV
AGYNPLNEPSDEQHVRLVAFYNRIEKAIRAIDSNHILFLDGKLTANCYGFPNPPSLYEGSKEQIQ
FHVDSYNGKTEYMRKHGSPVWVGEFGPVYQTSEDGYPDWKHINDTRFDVLQLQLDIYAKARASWS
IWLYKDIGFQGMIYAGEDTAYVKLLKEFLHKKKVAAADKWGADDRAVRAMFAPLESWLLETVPSI
SDRYPQDWSVGEHLSRLVRNMLLSEELVKEYAEHFRGKSHKELDELAKSFKFSNCTQRKRLNDVL
KSGSERGIDEKKSLWQVGEKV

***Cryptococcus* sp. S-2 ABP02069.1**

MKTATLLASLSVLAGALAAPLAGESALHRRTLPRLGGVNLAGCDFGIDIYGNSGTPACPGTEQVG
HFIADGANLFRLPAGWQYLVGNNQASTSLAPDFFAQYDALVQAVISKGAYAIIDVHNYARWNGAI
IGQGGPSNQDFANLWTLLATKYKNDPNVIFGLMNEPHDLDVPTWAGSVQAAVNAIRAAGATSQYI
LVPGTGFTNANAWFEGQDNALLGVTDPVGGTDKLLLDVHRYNDVDFSGTHAECTTNSLDVLSSLD
SWLQGNGRKAIVSETGGGHTTSCETDLGEFLHGIKEDYPSVLGFAVWAAGSFDPSYVLSITPTNG
VDNQLFDIAVKPNLP

***Cystobacter fuscus* WP_002630490.1**

MQINDSSYQGEKGTWRRGLFAGAAFGLAALCGFSPSEAHALNMLKTSGRNIVDSTTGAVVRLRGV
NLGGWLVQERWMTPLDSGNLPDTYSVMKELDRRFGVATEQSLMKTYQDNWITTTDLDNIRAGGYN
VVRVPVWWGNFYALDNVSNSGWRADAFEKLDWIVNNAGARGLYVIIDMHGVVGGQSLSDTTGQAN
RNEYWVNGSHQGNTAWMWWQIANHFKGNGTVAGYDLINEPIGAPSASAVWSAYDSLYKSVRSVDP
AHMIFLEGAYGNWNWNMLPNPAQYGWTNVVYEMHEYQYNGSAAQVQQGATNQVTDFNNHASWNVP
GYIGEFNSMGTGASTWAATKKTYDNAGLSWTMWSYKATHGLVPDSWGWYDPTWWPATPNISTDSA
ATISSKWQQWKTTTSFGKNTSISM

***Granulicella mallensis* YP_005059433.1**

MNRITAAHRTSRLLVRMALRLAACLLVILACSHSALAQLSMLHTSGRSIVNANGNIVQLKGVNLG
GFMVMEPWMCPADSGGLPDTYSIISELDSRFGVAEEQALIRGYQQAWITSADFANIKAAGFNAVR
VPVWWGNFYPIANVSNASWRADAFTELDWVVSQAAAQGIYVIIDMHGVVGGQSTSDDTGQQNQNQ
YWTNGNDQGNTAFMWWQIANHYKGNPTIAGYDLINEPMNAPSNSAVISANAGLYNSVRSIDPSHI
IIIEGTWGNWDWSMLPNPSTEGWTNVVYEMHEYQWNASQSVVAQGSVNQVNDFNNHSSYNVPGYI
GEWNDFQYSSSVWQGSVSDYNNGGESWTFWAYKATSGLNPNGWGLYNPTHWATTPNVSTDSAATI
LADWQQWTTANSFAFNSSLGFN

***Hypocrea jecorina* CAA71999.1**

MIQKLSNLLVTALAVATGVVGHGHINDIVINGVWYQAYDPTTFPYESNPPIVVGWTAADLDNGFV
SPDAYQNPDIICHKNATNAKGHASVKAGDTILFQWVPVPWPHPGPIVDYLANCNGDCETVDKTTL
EFFKIDGVGLLSGGDPGTWASDVLISNNNTWVVKIPDNLAPGNYVLRHEIIALHSAGQANGAQNY
PQCFNIAVSGSGSLQPSGVLGTDLYHATDPGVLINIYTSPLNYIIPGPTVVSGLPTSVAQGSSAA
TATASATVPGGGSGPTSRTTTTARTTQASSRPSSTPPATTSAPAGGPTQTLYGQCGGSGYSGPTR
CAPPATCSTLNPYYAQCLN

***Irpex lacteus* BAD67544.1**

MKSLLLSAAATLALSTPAFSVSVWGQCGGIGFTGSTTCDAGTSCVHLNDYYFQCQPGAATSTVQP
TTTASSTSSAAAPSSSGNAVCSGTRNKFKFFGVNESGAEFGNNVIPGTLGTDYTWPSPSSIDFFV
GKGFNTFRVPFLMERLSPPATGLTGPFDSTYLQGLKTIVSYITGKGGYALVDPHNFMIYNGATIS
DTNAFQTWWQNLAAQFKTDSHVVFDVMNEPHDIPAQTVFNLNQAAINRIRASGATSQSILVEGTS
YTGAWTWTTTSGNSQVFGAIHDPNNNVAIEMHQYLDSDGSGTSPTCVSPTIGAERLQAATQWLQQ
NNLKGFLGEIGAGSNADCISAVQGALCEMQQSDVWLGALWWAAGPWWGDYFQSIEPPSGVAVSSI
LPQALEPFL

***Melampsora larici-populina* EGG08561.1**

MLLHRALCLLSLSASFATQALDSVTPNTHQLISRSSIQSHTRKIQDFNLRRHIDPSFSKRDQEIK
PSFDYAKYKIRGVNLGGWLVTEPWITPSLYNTGNDKIVDEYTLCEQLGQKAATELLRAHWESFYK
EEDFQRISSYGLNHVRIPIEGIPTFMILGYWAFDILTDEPYVQGQLEYLHRAVGWAQGAGLKVMI
DLHGAPGSQNGFDNSGKRGEINWASEDSNVARTRQALVLLAQEFSQPKYAGTVTSLESLNEPAGF
ANEKTLKTTRQYYYDGYGIVRYPIPQGSQSNLLYAIHDAFQPLDAWTHSFPAPKWQGVALDTHIY
TVFNNTQLKMTDDERVKSYCDLTESLVQSDSSLWTFVGEFTPAPTDCAPRLNGQGIGSRYDATFK
DSPRLGSCEGKSGSMSSFSEEYKASLGRFFEVQTHVFEKASGWFMWTFKAENSDDWSYDAGVKGG
WIPRDPGFKAHGNPCG

***Melampsora larici-populina* EGG09724.1**

MPPLQATPILLWATFLFSIQVSLATHSSSPNSRDSHHHVARARADQVPIQVDLHSRGFESAEGNS
PVTSTHQKRDEPINPGFDYARQKIRGVNIGGWLVTEPWITPSLYNTGNSKIIDEYTYCGQLGRSE
ATKRLHAHWESFYKEGDFHTIKSYGLNHVRIPIGYWAFDISAGEPYVQGQFEYLKKGVEWARRAG
LKVMIDLHGAPGSQNGFDNSGRKGPINWATDPKNLVRTKQALAKLAKEFTQPKYAGTVTSLEALN
EPAGFANDGHKTLNAAKQYYYDGYTIVRHPNGQGPQSNVLYAIHDAFQPLDTWSTAFPQPKYQGL
ALDTHIYTVFDTPSLQKNDDARVATYCGMASGLARSNSAIWTFVGEFTPAPTDCAPRLNGQGTGA
RYDGTFMDSQRLGSCQGKSGSAKNFSKEYKTSLARFFEVQTTVYEKASGWFMWTFKAENADDWSY
DAGVKGGWIPRDPGSKPHGHPCS

***Melampsora larici-populina* EGG09755.1**

MHLLQATPILLWAAFFLSFEISITTQSLWSDISSDIFGTQNEFSGDQTDTAPADTAIRSQDSDSS
EGTTPIIDSREKYTAASTNTGFEYGKDKIRGVNIGGWLVTESWLTPTLYRTGNSKIVDEYTFCQY
LGREEASKRLRAHWDSFYTESDFQAMKSYGLNHVRIPIGYWAFDISGGEPYVQGQYEYLKQAVEW
SRRAGLKVMIDLHGAPGSQNGFDNSGRKGPINWPNDPKNILRTKQTLAEITKEFSQAKYGNLEAL
NEPAGFANDGGKTLNTAKQFYHDAYDIVRYPNNETLQSDLLYVVHDSFQPIETWSNSFPSPKYQS
VALDTHIYTIFDKISIEKSDDERVATYCAMANSLEKSNQAILTFVGEFAPSPTDCANSVNHQPSG
SRYDGTYTGFQKIGSCIGKSGSRETFSEEYKLSLGRLFEVQTTVYEKASGWIMWTFKAENADDGS
YDAGVKGGWIPKDPTFKAHGNQCN

***Metarhizium anisopliae* ARSEF 23 EFY97537.1**

MTPSAVQNAPPPSPSPGGPSSGGPGTSKHANRPCAMLRVSGTRIVDPSGDEVVLKGAGLGGMLNM
ENFITGYSGHEHEHRAALAEVLGQEKADFYFSRLLHHFFGEADAALLASLGLNCLRVPFNYRHFM
DDDNPDVIKASGFRLLDRIVDICGRHNIYVVLDLHAVPGGQNQDWHSDSGLSRALFWEFRDFQDR
AVQLWTAIAAHYAGNPVVAGYNPLNEPADPRHTRLVAWYERVEAAIRAVDPDHMLFLDGNTYAMD
FSAFDPARTLPNAVYSVHDYSTLGFPLPEQYEATDAQRARLRASFARKARFMREAGVPVWNGEFG
PVYQDPRTDPDAAATNAKRFALLREQLAVYRECGGVSWSIWLYKDVGYQGMVYLDPESAHMRLVR
PFIEKKQRLGLDFWACADKSLVDAQVYRPLIDKLKDMVPEHLRRKKYPKTWTFDRQVERVVRECL
MSEYLGWELAEAFAGKTEDELEELAASFALDKCLRRDELNWILQLDTRGEL

***Phaffia rhodozyma* AAC17104.1**

MHLANVLLTLLPVSLLATESLAGSSSHSAHALPARRRHNKGRALSPIKASNSSSEHETNRIASAG
ASADDFSPVTGRRVSKRAQCGVSSPATSSKTSSTITVGAAVVPTAAATSSSKWKLDLEAKGNSFF
DTFNFWAYDDPTHGTVTYVSQDEATKSNLATVNGKGNAVLAVDTTQNVQKGRKAVRLHSSYIFNG
GLILADIVHMPTGCGTWPAWWSNGPDWPNKGEIDILEGTHSWDRNQVSVHTSDGCTIPSNYGASA
VLTTGSFVNTNCASYATSNQGCGQRESASHQAYGEPFNQNGGGVYAMKWDTSGISVYFFPRNAIP
ADITQGVPLPETWGTPMGNFPSTSCEPFKFFKDHHTIINTTFCGDWANSDWWTAGSAGNGQSCAA
KTGYNSCSDYVLNNGDKFHEAYWEFASVKYYQPK

***Phanerochaete chrysosporium* CAZ65735.1**

MRPTLTSFVALAYCLSGALAGSYTLIDNYVGSTFLSAFVHEAIADPTHGRVNYVNQATAVAKNLT
FASGNTLILRADDTTVLSPSGPGRNSVRIRSVKAYTTHVAIIDVRHMPQGCGTWPAFWETDGSNW
PNGGEVDIIEGVNDQSPNAMTLHTGANCNMPASRAETGTPTGLNCDVNTDGNTGCGVQAPTANSY
GPALNAIGGGWYAMERTNNFIKVWFFPRNGNTPSDLKNGASSINTDNWGTPTAFFPNTNCDIGSH
FDQNNIIINLTFCGDWAGAVYGNSGCPSTCVDYVNNNPSAFKNAYWDIAAVRVYE

***Puccinia graminis f. sp. tritici* CRL 75-36-700-3 XP_003337030.1**

MAVKQTPFWQGLFTLFSILSFFSGAFADETAPGPKPGTADSSKSAIPRLVGINLPGFEFGAQTDG
QYTAGTNPAMPPPESQIAHFLSQNVNFFRVPVAWEFLQPEMNGKLNEVNLKAYQTFIEKITTHGA
YVAIDLHAFARYKGQIVGESPSTPASALVSLWTQLGAVFKDNPLVMFGISNEPHDLVITTWATTV
QKVVTALREKKIENILLIPGTDYTAMKSFPEWYKAMKVVKNPDGSFDGLVMEVHRYLDTDNSGKS
RDCTASHADEVAKAVELLKADGRQVILGETGGGSTDTCMKFLPELAAAVTDAYPVFLGFAMWAAG
SFDANYELVTTVKDESSPTGWKDQGNWNSIKKFIPAKGSEKSAKSPNSKPKTKKKAACKRRAMI

***Puccinia graminis f. sp. tritici* CRL 75-36-700-3 XP_003337035.1**

MAFNQYTFWQGLFMLLSAMSAISTTAPKSDSNPGMNENTVLPRLAGAPSKLLIISPVHYISAWLF
QMALFQSWDFGAQTSGQYTPGSTGVAPPKTQITHFLKQNVNLLRVPVAWEFLQPERNTKLNATNV
KVYREYIEEITSQGAYAIIDLHAYARYKSEIVGESPNMPASVLVNLWLQLGGLFKDNKKVLLGLS
NEPHDQDIDKWAVTVQKVVTALRKDGIDNIILLSGTDYSSLKAFPEWYKSMKTVKNPDGSFEGLW
FEVHRYLDTDNSGKSTECVASHADEVVNVAKMLKADGRQVLLGETGGGSTESCMTYLPELVQAVV
EAYPVFSGFAIWSAGAFGATYELVVTVQDDSSPTGWKDQGNWLSIKKFLPNKASLAHRNKPSTGG
AKKNARRSIVSN

***Schizophyllum commune* XP_003037324.1**

MALLRTVLALALLAQSLAISPGFNYGGTKVRGVNLGGWLVLEPWITPSLFDATGNDAIVDEYTFC
AYQSRDVAASALYNHWNTFITEDDFAQIAAAGLNHVRLPIGYWAFDVRDEPYIQGQVEHLNNAVT
WASNHGLKVIVDLHGVPGSQNGFDNSGQRMDYPTWHTQQSNIDRSNAIIKTLENMFKDRTDTVTV
IAPLNEPAGFHGSDVLAATRQFWLDSYGNIRYPFGSSRKSNTVELIHDAFQDLSYWNGFMTSGFE
GVAIDTHIYTIFSNAEAAMSFNQHVSTVCNKQGALSSFDLWTIVGEWTPAYTDCARYLNGRGIGA
RYDGSYPGSSRIGSCSSKTGTGDTFSNEYKSRLRSFWEAQVISYEKGAGWIMWTWKAEEAHEWSY
QAGLDFGWIPWNPTDIQNRRICG

***Serpula lacrymans* var. *lacrymans* EGN93093.1**

MPFLRHVAFALLGLSLAPSALSFSPGFPYGSQKVRGVNLGGWLVLEPWITPSLFDNTGNSAIVDE
WTFGQHQNRVVAEATLQTHWNTWITESDFANIAAAGLNHVRLPIGYWAFEVGPGEPYIQGQLPYL
QKAVTWAGNHGLKLIIDLHGAPGSQNGFDNSGQRMSFPHWQSNQTNIDRTNAVMKTIATMFTSNP
NVIPIIAPLNEPAGFDGAAVLNATRDYWGSSYQSIRYPHGQSQSPSNIVELIHDAFQPPSYWKGF
EVAPNFQGVAMDTHIYQVFSDSEVAMSYQQHIKTACQTQSTLSSYDLWIIVGEWASAPTDCAKYL
NGRGVGARYDGSYSGSTKVGSCSGMTGSASSFSSSYKTFLGQFWEAQVISYEKSGDGWIQWTWKA
ENADDWSYEAGLANGWIPKDPTKLQYPNICN

***Serpula lacrymans* var. *lacrymans* EGN93094.1**

MSFCSFLATALLGLSLTQSALSISPGFPYGSQKVRGVNLGGWLVLEPWITPSIFDNTGNSAIVDE
WTFGQLQDSNTATSVLQSHWNTWITESDFAAIANAGLNHVRLPIGYWAFEVGPGEPYIQGQLPYL
QKAVTWAGNYGLKVIVDLHGAPGSQNGYDNSGHRISFPEWQSNQTNVDRTDAIIKTIASMFDGQT
NVVPIIAPLNEPAGYDGEQMLEVVTQYWYDSYGNIRYPYGTSQESNTVVLLHDAFQPLSYWDGFQ
TPPNYQGVAMDTHIYQVFSDSDVAMSYQDHINTACATQSSLSSFDLWTIVGEWTPAATDCATYLN
GRGIGARYDGSYPGSTYVGRSSFSSTYKTFLRQFWEAQVISYEKGAGWLQWLWKAENADEWSYQA
GLANGWIPQDPTALMYPSICG

***Stigmatella aurantiaca* YP_003950750.1**

MENHGSSPQEVKRLSRRGILAGAALGLAVLCGFSPAPAHALNMLRTNGRNIVDSTTGAVVRLRGV
NLGGWLVMEKWMTPMDSGNLVDTYAVMQELNRRFGVATQQSLMKTYQDNWITTTDLDNIRAGGYN
VVRVPVWWGNFYALDNVSNSGWRSDAFTQLDWIVNNAGARGLYVIIDMHGVVGSQSLSDTTGQAN
RNEYWSNGNHQGNTAWMWWQIANRYKGNGTVAGYDLINEPIGAPTSAAVWSAYDSLYKSVRSADP
NHIVIMEGAYGNWNWNMLPNPAQYGWTNVVYEMHEYQFNGTAAQVKQGATNQVTDFNNHASWNVP
GYIGEFNSMGTGAATWAETKRLYDNAGLSWTMWSYKATHGLVPDSWGWYDPTYWPATPNISTDSS
ATISSKWQQWKTTTSFGKNTSISM

***Trametes hirsuta* BAD01163.1**

MKAILSLAAALLSAAPAFSTAVWGQCGGIGFSGDTTCTASTCVKVNDYYSQCQPGASAPTSTASA
PGPSACPGSRTKFKLFGVNESGAEFGNNVIPGALGTDYTWPSPTSIDFFLDQGFNTFRIPFLMER
VSPPSTGGLTGPFNNTYLDGLKQTVSYITGKGGFAIIDPHNFMIFNGATITSTSQFQAWWQKLAA
EFKTNNNVIFDLMNEPHDIPAQTVFQLMQAAVNGVRASGATSQLILVEGTSWTGAWTWTTSGNSD
AFGAITDPNNNVAIQMHQYLDSDGSGTSPTCVSSTIGAERLQAATQWLQQKGLKGFLGEIGTGNN
TQCVTALQGALCEMQQAGGTWLGALWWAAGPWWGDYYQSIEPPNGDAITNILPALKAFL

***Trametes hirsuta* BAD01164.1**

MKAILSLAAALLSAAPAFSTAVWGQCGGIGFSGDTTCTASTCVKVNDYYSQCQPGASAPTSTASA
PGPSACPGSRTKFKLFGVNESGAEFGNNVIPGALGTDYTWPSPTSIDFFLDQGFNTFRIPFLMER
VSPPSTGGLTGPFNNTYLDGLKQTVSYITGKGGFAIIDPHNFMIFNGATITSTSQFQAWWQKLAA
EFKTNNNVIFDLMNEPHDIPAQTVFQLMQAAVNGVRASGATSQLILVEGTSWTGAWTWTTSGNSD
AFGAITDPNNNVAIQMHQYLDSDGSGTSPTCVSSTIGAERLQAATQWLQQKGLKGFLGEIGTGNN
TQCVTALQGALCEMQQAGGTWLGALWWAAGPWWGDYYQSIEPPNGDAITNILPALKAFL

Tremella mesenterica EIW65879.1

MSPLQREVEFLRVEGSNITLSGKPIVLKGAGLGGWMNMENFITGYPGHEHQMRAALRTTMGQDKY
EFFFDKATFLEYFFQKADAAFIASLGLNCLRLPVNYRHFEDDSNPRVFKSDGLKHLDRVIDLCAK
HGIYTIIDLHSAPGGQNIDWHCDAGNHQANFWVHKDFQDRAIAIWEHLAEHYKGNTWVAGYNPLN
EPTDSEHVRLLSFYQRVEKAIRAVDPDHILFLDTFGEDLSRFGDPLPNCVYACHDYSMYGKPDQV
AHHRKSFDRKVEYMRRIGGPIWNGEFGPVYASSSDSNHEQINQSRYAVLEHQLSIYAQAKASWSI
WLYKDIGFQGKMYVNPDTEYMKLLGPFLDKKKRLAVDAWAVDEENVKHIFDPLNNWLEENISNVN
HEKRYPPMWKMSRHVSRMVREILLSEELVHEYAQYFCDKSKDELEELAKSFAFENCIQRERLNDI
LKKDAGGEVMIAA

Tremella mesenterica EIW68174.1

MLRSFTLLLLSLLPLLPLTLSLPTGEHFGGTGSTNHSQLNIQQRDVNVGWQYGDWKIRGVNIGGW
LVLEPFITPSLFQNTGNDDIVDEYTFCKLQNRGKAQAALRQHWDTWITESDFAAIAAAGLNHVRI
PIGFWAYDVSGGEPYIQGAAAYLDRAIGWARNHGLKVMIDLHGAPGSQNGYDNSGRRGNALWATN
SNNVLRTKNIIQSLSQKYSDSSYYQVVTALGLLNEPATYLNQQLLSTTRQYWYDAYGAARYPWAS
QGSGSKSGLVLVIHDGFQPLNTYNNYMSQPTYEDVMIDHHSYQIFDQPTNEWTWDQHIQGICQQS
STFDGSPLWLVNGEWTVASTDCALWLNGRGTGARYDGTLPGSSYVGDCSTKTGDGSSFSAEYKDF
MQRFWDVQTQTYENHGQGWIYWTWKTENAAEWSYSAGMAGGWIPRDAWYHKYSIQQLCG

Tremella mesenterica EIW69846.1

MSIFATLAALSLSLSFSVLPVIAQNVTSYAISPAPFTDLCVAPSSSSEGAQLVLTNCNDDLVAWT
YSAGSLVNTATNMCLDLTDGGAWSGNKLQVWGCYSWNPNQQWDLSGENIKWDGQNLCMDLTDGDG
SDGTVLQVWQCYDGNSNQEWVFTEIEEVGDDDECVTASPSGPTATASTASSSTASPSFIADAVNA
TDLLATPTASATADSASDTTSVIGGELWGNGGGNNHTSSSSASDSWNTGGSSDSWGSSTSASWAS
ATASATFTSSSASSSSTSSSSGSVSNSGILQTSGTKIVDPSGNEVVLRGVNIGGWLVLEDWMCGI
NDNSGSGDRFAQDTLENRFGVDATAALIETWQDNYMVESDFDNIAAMGFNVMRMPFSYRTVQWAN
GSWRDDAFTKMDWAIAQGKKRGIYCIPTFHIWDSQKDDYSLISEDSDGGQSSRDAAGEIWKKVAA
HWMGETAIAAYDAINEPTGSAGDKLQQDLYNAIRSVDANRIIIMESISTDPSTYGWTQVVYSMHE
YLMMTDDVGANQAAWSGAQGDIDTWNGYQIPTYIGEFMAHEDTLPYMLDQLNNNRVSWSFWSYKS
VNMGGWSVYIFPANPVDVSNDDYNSIMNAWSNFGTPTQNSDIYQTYVNAAGGNANLKKREERFVK
VSQRAGAESARRSWGSHRRAGRFSSHGRSK

***Trichoderma viride* ADJ57703.1**

MIQKLSNLLVTALAVATGVVGHGHINDIVINGVWYQAYDPTTFPYESNPPIVVGWTAADLDNGFV
SPDAYQNPDIICHKNATNAKGHASVKARDTILFQWVPVPWPHPGPIVDYLANCNGDCETVDKTTL
EFFKIDGVGLLSGGDPGTWASDVLISNNNTWVVKIPDNLAPGNYVLRHEIIALHSAGQANGAQNY
PQCFNIAVSGSGSLQPSGVLGTDLYHATDPGVPINIYTSPLNYIIPGPTVVSGLPTSVAQGSSAA
TATASATAPGGGSGPTSRTTTTARTTQASSRPSSTPPATTSAPAGGPTQTLYGQCGGSGYSGPTR
CAPPATCSTLNPYYAQCLN

***Trichosporon asahii* var. *asahii* EKD01579.1**

MENFITGYPGHEHEMRRALKAVLGPEKYEYFFERFLTYFFDEADAAFFASLGLNCLRLPVNYRHF
EDDMNPRVFKEEGLRHLDRVVDLCARHGIYTIIDLHAAPGGQNVDWHSDSGIAKALFWGHKDFQD
RTVLIWEKLAQHYKGNPWVAGYNPLNEPTDVEHTRLLAFYERVEKAIRAIDAEHILFLDGNTFGA
DFSRFGKPLPNSVYACHDYSNYGFPNPPEPFTRSEKQIATLERQFERKIKYMREIGGPVWNGEFG
PVYASPEDGDDYEKTNDERYAVLEEQLKIYARVNASWSIWLYKDIGFQGMVYVDPETPYMKLLKP
FLEKKKQTAVDAWGCDDTPVRDVFVPMERWLTESAPGIEKLYPPMWRAHKHLFRRVRNCLLGEVM
CDEYAEYFRDKTTEELDELAKSFALANCQKRDRLNHILTEDGKREVNE

Appendix B

Amino Acid Abbreviations

Table 1. Standard three and one letter abbreviations for both essential and non-essential amino acid residues

Amino Acid	Three letter abbreviation	One letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Iso-leucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V