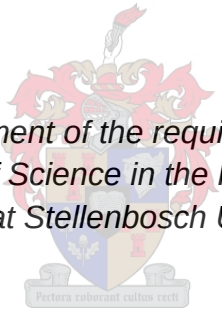
Urease activity of the systemic fungal pathogen *Emergomyces africanus*

By

Barbra-Ann Lerm



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Abstract

A novel species of an *Emmonsia*-like fungus, recently named *Emergomyces africanus* Dukik, Kenyon, Govender *et* de Hoog, was identified as a cause of disseminated mycosis in HIV-infected persons in South Africa. During the disease process, the yeast-like cells of *E. africanus* are known to disseminate and colonize various organs of the human body. The ability to cause disseminated AIDS-related mycosis is also characteristic of another unrelated fungus, *Cryptococcus neoformans,* an opportunistic pathogen responsible for the AIDS-defining illness, cryptococcal meningitis. During the latter disease progression, entry of cryptococcal cells into the brain is facilitated by virulence factors that include urease enzyme activity. However, in contrast to *C. neoformans*, the enzymes produced by *E. africanus*, some of which may be involved in pathogenesis, have not been described. Using a clinical isolate of *C. neoformans* as a reference, the aim of this study was to confirm, characterize and quantify the urease activity of *E. africanus* clinical isolates. Urease activity was tested using Christensen's urea agar, after which the presence of a urease gene in the genome of *E. africanus* was confirmed by gene sequence analysis. Subsequent evaluation of colorimetric enzyme assay data, using Michaelis-Menten enzyme kinetics, revealed similarities between the substrate affinity of the urease enzyme produced by *E. africanus* (K_m ca. 26.0 mM) and that of *C. neoformans* (K_m ca. 20.6 mM). However, nutrient conditions were found to affect the urease activity of these two fungi differently.

Colorimetric enzyme assays revealed that the addition of 2.5 g/l urea to the culture medium stimulated the urease activity of *E. africanus*, whereas nutrient limitation notably increased cryptococcal urease activity. The significant enhancement of urease activity in *C. neoformans* CAB 1055 under conditions of nutrient limitation was also confirmed by relative real-time quantitative PCR (RT-qPCR) analyses. Unlike that observed for *E. africanus* JX398293, the urease gene expression of *C. neoformans* CAB 1055 was significantly up-regulated by a mean fold change of 2 ± 0.15 in response to nutrient-limited conditions.

In addition to the work on the effect of different nutrient conditions, a preliminary study was conducted to investigate the effect of pH on the urease activity of *E. africanus* JX398293 and *C. neoformans* CAB 1055. For both fungi, analysis of

colorimetric enzyme assay data revealed that there was no significant difference in the urease activity of crude protein extracts originating from yeast cells exposed to an environment with a pH of 5 and 8, respectively. Thus, indications are that environmental pH does not play a role in the regulation of urease activity in *E. africanus* JX398293 and *C. neoformans* CAB 1055.

Overall, this study has confirmed the presence of an active urease enzyme in the novel *E. africanus* species. Furthermore, we demonstrated novel mechanisms of urease regulation in both *E. africanus* JX398293 and *C. neoformans* CAB 1055. Future studies should aim at the development of a urease knock-out mutant strain of *E. africanus*, which can be used to investigate the potential role of urease in the pathogenesis of *E. africanus* and its survival in the natural environment.

Opsomming

‘n Nuut-ontdekte spesie van ‘n *Emmonsia*-agtige fungus, wat onlangs die naam *Emergomyces africanus* Dukik, Kenyon, Govender *et* de Hoog ontvang het, is as ‘n oorsaak van verspreide mikose in HIV-geïnfekteerde persone in Suid-Afrika geïdentifiseer. Dit is bekend dat die gis-agtige selle van *E. africanus* gedurende die verloop van die siekte versprei en verskillende organe van die menslike liggaam koloniseer. Die vermoë om verspreide VIGS-verwante mikose te veroorsaak is ook kenmerkend van ‘n ander onverwante fungus, *Cryptococcus neoformans,* ‘n opportunistiese patogeen verantwoordelik vir die VIGS-definiërende siekte, cryptokokale meningitis. Gedurende die verloop van dié siekte word die binnedringing van cryptokokkale selle in die brein gefasiliteer deur virulensiefaktore wat urease ensiemaktiwiteit insluit. In teenstelling met *C. neoformans*, is die ensieme wat deur *E. africanus* geproduseer word - sommige wat by patogenese betrokke mag wees - egter nog nie beskryf nie. Deur ‘n kliniese isolaat van *C. neoformans* as verwysing te gebruik, was die doel van dié studie om urease-aktiwiteit in kliniese isolate van *E. africanus* te bevestig, te karakteriseer en te kwantifiseer. Urease-aktiwiteit is met behulp van Christensen se ureum-agar getoets, waarna die teenwoordigheid van 'n ureasegeen in die genoom van *E. africanus* deur geenvolgordeanalise bevestig is. ‘n Daaropvolgende evaluasie van kolorimetriese ensiemtoetsdata, wat deur Michaelis-Menten ensiemkinetika verkry is, toon ooreenkomste tussen die substraataffiniteit van die urease-ensiem wat deur *E. africanus* geproduseer word (K_m ca. 26.0 mM), en dié wat deur *C. neoformans* geproduseer word (K_m ca. 20.6 mM). Daar is egter vasgestel dat nutriëntkondisies die urease-aktiwiteit van hierdie twee fungusse verskillend affekteer.

Kolorimetriese ensiemtoetse het getoon dat die byvoeging van 2.5 g/l ureum by die kultuurmedium die urease-aktiwiteit van *E. africanus* stimuleer, terwyl nutriëntbeperking die cryptokokkale urease-aktiwiteit merkbaar verhoog. Die beduidende bevordering van urease-aktiwiteit in *C. neoformans* CAB 1055 onder nutriëntbeperkende omstandighede is deur relatief-intydse-kwantitatiewe- PKR (RT-kPKR) analise bevestig. In teenstelling met *E. africanus* JX398293, is die urease-geenuitdrukking van *C. neoformans* CAB 1055 beduidend in reaksie op nutriëntbeperkte toestande verhoog deur 'n gemiddelde verandering van 2 ± 0.15.

Benewens die navorsing op die uitwerking van verskillende nutriëntkondisies, is ‘n voorlopige studie gedoen om die uitwerking van pH op die urease-aktiwiteit van *E. africanus* JX398293 en *C. neoformans* CAB 1055 te bepaal. Analises van kolometriese ensiemtoetsdata van beide fungusse het gewys dat daar geen beduidende verskil in die urease-aktiwiteit van ru-proteïenekstrakte van gisselle wat aan omgewings met ‘n pH 5 en 8 blootgestel is nie. Daar is dus aanduidings dat omgewings-pH nie ‘n rol in die regulering van urease-aktiwiteit in *E. africanus* JX398293 en *C. neoformans* CAB 1055 speel nie.

In geheel bevestig hierdie studie die teenwoordigheid van ‘n aktiewe urease-ensiem in die nuwe *E. africanus* spesie. Ons het verder nuwe meganismes vir die regulering van urease-aktiwiteit in beide *E. africanus* JX398293 en *C. neoformans* CAB 1055 gedemonstreer. Toekomstige studies moet fokus op die ontwikkeling van ‘n mutante stam van *E. africanus* waarvan die ureasegeen uitgeslaan is en wat gebruik kan word om die moontlike rol van urease, in die patogenese en oorlewing van *E. africanus* in die natuurlike omgewing, te ondersoek.

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Motivation

Recently, representatives of a novel fungal genus and species, named *Emergomyces africanus* Dukik, Kenyon, Govender *et* de Hoog (Dukik *et al*., 2017), were identified as causative agents of disseminated mycosis in HIV-infected individuals in South Africa (Kenyon *et al*., 2013; Schwartz *et al*., 2015b). This thermally dimorphic pathogen is known for its ability to undergo a morphological transition from a hyphal phase at 26 ºC to a yeast-like phase at 37 ºC (Kenyon *et al*., 2013). The yeast-like cells of *E. africanus* have been found to affect numerous organ systems of immunocompromised individuals, particularly HIV-infected patients (Schwartz *et al*., 2015a and 2015b). The ability to cause severe disseminated AIDS-related mycosis is also characteristic of the neurotropic basidiomycetous yeast *Cryptococcus neoformans* (Meyohas *et al*., 1995; Wang *et al*., 1995; Kambugu *et al*., 2008; Park *et al*., 2009). In patients with advanced HIV-infection, *C. neoformans* is commonly responsible for the AIDS-defining illness cryptococcal meningitis (Fries and Cox, 2011). During the disease process, entry of cryptococcal cells into the brain is facilitated by numerous virulence factors, including urease enzyme activity (Cox *et al*., 2000; Dromer and Levitz, 2011). In contrast to *C. neoformans*, the enzymes produced by *E. africanus*, some of which may be involved in pathogenesis, have not yet been described.

Using *C. neoformans* as a reference, the aim of this study was to confirm, characterize and quantify the urease activity of the novel species, *E. africanus*. In order to achieve this, clinical representatives of *E. africanus* were first screened for urease activity using Christensen's urea agar, whereafter the presence of the urease gene in the genome of *E. africanus* was confirmed using gene sequence analysis (Objective 1, Chapter 2). Using colorimetric enzyme assays and Michaelis-Menten enzyme kinetics, the urease enzyme substrate affinity of *E. africanus* was then determined and compared to that of a clinical *C. neoformans* strain (Objective 2, Chapter 2). Thereafter, the effect of different nutrient conditions on the urease activity of both *E. africanus* and *C. neoformans* was studied using colorimetric enzyme assays and real-time quantitative PCR (Objective 3, Chapter 2). Finally, colorimetric enzyme assays were also used in a preliminary study to determine the

effect of pH on the urease activity of the *E. africanus* and *C. neoformans* strains (Objective 4, Chapter 3).

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CHAPTER 1

Literature Review

1.1 INTRODUCTION

According to the World Health Organisation (WHO), human immunodeficiency virus (HIV) remains a global public health issue, claiming over 35 million lives to date (WHO, 2016). Sub-Saharan Africa is the most affected region with 25.6 million HIV-infected individuals in 2015. South Africa in particular has the highest prevalence of HIV with 7 million infected individuals (UNAIDS, 2015). Due to a compromised immune system, HIV-infected patients are susceptible to various opportunistic infections (Ruhnke, 2004). A common bacterial lung infection amongst individuals suffering from acquired immunodeficiency syndrome (AIDS) is tuberculosis (TB), caused by *Mycobacterium tuberculosis*. In 2010, 13 % of the total incident cases of TB were co-infections with HIV (Gray and Cohn, 2013). In addition to TB, specific fungal diseases have been described as AIDS-defining opportunistic illnesses (Ruhnke, 2004; Govender *et al*., 2011). The two most common fungal opportunistic pathogens are *Candida albicans* and members of the genus *Cryptococcus* (Holmes *et al*., 2003). It is contended that the natural reservoirs for *C. albicans* are the mucosal surfaces of humans (Moran *et al*., 2012) and fecal polluted environments (Stone *et al*., 2012), while that of pathogenic cryptococci are decaying trees and bird droppings (Lazéra *et al*., 1998; Zarrin *et al*., 2010; Abulreesh *et al*., 2015).

Cryptococcus neoformans, syn. *Cryptococcus neoformans* var. *grubii* (Hagen *et al*., 2015), is the predominant causative agent of cryptococcosis and is the most prevalent species with a global distribution (Antinori, 2013). Two molecular types within this species, VNI and VNII, are responsible for 98 % of cryptococcal infections amongst HIV/AIDS patients (Meyer *et al*., 2011; Sabiiti and May, 2012). Cryptococcal infection usually commences in the lungs of immunocompromised individuals, followed by the dissemination of yeast cells into the central nervous system (CNS; Chang *et al*., 2004). Cryptococcal meningitis is the most common form of cryptococcosis and is responsible for numerous cases of morbidity and mortality amongst HIV-infected patients (Ruhnke, 2004; Fries and Cox, 2011). Although not as lethal as *C. neoformans*, the commensal and opportunistic pathogen *C. albicans* is the causative agent of the common yeast infections, oral and vaginal candidiasis (Ruhnke, 2004). More specifically, this fungus is responsible for the AIDS-defining illness esophageal candidiasis (Kim and Sudbery, 2011). In a recent study conducted on oral lesions in HIV-infected patients in South Africa, *C. albicans*

accounted for 41.8 % of all lesions (Moodley and Wood, 2015). Both cryptococcosis and candidiasis can co-occur with a variety of other fungal diseases in HIV/AIDS patients. The list of these fungal diseases is ever-increasing as more emerging fungal pathogens are identified.

Recently, representatives of an *Emmonsia*-like dimorphic fungal genus were found to be the causative agents of disseminated mycoses in a number of HIV-positive individuals in South Africa (Kenyon *et al*., 2013; Schwartz *et al*., 2015). In a recently published paper (Dukik *et al*., 2017) these fungi were classified into a novel genus and species, namely *Emergomyces africanus*. This genus also includes another pathogenic species, *Emergomyces pasteurianus,* previously called *Emmonsia pasteuriana* (Dukik *et al*., 2017). Members of *Emergomyces* are characterized by their ability to form yeast-like cells in response to a change in temperature (Kenyon *et al*., 2013). This phenomenon is termed thermal dimorphic switching and involves a morphological transition from a hyphal phase at 26 °C (Fig. 1.1a and b) to a yeast-like phase at 37 °C (Fig. 1.1c and d). Thermal dimorphism is characteristic of many other opportunistic fungi and in some cases has been found to serve as a virulence factor (Hogan *et al*., 1996).

Known virulence factors of the well-studied opportunistic pathogen, *C. albicans*, include dimorphism, phenotypic switching as well as the production of enzymes such as proteinases and phospholipases (Mayer *et al*., 2013). The latter enzymes are also known virulence factors of the human pathogenic fungus *C. neoformans* (Alspaugh, 2015). The specific role of phospholipases in the virulence of both *C. albicans* (Schaller *et al*., 2005) and *C. neoformans* (Cox *et al*., 2001) is not yet known; however, various hypotheses have been suggested. For example, disruption of the phospholipase gene in *C. albicans*, ca*PLB1*, led to a decrease in host cell penetration in murine models of candidiasis (Leidich *et al*., 1998). As a result, it has been suggested that during cryptococcal infection, secreted phospholipases may assist in the penetration of phospholipid-rich membranes and surfactant (Cox *et al*., 2001). Other factors found to be associated with the virulence of *C. neoformans* include capsule formation, melanin production and urease activity. Urease is a well-known fungal virulence factor and has been extensively studied during past decades (Rutherford, 2014).

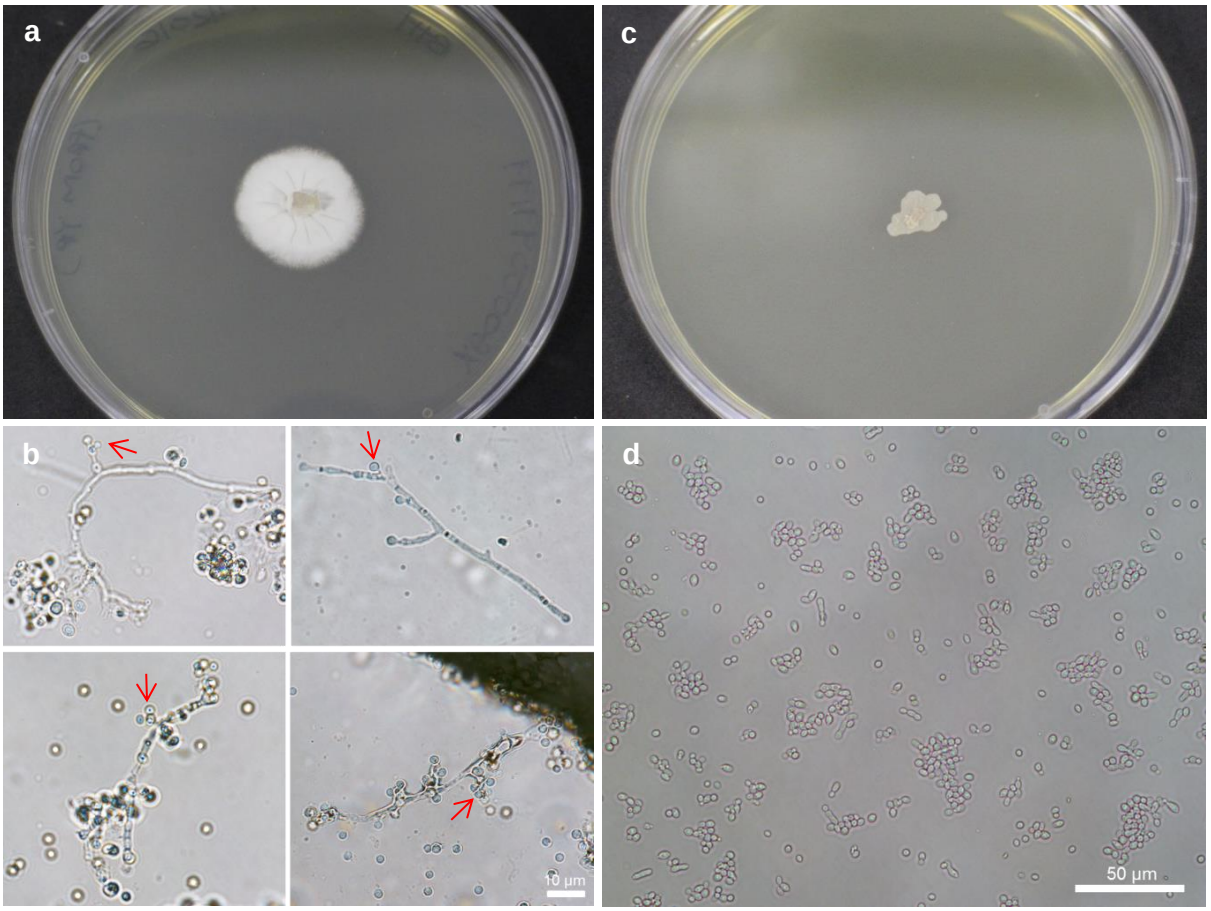

Figure 1.1 Filamentous and yeast-like phase of a representative *E. africanus* strain cultured at 26 and 37 °C respectively. (a) Filamentous growth on brain heart infusion (BHI) agar plates at 26 °C for 14 days. The surface of the colony is cerebriform and powdery. (b) Light microscopy images of hyphal fragments of the filamentous phase with red arrows pointing towards conidia arranged singularly or in clusters along hyphae. Scale bar represents 10 µm. (c) Yeast-like colony obtained by repetitive culturing on BHI agar plates at 37 °C for 21 days. (d) Light microscopy image of spherical or oval yeast-like cells cultured in BHI broth for three days. Scale bar represents 50 µm. All light microscopy images were captured with a Nikon eclipse E400 microscope.

1.2 UREASE

Urease is the first described Ni-metalloenzyme found to be responsible for the catalysis of urea decomposition (Sirko and Brodzik, 2000). This enzyme was first isolated from urine in 1874, but has since been identified in a variety of organisms including algae, invertebrates, plants as well as bacteria and fungi (Sirko and Brodzik, 2000; Krajewska, 2009).

1.2.1 Structure and function of ureases

Ureases are ubiquitous enzymes that possess a single catalytic function, namely the degradation of urea into ammonia (NH_3) and carbon dioxide (CO_2) (Sirko and

Brodzik, 2000; Krajewska, 2009). More specifically, urea is hydrolysed to NH_3 and carbamate (Krajewska, 2009). The resulting carbamate is hydrolysed spontaneously, producing another NH_3 molecule as well as carbonic acid (see Reactions 1 and 2 below). This reaction is common to all ureases, despite slight differences in protein structure (Mobley *et al*., 1995). The ureases of both plants and fungi consist of identical subunits and are therefore defined as homo-oligomeric proteins (Mobley *et al*., 1995; Sirko and Brodzik, 2000). In contrast, bacterial ureases are composed of up to three different subunits, namely UreA, UreB and UreC, and are therefore described as multimers. A common feature of bacterial, fungal and plant ureases is the presence of an active site, which can incorporate two nickel (Ni) ions (Krajewska, 2009).

$$\underset{\text{Urea}}{CH_4N_2O} + \underset{\text{Water}}{H_2O} \xrightarrow{\text{Urease}} \underset{\text{Ammonia}}{NH_3} + \underset{\text{Carbamate}}{CH_3NO_2} \qquad \text{(Reaction 1)}$$

$$\underset{\text{Carbamate}}{CH_3NO_2} + \underset{\text{Water}}{H_2O} \rightarrow \underset{\text{Ammonia}}{NH_3} + \underset{\text{Carbonic acid}}{H_2CO_3} \qquad \text{(Reaction 2)}$$

1.2.2 Sources of urease

1.2.2.1 Plant ureases

Urease of plant origin was first isolated from soybean in 1909 and the first demonstration of enzyme crystallization was conducted with urease isolated from jack bean in 1926 (Krajewska, 2009). Later studies revealed the presence of this enzyme in a variety of plants, thereby demonstrating the ubiquity of plant ureases (Hogan *et al*., 1983). Within plant cells, urease is responsible for the assimilation of organic nitrogenous compounds such as urea (Krajewska, 2009). Urea may be produced within the plant itself, or it may enter the plant roots from surrounding soil. Production of urea within plant cells can be a result of two metabolic processes, the first being the catabolism of arginine (see Reaction 3 below) and the second being the breakdown of ureides (see Reaction 4 below) originating from purine degradation (Polacco and Holland, 1993; Muñoz *et al*., 2016). Urease activity within plant cells results in the formation of NH_3, which can be readily used by the plant as a nitrogen source (Sirko and Brodzik, 2000).

$$\underset{\text{Arginine}}{C_6H_{14}N_4O_2} + \underset{\text{Water}}{H_2O} \xrightarrow{\text{Arginase}} \underset{\text{Ornithine}}{C_5H_{12}N_2O_2} + \underset{\text{Urea}}{CH_4N_2O} \qquad \text{(Reaction 3)}$$

$$\underset{\substack{\text{Ureide}\\(\text{Allantoate})}}{C_4H_8N_4O_4} \xrightarrow{\substack{\text{Allantoate}\\\text{aminohydrolase}}} \underset{\text{Ureidoglycolate}}{C_3H_6N_2O_4} + \underset{\text{Urea}}{CH_4N_2O} \xrightarrow{\substack{\text{Ureidoglycolate}\\\text{urea-lyase}}} \underset{\text{Glyoxylate}}{C_2H_2O_3} + \underset{\text{Urea}}{CH_4N_2O} \qquad \text{(Reaction 4)}$$

Increasing the availability of nitrogen in plants is particularly important for the germination of seedlings (Sirko and Brodzik, 2000). Urease is abundant in the seedlings of leguminous plants such as soybean and jack bean. Transport of nitrogen within legumes is predominantly in the form of ureides, which can be catabolised to urea and subsequently hydrolysed by urease. In addition to leguminous plants, urease has been found to play a role in the germination of *Arabidopsis thaliana* seedlings (Zonia *et al*., 1995). This was evident by the inhibition of seedling germination as a result of urease inactivation. Inhibition was found to be alleviated upon the addition of nitrogenous compounds. Later studies aimed at investigating the activation of plant ureases with reference to the well-described urease activation process of the bacterium *Klebsiella aerogenes* (Freyermuth *et al*., 2000; Witte *et al*., 2001; Follmer, 2008).

1.2.2.2 Microbial ureases

Initial studies on microbial ureases begun with the isolation of the ureolytic bacterium *Micrococcus ureae* in 1864 (Krajewska, 2009). Ureases have since been isolated from a variety of bacteria including members of the genera *Helicobacter*, *Klebsiella*, *Proteus*, *Pseudomonas* and *Staphylococcus* (Mobley and Hausinger, 1989). In addition, urease production was found to be a characteristic of many fungi, both unicellular and filamentous. Fungi capable of urease production include members of the genera *Aspergillus*, *Blastomyces*, *Coccidioides*, *Cryptococcus, Histoplasma* and *Paracoccidiodes* (Mobley and Hausinger, 1989; Rutherford, 2014). The ureases produced by many fungal and bacterial species within the abovementioned genera

are known to play important roles in pathogenesis, ruminant metabolism, as well as environmental conversions of nitrogen (Mobley and Hausinger, 1989).

Nitrogen commonly enters the environment in the form of urea present within the excretion products of animals, including birds (Tomassen *et al*., 2005; Jauffrais *et al*., 2015), earthworms (Bityutskii *et al*., 2007; McDaniel *et al*., 2013), insects (Scaraffia *et al*., 2008; Mishra *et al*., 2013) and mammals (Lobley *et al*., 2000; Pedersen and Boisen, 2001). Within the human body, urea is formed in the liver as a result of amino acid decomposition (Krajewska, 2009; Rutherford, 2014). From the liver, urea is transported in blood serum to the kidneys and ultimately excreted in urine (Krajewska, 2009). A portion of urea remains within the human body in blood serum as well as the gastrointestinal tract. The presence of urea in various regions of the human body is known to assist in the pathogenesis of both bacterial and fungal pathogens. For example, the pathogenic bacterium *Helicobacter pylori* is able to utilize urea as a means to survive the acidic conditions present within the abdomen and intestine of humans (Eaton *et al*., 1991; Mobley, 1996; Kusters *et al*., 2006). This is achieved through the production of NH_3 as a result of urease activity, leading to a localized increase in pH, thereby promoting *H. pylori* colonization and ultimately the development of gastritis and peptic ulcers.

In contrast, microbial urease activity is beneficial to a group of mammals with a unique digestive system, known as ruminants (Mobley and Hausinger, 1989). In brief, the digestive system of ruminants is characterized by the presence of four chambers, namely the rumen, reticulum, omasum and abomasum (Peng *et al*., 2015). Of these chambers, the rumen is the primary site of microbial fermentation and harbours a microbiome that mainly consists of various populations of bacteria, fungi and protozoa, which are able to hydrolyse and ferment plant carbohydrates (Lee *et al*., 2000; Belanche *et al*., 2012). In addition to the degradation of plant material, rumen microorganisms serve as the predominant source of protein for the ruminant (Leng and Nolan, 1984; Belanche *et al*., 2012). Microbial proteins are synthesized from available peptides, amino acids as well as NH_3 (Leng and Nolan, 1984). Interestingly, so-called urease-positive microorganisms within the rumen are able to produce their own NH_3 from urea (Mobley and Hausinger, 1989). The latter is commonly added as nitrogen supplement to animal feed. In addition, some urea is derived from the animal itself as a result of mammalian protein metabolism, and may

enter the rumen through saliva or direct diffusion across the rumen wall. Once urea enters the rumen, it is hydrolysed by microbial ureases, thereby producing NH_3 and CO_2. Any urea that is not metabolised may ultimately enter the environment within ruminant excreta (Dai and Karring, 2014).

1.2.2.3 Soil ureases

Animal waste, plant material and soil microorganisms are all potential sources of urease activity in soil (Dharmakeerthi and Thenabadu, 1996). Regardless of their origin, all soil ureases catalyse the same reaction - the hydrolysis of soil urea into NH_3 and CO_2. Urea is a common nitrogen source of plants and has been included in nitrogen fertilizers for decades across the world (Glibert *et al*., 2006; Witte, 2011). Application of these fertilizers for agricultural purposes results in a high concentration of soil urea and ultimately an increase in the formation of NH_3, which is readily assimilated by plants (Witte, 2011). However, rapid NH_3 production as a result of soil urease activity can be toxic to plants (Dharmakeerthi and Thenabadu, 1996). Within soil, an equilibrium usually exists between ammonium ions and gaseous NH_3. The use of urea fertilizers can lead to a disruption of this equilibrium resulting in an increase in gaseous NH_3, which not only damages plants, but also results in a tremendous loss of N. To overcome this drawback, various research projects were conducted to study urease activity and the inhibition thereof within soil (Bremner and Douglas, 1971; Gioacchini *et al*., 2002; Sanz-Cobena *et al*., 2008).

1.2.3 Urease activation

Since urease is a Ni-metalloenzyme; the incorporation of Ni ions into the active site of this protein is essential for enzyme activity.

1.2.3.1 Environmental sources of Ni

The transition metal Ni is the 24th most abundant element on Earth and it is present within air, soil, water and biological material such as food (Barceloux, 1999; Cempel and Nikel, 2006). This widespread occurrence of Ni within the environment is due to both natural processes and anthropogenic activities. Nickel enters the atmosphere predominantly through industrial processes such as the incineration of waste and the combustion of coal and oils. In addition, atmospheric Ni levels may be affected by dust formation originating from natural processes such as volcanic emissions and the weathering of rocks and soil. Nickel is found in all soils at concentrations that

vary depending on local geology and levels of anthropogenic activity. Due to natural processes such as erosion, the Ni present within soil may end up in various water sources. Nickel present within aquatic ecosystems, such as lakes and oceans, may also originate from the dissolution of surrounding rocks. Wastewater effluent is another source of Ni in both freshwater and marine ecosystems. According to the WHO, drinking water may also contain traces of this chemical element due to leaching from metal pipes and fittings. Consequently, ingestion of drinking water results in trace amounts of this metal ion in human tissues. In addition, Ni is known to be present within crops (Gupta *et al*., 2008) and livestock (Bolan *et al*., 2004) and therefore diet may serve as another source of this element within the human body.

1.2.3.2 Nickel uptake mechanisms

Various high-affinity Ni transport systems highlight the cellular requirement of Ni for urease activation. This is especially evident from studies that found a direct association between the presence of Ni transport proteins and enhanced urease activity. For example, homologs of the well-studied Ni transport genes in *Escherichia coli*, *nikABCDE*, were identified in the genome of the urease-positive bacterium *Brucella suis* (Jubier-Maurin *et al*., 2001). Furthermore, it was found that *nikA* mutant strains demonstrated suboptimal urease activity, which was restored following the addition of excess Ni. The ATP-dependent NikABCDE transport system consists of five proteins necessary for the transportation of Ni across the cell membrane (Navarro *et al*., 1993). NikA is a periplasmic binding protein and transports Ni from the periplasm to NikB and NikC. The latter proteins form a heterodimeric transmembrane core for the uptake of Ni into the cell. Lastly, NikD and NikE are the ATP-binding components responsible for the hydrolysis of ATP, thereby supplying energy for Ni transport into the cell.

Similar to *B. suis*, homologs of the NikABCDE transport system have been identified in other bacteria such as *Vibrio parahaemolyticus* (Park *et al*., 2000) and *Yersinia pseudotuberculosis* (Sebbane *et al*., 2002). The genome of *Y. pseudotuberculosis* was found to contain a *yntABCDE* operon upstream from its urease coding region (Sebbane *et al*., 2002). In addition, another gene encoding the Ni permease, UreH, was found to be situated downstream of the urease gene cluster. UreH is a single-component Ni transporter and was found to share homology with nucleotide sequences of the nickel-cobalt transporter family. Deletion of the *ynt* gene cluster of

Y. pseudotuberculosis resulted in a dramatic loss of urease activity, whereas the deletion of *ureH* had no effect. Both *ynt* and *ureH* mutants demonstrated altered Ni uptake rates. In addition to UreH, another single integral membrane protein, termed HoxN, was found to be important for low-capacity Ni uptake into bacterial cells (Wolfram *et al*., 1995; Mulrooney and Hausinger, 2003). This was evident from a study conducted on the HoxN protein of *Alcaligenes eutrophus* (Wolfram *et al*., 1995). Co-expression of *A. autrophus hoxN* and *Klebsiella aerogenes* urease operon in *E. coli* resulted in a 10-fold increase in urease activity, which was absent in strains that lacked a functional *hoxN* gene.

In contrast to the myriad of information on bacterial Ni uptake, available literature on fungal Ni uptake mechanisms is limited. One of the few known Ni transport mechanisms of yeasts is that of *Schizosaccharomyces pombe* (Eitinger, 2000). The genome of this fission yeast contains an open reading frame, identified as Nic1p, which encodes for the production of a plasma-membrane Ni transporter. Urease activity of a *nic1* mutant strain was below the assay threshold, thus indicating the role played by Nic1p in urease biosynthesis. Similarly, the genome of *C. neoformans* was found to possess a Ni permease homologous to the genes encoding HoxN and Nic1p (Singh *et al*., 2013). Interestingly, *nic1* mutant strains were avirulent in murine models of cryptococcal infection despite evidence of delayed urease activity on Christensen's urea agar.

1.2.3.3 Identification of Ni-associated urease accessory proteins

Incorporation of Ni ions into the active site of urease is facilitated by the combined action of various accessory proteins. Respective studies conducted on the urease-positive bacteria *K. aerogenes* and *Proteus mirabilis*, identified four accessory proteins involved in Ni-associated urease activation, namely UreD, UreE, UreF and UreG (Jones and Mobley, 1989; Lee *et al*., 1992). Homologous genes encoding these proteins have also been identified in other bacterial species such as *Helicobacter pylori* and *Yersinia enterocolitica* (Mobley *et al*., 1995). For example, urease activation in *H. pylori* requires the accessory protein UreH, which is a homolog of UreD in other bacterial species (Maier *et al*., 2007; Fong *et al*., 2011). Furthermore, homologs of the bacterial urease accessory genes were identified in the eukaryotic genome of the yeast *C. neoformans* (Singh *et al*., 2013). More specifically, it was found that the Ure4, Ure6 and Ure7 accessory proteins of the

C. neoformans H99 strain were homologous to the UreD, UreF and UreG bacterial accessory proteins, respectively. Interestingly, the plant *Arabidopsis thaliana* was also found to possess three urease accessory proteins essential for urease activation, namely *At*UreD, *At*UreF and *At*UreG (Witte *et al*., 2005). However, it was not determined whether these proteins show homology with known bacterial accessory proteins.

1.2.3.4 Function of Ni-associated urease accessory proteins

The bacterial urease accessory proteins UreD, UreE, UreF and UreG are believed to play a role in the assembly of the Ni metallocenter within the urease active site. The specific function of UreD remains unknown due to its insolubility when overexpressed alone; however, hypotheses have been formulated based on results obtained in various studies conducted on *K. aerogenes* (Park *et al*., 1994; Carter and Hausinger, 2010). For example, once UreD was made soluble by fusion to a maltose binding protein (MBP), experiments revealed that this protein is likely to participate directly in the transfer of Ni to the urease apoprotein (Carter and Hausinger, 2010). Similarly, formation of the UreEF fusion protein produced a soluble form of UreF, thereby further enabling its characterization (Kim *et al*., 2006). A later study revealed the possible role of UreF in the GTPase activity of UreG (Boer and Hausinger, 2012). It was hypothesized that UreF assists UreG in the coupling of GTPase activity to the incorporation of Ni. Together, the accessory proteins UreD, UreF and UreG form the UreD-UreF-UreG-urease apoprotein complex, which functions as a molecular chaperone (Soriano and Hausinger, 1999). Nickel-dependent activation of this complex relies on the GTPase activity of UreG. Lastly, UreE has been identified as a metallochaperone, with the specific function of transporting Ni to UreG within the apoprotein complex (Soriano *et al*., 2000). The genes encoding the abovementioned urease accessory proteins of *K. aerogenes* are present within the same operon and are regulated in response to nitrogen availability (Macaluso *et al*., 1990).

1.2.4 Urease regulatory factors

Urease gene expression may be constitutive or regulated in response to various environmental conditions, such as pH, urea availability and nitrogen limitation (Mobley *et al*., 1995).

1.2.4.1 Role of nitrogen-limited conditions

Under nitrogen-limiting conditions, urease activity may serve as a means of supplying microorganisms with their preferred source of nitrogen, namely ammonia (Mobley and Hausinger, 1989). A well-studied example is the urease gene cluster of *K. aerogenes*, which is controlled by the bacterium's nitrogen regulatory (ntr) system (Friedrich and Magasanik, 1977; Macaluso *et al*., 1990; Collins *et al*., 1993). Mutations in either of the two *ntr* genes, namely *ntrA* and *ntrC*, resulted in a urease-negative phenotype, which could be restored following the addition of recombinant plasmids containing the corresponding functional *ntr* gene (Collins *et al*., 1993). Similarly, a reduction in urease synthesis was observed when the bacterium *Saccharopolyspora erythraea* was grown in a medium supplemented with ammonium salt (Flores, 1996).

1.2.4.2 Effect of urea on cellular urease activity

The urease activity of microorganisms can also be regulated in response to the availability of the enzyme's substrate, urea. The genome of the bacterium *Proteus mirabilis* contains a nucleotide sequence, *ureR*, which is required for transcriptional regulation of the urease operon in the presence of urea (Nicholson *et al*., 1993). When urea is available, the transcriptional activator, UreR, positively activates expression of the urease gene cluster (D'Orazio and Collins, 1993; Dattelbaum *et al*., 2003). Similarly, the native urease of *Providencia stuartii* was found to be induced fourfold when grown in a medium supplemented with 0.1 % urea (Mobley *et al*., 1986). Microbial urease activity, however, is not always regulated in response to urea. For example, urea supplementation of growth media did not result in the up-regulation of genes within the urease operon of *Bacillus cereus* (Mols and Abee, 2008). Furthermore, urease operon genes were not induced under acidic conditions and *B. cereus* urease was therefore suggested to be important for cellular nitrogen metabolism under ammonia-limited conditions.

1.2.4.3 Role of pH in cellular urease activity

Urease-positive microorganisms may regulate urease gene expression in response to changes in environmental pH (Li *et al*., 2000; Scott *et al*., 2000; Merrell *et al*., 2003). For example, in *Streptococcus salivarius* biofilms, acidic conditions resulted in the up-regulation of genes within the urease operon (Li *et al*., 2000). The production of urease-derived ammonia (NH_3) results in the alkalinization of the surrounding

environment, thereby protecting *S. salivarius* from acidic conditions encountered within the oral cavity. Similarly, a low pH was found to activate the ureases of both *Morganella morganii* and *Y. enterocolitica in vitro* (Young and Amid, 1996). Interestingly, the cytoplasmic urease of *Y. enterocolitica* was activated 780-fold under acidic conditions. In addition to the abovementioned bacteria, pH-induced urease expression is characteristic of *H. pylori.* Transfer of *H. pylori* to an acidic environment resulted in the up-regulation of six genes within the urease operon, namely those encoding the structural proteins UreA and UreB as well as the accessory proteins UreF, UreG, UreH and UreI (Merrell *et al*., 2003). This is not surprising as it is well-known that urease is essential for the survival of this gastric pathogen within the acidic environment of the stomach (Eaton *et al*., 1991; Ferrero *et al*., 1992; Tsuda *et al*., 1994).

1.2.5 Role of urease in pathogenicity

A range of microorganisms are able to produce ureases with important pathogenic implications (Rutherford, 2014). Initially this was evident from studies conducted on urease-positive bacteria such as *H. pylori* (Mobley *et al*., 1988). As briefly mentioned earlier, this bacterium relies on urease activity in order to survive the acidic conditions of the human stomach. Urease-derived NH_3 neutralises the gastric mucosa, thereby facilitating *H. pylori* colonization and ultimately the development of gastritis and peptic ulcers (Eaton *et al*., 1991; Mobley, 1996; Kusters *et al*., 2006). Furthermore, the production of NH_3 by *H. pylori* urease may have a direct toxic effect on gastric epithelial cells (Mobley, 1996). Interestingly, urease was also found to play an important role in the motility of *H. pylori* within viscous environments such as the gastric mucosa (Nakamura *et al*., 1998). In addition to *H. pylori*, other pathogenic bacteria with urease activity include members of the genera *Klebsiella*, *Proteus* and *Staphylococcus* (Burne and Chen, 2000). Production of urease within the human body by representatives of these genera may result in calculi (stone) formation (Mobley and Hausinger, 1989). For example, infection stones are characteristic of urinary tract infections caused by *Proteus mirabilis* (Mobley and Warren, 1987). The role of ureases in the pathogenicity of bacterial species is therefore well-studied; however, it is only recently that studies were conducted to understand the role of urease in fungal pathogenesis (Rutherford, 2014).

Urease activity is characteristic of various medically-important fungi (Mirbod-Donovan *et al*., 2006), including members of the genera *Aspergillus*, *Blastomyces, Coccidioides*, *Cryptococcus*, *Histoplasma* and *Paracoccidioides* (Mirbod-Donovan *et al*., 2006; Rutherford, 2014). Interestingly, the effect of urease activity on the virulence of certain species within these genera has only been described for *Coccidioides immitis*, *Coccidioides posadasii* and *C. neoformans* (Cox *et al*., 2000; Mirbod *et al*., 2002; Mirbod-Donovan *et al*., 2006).

The respiratory pathogens *C. immitis* and *C. posadasii* are the causative agents of coccidioidomycosis, commonly known as valley fever (Galgiani *et al*., 2005). Infected host tissue is characterised by an increase in pH, which can be partly attributed to urease activity (Mirbod *et al*., 2002; Mirbod-Donovan *et al*., 2006). In addition, murine models of infection with *C. posadasii* demonstrated elevated host inflammatory responses as a result of urease activity (Mirbod-Donovan *et al*., 2006). This immune response, together with host tissue alkalinization, has been suggested to increase host tissue damage. The same may be true for *C. immitis*, which is also known to release urease-derived NH_3 into its surrounding environment (Cole, 1997; Mirbod *et al*., 2002). More specifically, following host tissue colonization with *C. immitis*, abscesses are formed with elevated internal pH levels (Cole, 1997). Interestingly, further support for the role of urease in the virulence of *C. immitis* was obtained in an *in vivo* study where mice were immunized with recombinant urease protein and an expression vector containing urease DNA (Li *et al*., 2001). Both immunization methods elicited an immunoprotective response in mice, suggesting the potential use of *C. immitis* urease as a vaccine against coccidioidomycosis.

In vivo studies have commonly been used to demonstrate the role of urease in the virulence of *C. neoformans* (Cox *et al*., 2000; Olszewski *et al*., 2004; Osterholzer *et al*., 2009). Initial findings by Cox *et al*. (2000) revealed that mice infected with the urease-positive *C. neoformans* H99 strain had lower survival rates than those infected with the mutant *ure1* strain. This study therefore provided evidence for the involvement of urease in the pathogenesis of *C. neoformans*. In 2004, another murine model revealed the potential mechanism by which urease enhances the pathogenicity of *C. neoformans* during CNS invasion (Olszewski *et al*., 2004). It was found that urease activity contributed to microvascular sequestration of yeast cells, thereby promoting transmigration of *C. neoformans* across the blood-brain barrier. In

addition to the brain, cryptococcal urease has been shown to contribute to pathogen virulence within the lungs of murine models (Osterholzer *et al*., 2009). Mice infected with the urease-positive H99 cryptococcal strain had elevated levels of immature dendritic cells in the lungs as well as an enhanced T2 immune response. Furthermore, this immune response was ineffective at clearing cryptococcal cells from the lungs.

1.3 CONCLUSIONS

C*andida albicans* and members of the genus *Cryptococcus* are well-known causative agents of AIDS-defining opportunistic illnesses. Recently, the opportunistic pathogen *E. africanus* was also discovered to be associated with HIV-positive individuals. Similar to *C. neoformans*, the novel species *E. africanus* was found to cause severe disseminated disease in HIV-infected patients. Fungal pathogenicity is known to be facilitated by a series of virulence factors, such as the urease enzyme of *C. neoformans*, which promotes the entry of cryptococcal cells into the central nervous system. The ability of *E. africanus* to produce urease, however, has not yet been determined. The aim of this study was therefore to confirm, characterize and quantify the urease activity of the novel species *E. africanus*.

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CHAPTER 2

Urease activity of the systemic fungal pathogen *Emergomyces africanus*, compared with that of the neurotrope *Cryptococcus neoformans*

Chapter 2 is compiled according to the journal format of FEMS Yeast Research

2.1 INTRODUCTION

Recently, novel *Emmonsia*-like fungi have emerged as a cause of human disease across the globe (Kenyon *et al*. 2013; Schwartz *et al*. 2015b). Over the past five years, the number of human cases has escalated drastically with the majority of cases being South African HIV-infected individuals (Schwartz *et al*. 2015b). The *Emmonsia*-like fungi that primarily cause AIDS-related mycoses are characterized by small yeast-like cells *in vivo* (Schwartz *et al.* 2015b) and have recently been placed in a novel genus, *Emergomyces* (Dukik *et al*. 2017). The predominant species that causes disease in South Africa has been named *Emergomyces africanus* Dukik, Kenyon, Govender *et* de Hoog (Dukik *et al*. 2017) and is associated with mortality rates of around 48% (Schwartz *et al*. 2015b).

Members of the novel genus *Emergomyces* (including *E. africanus* and *Emergomyces pasteurianus,* previously called *Emmonsia pasteuriana*) (Dukik *et al*. 2017) are classified as thermally dimorphic due to their ability to undergo a morphological transition from a mycelial phase at 26 °C to a yeast-like phase at 37 °C (Kenyon *et al*. 2013). Although the primary environmental source of *E. africanus* is unknown, its initial portal of entry is presumed to be inhalational and the main clinical manifestation of disease is the presence of widespread skin lesions (Schwartz *et al*. 2015a). Early clinical data, however, suggests that virtually all organ systems can be affected (Kenyon *et al*. 2013; Schwartz *et al*. 2015a; Schwartz *et al*. 2015b).

Clinical studies have established that *E. africanus* is an opportunistic pathogen that primarily causes an AIDS-related mycosis, as all patients diagnosed to date have been profoundly immunocompromised (Kenyon *et al*., 2013; Schwartz *et al*. 2015a). This is similar to the neurotropic basidiomycetous yeast *Cryptococcus neoformans*, which is known for its ability to cause severe disseminated disease in immunocompromised individuals, particularly HIV-infected patients (Meyohas *et al*.1995; Wang, Aisen and Casadevall 1995; Kambugu *et al*. 2008; Park *et al*. 2009).

Cryptococcus neoformans, syn. *Cryptococcus neoformans* var. *grubii* (Hagen *et al*. 2015), is the primary causative agent of cryptococcosis in persons with advanced HIV infection (Meyer *et al*. 2011; Cogliati 2013). Cryptococcal infection commences in the lungs, following the inhalation of basidiospores or yeast cells into the alveolar

spaces of the human host (Giles *et al*. 2009; Velagapudi *et al*. 2009; Nielsen and Kwon-Chung 2011). From there, *C. neoformans* can disseminate to the central nervous system (CNS) (Chang *et al*. 2004). More specifically, cryptococcal cells are ingested by blood phagocytes, which act as a vehicle for fungal dissemination to the CNS (Santangelo *et al*. 2004; Charlier *et al*. 2009). The AIDS-defining illness, cryptococcal meningitis (Fries and Cox 2011) is one of the leading causes of mortality amongst HIV-positive individuals, particularly in the developing world (Lawn *et al*. 2008).

Upon entry into the CNS, *C. neoformans* may employ multiple virulence factors to assist in its migration across the blood-brain barrier (Dromer and Levitz 2011). Virulence factors that have been linked to *C. neoformans* brain invasion include the presence of mating types with varying levels of virulence, capsule formation, phospholipase B and laccase secretion, as well as the production of urease (Dromer and Levitz 2011). The nickel-requiring enzyme urease is responsible for the enzymatic hydrolysis of urea into ammonia and carbonic acid (Mobley, Island and Hausinger 1995). This enzyme was first implicated in the pathogenesis of *C. neoformans* during a study conducted on experimental cryptococcosis in murine models (Cox *et al*. 2000). It was found that mice infected with a urease-negative *C. neoformans* mutant showed increased survival compared to mice infected with the wild-type strain. The reason for this enhanced pathogenesis was later suggested by Olszewski *et al*. (2004), who found that urease activity promoted microvascular sequestration of *C. neoformans* cells in the CNS. While the mechanism behind this process is still unknown, it was suggested that the phenomenon can be ascribed to the effects of urease-derived ammonia, such as endothelial cell toxicity.

To date, the ability of *E. africanus* to produce urease has not been determined. This may be of clinical utility as similar to that seen in patients with cryptococcosis, neurological symptoms have been observed in patients infected with *E. africanus* (Schwartz *et al*. 2015a). Using a clinical isolate of *C. neoformans* as a urease-positive reference strain, the aim of this study was therefore to confirm, characterize and quantify the urease activity of *E. africanus*. To achieve this, clinical isolates of *E. africanus* were screened for urease production using a conventional plate assay. Thereafter, polymerase chain reaction (PCR) amplification was used to confirm the presence of the urease gene within the genome of *E. africanus*. The substrate

affinity of urease within crude protein extracts of a representative strain was then determined using Michaelis-Menten enzyme kinetics. This was followed by an investigation of the effect of different nutrient conditions on the urease activity of crude protein extracts. Finally, the effect of nutrient limitation on urease gene expression was studied using relative real-time qPCR (RT-qPCR) analysis.

2.2 MATERIALS AND METHODS

2.2.1 Strains and maintenance

The clinical strains, and their origins, used in this study are listed in Table 2.1. Both *E. africanus* isolates were maintained in a filamentous phase by periodic transfer to brain heart infusion (BHI) broth (pH 7.4; Merck, Darmstadt, Germany) supplemented with 2 % (w/v) bacteriological agar (Merck) and incubated at 26 ºC. The yeasts *Candida albicans* and *C. neoformans* were maintained at 26 ºC by periodic transfer to yeast extract-malt extract (YM) broth (pH 5; Yarrow 1998).

Table 2.1 Strains and their origins used in this study.

Species	Strain	Origin
Emergomyces africanus[a]	JX398291*	Clinical Isolate, NICD, RSA
Emergomyces africanus[a]	JX398293*	Clinical Isolate, NICD, RSA
Candida albicans[b]	CAB 397	Clinical Isolate, Tygerberg Hospital, RSA
Cryptococcus neoformans[b]	CAB 1055	Clinical Isolate, Tygerberg Hospital, RSA

*Identity of strains based on GenBank accession numbers for the sequences of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (Kenyon *et al*., 2013)

[a]Strains obtained from the culture collection of the National Institute for Communicable Diseases (NICD), RSA

[b]Strains obtained from the culture collection of the Department of Microbiology, University of Stellenbosch, RSA

2.2.2 Selection of urease-positive strains

Strains listed in Table 2.1 were screened for urease activity on Christensen's urea agar according to the method described by Kurtzman *et al*. (2011). Christensen's urea agar contains the pH-indicator phenol red, which changes colour from orange to deep pink under alkaline conditions. Urease-derived ammonia leads to medium alkalinization and a positive urease reaction is thus evidenced by the presence of a deep pink colour. In this study, inoculated agar plates were incubated at 26 ºC with

daily inspection for five days and all results were compared to the reactions obtained on control plates without urea. *Candida albicans* CAB 397 was included in the experimentation as a negative control.

2.2.3 Identification of urease genes and primer design

Predicted gene and protein sequences of a strain belonging to *E. africanus* were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Dukik *et al*. 2017). Amino acid sequences of predicted proteins were then compared and aligned to the amino acid sequences of known fungal ureases, obtained from the UniProt database (http://www.uniprot.org/), using BLAST+ (v2.2.31) with a minimum E-value of 1.0e-8 (Altschul *et al*. 1990). One of the predicted proteins had the greatest sequence identity (86 %) with the urease of *Blastomyces dermatiditis* ATCC 26199 (UniProt accession number T5BDP5) and was thus considered a putative urease of *E. africanus*. The CLC Main Workbench 7.6.4 (CLC Bio) was used to conduct a multiple sequence alignment where the putative urease amino acid sequence was aligned with the amino acid sequences of other known urease proteins available on GenBank (http://www.ncbi.nlm.nih.gov/genbank). Thereafter, a phylogenetic tree was constructed using the maximum likelihood approach with the JTT matrix and 100 bootstrap replicates (Fig. A1 in Appendix A). In addition, the same software was used to predict the open reading frame (ORF) within the gene encoding the putative protein. Thereafter, primers were designed to amplify the identified ORF within the genomes of the two *E. africanus* strains used in this study. The nucleotide sequences of the forward and reverse primers were 5'-CGATCGAATGAAATCATATGGATACCG-3' and 5'-CGTACATGTCGTTGCGGTTGTTCC-3', respectively. These primers were produced by Inqaba Biotechnical Industries (Pretoria, Gauteng, RSA).

2.2.4 DNA extraction and PCR amplification

Emergomyces africanus JX398291 and *E. africanus* JX398293 were cultured for two weeks on BHI agar plates at 26 ºC. Thereafter, genomic DNA (gDNA) was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Orange County, CA, USA) as per the manufacturer's instructions. PCR amplification of the urease gene was then performed using the abovementioned primer set. The reaction

components consisted of 1 µl gDNA, 1 µl of each primer (10 µM), 10 µl 2x KAPA Taq ReadyMix (KAPA Biosystems, MA, USA) and deionized water to give a volume of 20 µl. The amplification process was carried out in an Applied Biosystems 2720 thermal cycler (Foster City, CA, USA) with an initial denaturation step at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 45 sec and a final extension step at 72 °C for 7 min. Thereafter, the urease nucleotide sequences of the amplified regions were determined using the Applied Biosystems ABI3130xl genetic analyser and compared to known nucleotide sequences available on GenBank using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast).

2.2.5 Inoculum preparation for urease assays

Inoculum preparation differed depending on the fungal species. Inocula of *C. neoformans* CAB 1055 were obtained from five-day-old yeast cultures on YM agar plates incubated at 26 ºC, while yeast-like growth of *E. africanus* JX398293 was first obtained by repeated transfers to fresh BHI agar plates at 37 ºC. In the latter case, a loop-full of the yeast-like growth was aseptically transferred to a test tube containing 5 ml of BHI broth. The inoculated tube was incubated at 37 ºC on a TC-7 tissue culture roller drum (60 rpm; New Brunswick Scientific Co. Inc.) and the yeast-like phase was maintained by repeatedly transferring cells to test tubes containing fresh BHI broth. This yeast-like growth of *E. africanus* JX398293, obtained after three days of incubation at 37 ºC in BHI broth, served as inoculum for subsequent experiments.

2.2.6 Protein extract preparation for urease assays

A suspension of *E. africanus* JX398293 yeast-like cells was used as inoculum for two conical flasks (1 L) each containing 100 ml BHI broth. Following inoculation, flasks were incubated at 37 ºC for three days on an orbital shaker (Model G53, New Brunswick Scientific Co. Inc., Edison, NJ, USA) set at 200 revolutions per minute (rpm). For *C. neoformans* CAB 1055, four conical flasks (1 L) were each inoculated with a loop-full of cells obtained from cultures grown on YM agar plates. All four flasks were incubated with shaking (200 rpm) at 37 ºC for 16 hours. After the respective incubation periods, crude protein extracts were prepared according to a method described by Moller *et al*. (2016) with modifications. Cell suspensions were centrifuged at 10,000 g for 10 min at 20 ºC and the pellets washed three times with

distilled water (dH_2O). Pellets were then washed a final time (7000 g, 10 min, 4 ºC) in 400 µl chilled lysis buffer consisting of 40 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 10 % glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Subsequently, each pellet was re-suspended in 400 µl chilled lysis buffer and transferred to a 2 ml screw-cap microcentrifuge tube containing 0.5 ml acid-washed glass beads (400-600 µm; Sigma-Aldrich, St. Louis, MO, USA). Cells were then disrupted by vigorous mixing on a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) for 30 sec, followed by 1 min cooling on ice, for a total of seven cycles, before the removal of cell debris using centrifugation (13,000 g, 15 min, 4ºC). The protein content of the resulting supernatant was measured using the BioRad Protein Assay Kit II (BioRad Laboratories, Hercules, CA, USA) and a bovine serum albumin standard curve (Fig. A2 in Appendix A). Following quantification, the urease activity of the protein extract was determined immediately.

2.2.7 Urease activity assays

The protein extract was assayed for urease activity using urea concentrations ranging from 0.625 to 30 g/l. In brief, 100 µg protein was added to a glass spectrophotometer tube containing a specific concentration of urea (Sigma-Aldrich), 50 mM HEPES buffer (pH 7.4; Sigma-Aldrich) and dH_2O to a final volume of 1 ml. Thereafter, tubes were incubated at 37 ºC for 60 min on a G53 orbital shaker (200 rpm). Ammonia production within the reaction mixture was measured after 0, 10, 20, 30, 40 and 60 min using the phenol-hypochlorite assay of Weatherburn (1967) with slight modifications. At the respective time interval, 50 µl of reaction mixture was added to a spectrophotometer tube containing 500 µl of reagent A (10 g/l phenol [Merck] and 50 mg/l sodium nitroprusside [Merck]. An equal volume (500 µl) of reagent B (5 g/l sodium hydroxide [Merck] and 8.4 ml/l sodium hypochlorite [commercial bleach]) was subsequently added and the reaction mixture was mixed well before incubation at 37 ºC for 30 min. During this time, any ammonia present within the sample was allowed to react with both reagents, producing a blue colour. Absorbance at 625 nm was then measured using a SmartSpec Plus spectrophotometer (BioRad Laboratories Ltd., Johannesburg, RSA). Reaction mixture without urea served as the blank and reaction mixture lacking crude extract was used as a control. Absorbance values were converted to nanomoles (nmol) of ammonia using an ammonium chloride standard curve prepared with concentrations

up to 1000 µM (Fig. A3 in Appendix A). Thereafter, graphs of ammonia concentration [NH_3] versus time (t) for each urea concentration [S] were plotted on the same axis (data not shown). A linear regression analysis was then conducted to determine the initial reaction velocity (V_i) for each substrate concentration from the slopes of the straight lines that best fitted the plotted data points.

2.2.8 Michaelis-Menten constant (K_m) determination

A non-linear regression analysis, based on the principle of least squares, was used to fit the Michaelis-Menten function to constructed data plots of initial reaction velocities against corresponding urea concentrations. This analysis was performed on the Solver supplement of Microsoft Office Excel, which was used to generate estimates of the Michaelis-Menten enzyme kinetic parameters, V_{max} and K_m, by minimizing the sum of squares of the non-linear function below:

$$V_i = \frac{V_{max}[S]}{K_m + [S]}$$

2.2.9 Effect of different nutrient conditions on urease activity

The urease activity assay described above was conducted on protein extracts obtained from *C. neoformans* CAB 1055 and *E. africanus* JX398293 grown in BHI broth with and without supplemented urea. In addition, the effect of nutrient starvation in the presence and absence of urea was investigated by conducting the same assay on protein extracts obtained from cells that were transferred from BHI broth to a nutrient-limited medium. All experiments were conducted in triplicate. If an insufficient amount of protein extract was obtained for a biological repeat, multiple flasks were inoculated and the resulting lysate pooled.

For *C. neoformans* CAB 1055, a loop-full of growth from a YM agar plate served to inoculate each of two conical flasks (1 L), each containing 100 ml BHI broth, one of which was supplemented with 2.5 g/l urea. Following inoculation, both flasks were incubated with shaking (200 rpm) at 37 ºC for 16 hours. Similarly, a suspension of *E. africanus* JX398293 yeast-like cells was used to inoculate an additional two flasks containing the same two media as described above. Flasks inoculated with *E. africanus* JX398293 were subsequently incubated with shaking (200 rpm) at 37 ºC

for three days. After the respective incubation periods, log-phase cultures were harvested by centrifugation (10,000 g, 10 min, 20 ºC) and washed three times in dH_2O. Crude protein was then extracted and quantified according to the method described above in Section 2.2.6. Urease activity of 50 µg crude protein was assayed immediately using the protocol described in Section 2.2.7, but with a fixed urea concentration of 20 g/l. An ammonium chloride standard curve was used to calculate the amount of ammonia formed in a reaction volume of 1 ml at each time interval. Graphs of nmol ammonia against time were constructed and linear regression analysis was used to determine the initial reaction velocities (nmol ammonia/min).

Using similar techniques as above, the effect of nutrient limitation in the presence and absence of urea was investigated for both *C. neoformans* CAB 1055 and *E. africanus* JX398293. A prepared inoculum of each strain was added to two conical flasks (1 L), each containing 100 ml BHI broth. Flasks inoculated with *C. neoformans* CAB 1055 were incubated with shaking (200 rpm) at 37 ºC for 16 hours, whereas inoculated flasks containing *E. africanus* JX398293 were incubated with shaking (200 rpm) at 37 ºC for three days. After the respective incubation periods, the cells were harvested via centrifugation at 10,000 g for 10 min at 20 ºC. The resulting cell pellets were resuspended in 500 µl dH_2O and transferred to separate 1 L conical flasks, each containing 100 ml of a nutrient-limited medium (pH 6.8; 0.1 % [w/v] glucose, 0.91 % [w/v] monopotassium phosphate and 0.95 % [w/v] dipotassium phosphate), one of which was supplemented with 2.5 g/l urea. Thereafter, the four inoculated flasks were incubated under shaking conditions (200 rpm) at 37 ºC for three hours. Following incubation, cells from the respective flasks were harvested by centrifugation (10,000 g; 10 min; 20 ºC) and washed three times in dH_2O, followed by a final wash step (7000 g, 10 min, 4 ºC) in 400 µl chilled lysis buffer. Washed pellets were resuspended in another 400 µl chilled lysis buffer; whereafter crude protein was extracted and quantified. Urease activity of 50 µg protein was assayed in the same manner as above, again using a urea concentration of 20 g/l. As described previously, the initial reaction velocities (nmol ammonia/min) were calculated using an ammonium chloride standard curve and linear regression analysis.

Finally, the effect of added nitrogen on *C. neoformans* urease activity was tested using the abovementioned protocol with the following modifications. Yeast cells

grown for 16 hours in 100 ml BHI broth were transferred to a 1 L conical flask containing 100 ml of the nutrient-limited medium, supplemented with 4.086 g/l ammonium chloride (Kimix, Cape Town, RSA). The resulting nitrogen concentration was similar to that found in yeast nitrogen base without amino acids (Difco, Michigan, USA). The inoculated flask was subsequently incubated for three hours at 37 ºC under shaking conditions (200 rpm). Thereafter, crude protein was extracted, quantified and assayed for urease activity using methods described above.

2.2.10 Relative quantification of urease gene expression using RT-qPCR

The effect of nutrient limitation on the urease gene expression of *C. neoformans* CAB 1055 and *E. africanus* JX398293 was determined using RT-qPCR analysis. To achieve this, urease gene expression of cells cultured in BHI broth was compared to that of cells exposed to nutrient-limited conditions for three hours. All primer sequences used for RT-qPCR amplification are listed in Table 2.2 and were produced by Inqaba Biotechnical Industries. For *C. neoformans* CAB 1055, primer sequences were obtained from Benaducci *et al*. (2016) and were used to amplify a 66 and 54 bp region of the urease and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, respectively. The GAPDH gene served as a reference for the normalization of urease gene expression data obtained during this study.

For *E. africanus* JX398293, the CLC Main Workbench 7.6.4 was used to design primers for the amplification of a 100 bp region of the partial urease gene previously identified in this study (Section 2.2.3 and 2.2.4). Furthermore, using the same method as described in Section 2.2.3, the putative GAPDH gene of *E. africanus* was identified. To achieve this, amino acid sequences of predicted proteins obtained from the CBS, The Netherlands, were compared and aligned to amino acid sequences of known fungal GAPDH proteins, obtained from the UniProt database (http://www.uniprot.org/), using BLAST+ (v2.2.31) with a minimum E-value of 1.0e-8 (Altschul *et al*. 1990). One of the predicted proteins had the greatest sequence identity (93 %) with the GAPDH of *Histoplasma capsulatum* (UniProt accession number Q9HFX1) and was thus regarded as a putative GAPDH of *E. africanus*. The CLC Main Workbench 7.6.4 was again used to design primers to amplify a 106 bp region of the predicted GAPDH gene, which was used as a reference for data normalization.

Yeast cells of each strain were cultured in BHI broth and subsequently transferred to nutrient-limited conditions in the absence of urea for three hours (Section 2.2.9). All experiments were conducted in triplicate. Cells from before and after treatment were counted using a haemocytometer (Improved Neubauer, Marienfeld Superior, Germany) and in each case $3x10^8$ cells were subjected to total RNA extraction using the Ribopure™ Yeast Kit (Ambion, Austin, TX, USA). The concentration and purity of the resulting RNA was determined using the Nanodrop ND1000 Spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). Samples with A_{260}:A_{230} ratios less than 1.7 were purified by ethanol precipitation and the integrity of RNA samples was evaluated using microcapillary electrophoresis on the Agilent BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Thereafter, 1 µg of RNA was converted to complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's instructions. To screen for possible gDNA carry over, control reactions were performed under the same conditions, but without the addition of reverse transcriptase.

Resulting cDNA samples were used in subsequent RT-qPCR assays to assess primer efficiency and specificity. In brief, pooled cDNA samples of *E. africanus* were diluted 10-fold (10^{-1} to 10^{-6}) and 1 µl of each dilution was added to a reaction mixture consisting of 200 nM of each respective primer as well as 5 µl of Power SYBR Green PCR Master Mix (Life Technologies, Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 10 µl. The above setup was also performed for pooled cDNA samples of *C. neoformans*, but with primer concentrations of 800 nM each. Negative controls included one without template and one without reverse transcriptase. All reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the amplification conditions consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec, 60 °C for 1 min and a dissociation step of 95 °C for 15 sec, 60 °C for 30 sec and 95 °C for 15 sec. All data were analysed using the SDS version 2.4 software (Applied Biosystems) and a standard curve was used to determine PCR efficiency and linearity. In addition, a melt curve analysis was used to determine primer specificity.

For urease gene expression analysis, individual cDNA samples were diluted 1:20 in low-EDTA TE buffer and 1 µl of each diluted cDNA sample was used in subsequent

real-time PCR assays as described above. A 10-fold dilution series of cDNA standards was prepared as outlined in the previous paragraph and was included in each PCR assay. Negative controls and amplification conditions were identical to that described above. For each strain, the quantification cycle (C_q) values of the GAPDH reference gene were used to normalize the calculated C_q values of the urease gene amplified from the corresponding sample. The resulting ΔC_q values were used in further calculations to determine the mean fold change in urease gene expression compared to that of the control using the $2^{-\Delta\Delta Cq}$ formula (Livak and Schmittgen 2001).

Table 2.2 Primers used for RT-qPCR analysis.

Fungal strains	Gene	Primer name*	Primer sequence (5'-3')
E. africanus JX398293	Urease	URE-F	GAACTGCTATCCGGTTCGAG
		URE-R	CCATTGGCCATGAAACTGCC
	GAPDH	GAPDH-F	ACTGACTTGAACGGAGACCC
		GAPDH-R	CCCACTCGTTGTCGTACCA
C. neoformans CAB 1055	Urease	URE-F	TCGTATCGGTGAAGTCGTCACT
		URE-R	GGACCACGGAATTGCTTCAT
	GAPDH	GAPDH-F	TTTCCCGCGACTTTTTGG
		GAPDH-R	TCGCAGCCGAGTCTACGAT

*The suffix "F" indicates a forward primer, while the suffix "R" indicates a reverse primer.

2.2.11 Statistical analyses

All data are expressed as mean ± 1 standard error of the mean. One-way ANOVA was used for the analysis of variance in data sets obtained from the characterization of urease activity in *C. neoformans* CAB 1055 and *E. africanus* JX398293. Furthermore, mean values were compared using Fisher's LSD post hoc test for multiple comparisons. The abovementioned statistical analyses were also performed on the relative gene expression data of *C. neoformans* CAB 1055. For *E. africanus* JX398293, statistical significance of the RT-qPCR data was determined by the nonparametric Mann-Whitney U test. All statistical analyses were performed using the Statistica software package (Version 13, Dell, Round Rock, TX, USA) with a set significance level of $p < 0.05$.

2.3 RESULTS

2.3.1 Identification of urease-positive strains

All tested strains, except for *C. albicans* CAB 397, produced positive urease reactions on Christensen's urea agar after five days of incubation (Fig. A4 in Appendix A). In addition, the urease gene of *E. africanus* was partially amplified from gDNA of the two representative *E. africanus* strains (GenBank accession numbers KY241790 and KY241791; Appendix B). In both cases, the amplified region of the urease gene was found to share 86 % sequence identity with the partial urease messenger RNA (mRNA) of *Blastomyces dermatitidis* SLH14081 (GenBank accession number XM_002623809.1).

2.3.2 Michaelis-Menten enzyme kinetics

A typical hyperbolic curve was obtained when the Michaelis-Menten function was fitted to constructed plots of initial reaction velocities against corresponding urea concentrations (Fig. 2.1). Furthermore, calculated K_m values of *C. neoformans* CAB 1055 and *E. africanus* JX398293 were 1.24 g/l (ca. 20.6 mM) and 1.56 g/l (ca. 26.0 mM), respectively.

2.3.3 Urease activity in BHI broth with and without supplemented urea

Protein extracts of *C. neoformans* CAB 1055 cells cultured in BHI broth had a urease activity of 6.22 ± 0.16 nmol ammonia/min, which increased slightly but insignificantly with urea supplementation (Fig. 2.2a). In contrast, urease activity in protein extracts of *E. africanus* JX398293 cells increased significantly with the addition of urea from 6.24 ± 0.34 to 7.38 ± 0.37 nmol ammonia/min (p value = 0.02) (Fig. 2.2b).

2.3.4 Urease activity in a nutrient-limited medium with and without supplemented urea

Similar to the findings obtained when *C. neoformans* CAB 1055 was cultured in BHI broth, urea supplementation of the nutrient-limited medium was found to have no significant effect on cryptococcal urease activity (Fig. 2.2a). Likewise, the results obtained for the *E. africanus* strain with regard to urea supplementation in the nutrient-limited medium was similar to that obtained with BHI broth. Urease activity in cellular protein extracts of *E. africanus* JX398293 increased significantly with the

addition of urea, from 6.19 ± 0.18 to 7.34 ± 0.19 nmol ammonia/min (p value = 0.02) (Fig. 2.2b).

It must be noted, however, that in contrast to the results obtained with *E. africanus* JX398293 (Fig. 2.2b), cryptococcal urease activity was found to be significantly higher in cells originating from the nutrient-limited medium compared to those from the BHI broth (Fig. 2.2a). An activity of 17.6 ± 1.97 nmol ammonia/min was obtained for cryptococcal cells originating from the nutrient limited medium, which is more than double the enzyme activity of cellular protein extracts from *C. neoformans* CAB 1055 cultured in BHI broth alone (p value < 0.01).

2.3.5 Effect of ammonia addition on *C. neoformans* urease activity in a nutrient-limited medium

The supplementation of a nutrient-limited medium with ammonia did not affect the urease activity of cryptococcal protein extracts (Fig. A5 in Appendix A). An enzyme activity of 18.0 ± 0.63 nmol ammonia/min was obtained, which did not differ significantly from the resulting activities when cryptococcal cells were transferred to an ammonia-free nutrient-limited medium, both in the presence and absence of urea.

2.3.6 Relative quantification of urease gene expression under conditions of nutrient limitation

Upon transfer to a nutrient-limited medium, urease gene expression of *E. africanus* JX398293 showed a mean fold change of 1 ± 0.12, which was found to be insignificant (p > 0.05). In contrast, urease gene expression of *C. neoformans* CAB 1055 was significantly up-regulated (p < 0.01) by a mean fold change of 2 ± 0.15 in response to nutrient-limited conditions.

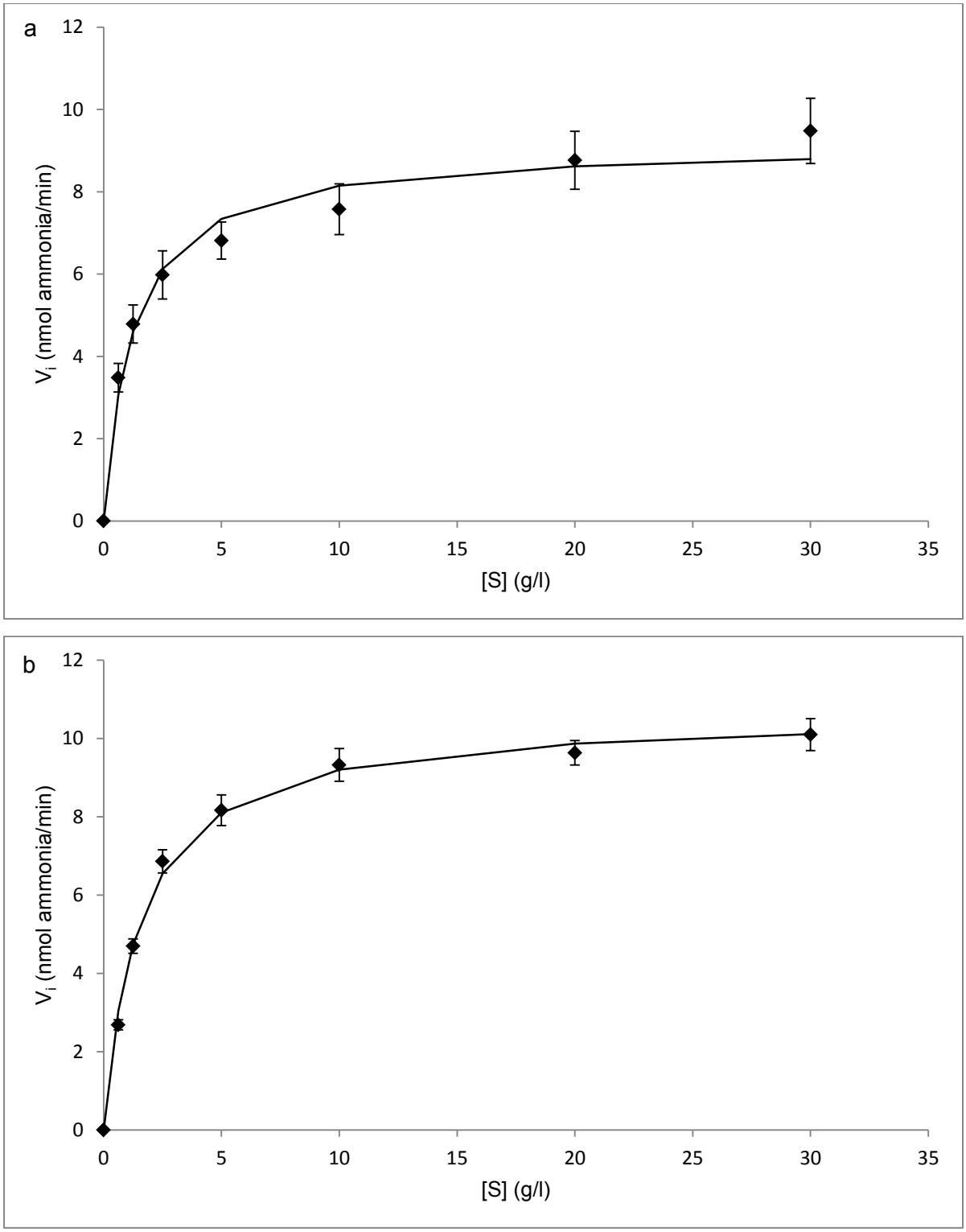

Figure 2.1 Plots of initial reaction velocity (V_i) versus substrate concentration [S] for *C. neoformans* CAB 1055 (a) and *E. africanus* JX398293 (b). Urease activity in 100 µg crude protein extract was assayed using a range of urea concentrations in a final reaction volume of 1 ml. The initial reaction velocity was calculated for each substrate concentration and expressed as nmol ammonia produced per min. Each data point represents the mean of four repetitions and whiskers indicate standard error.

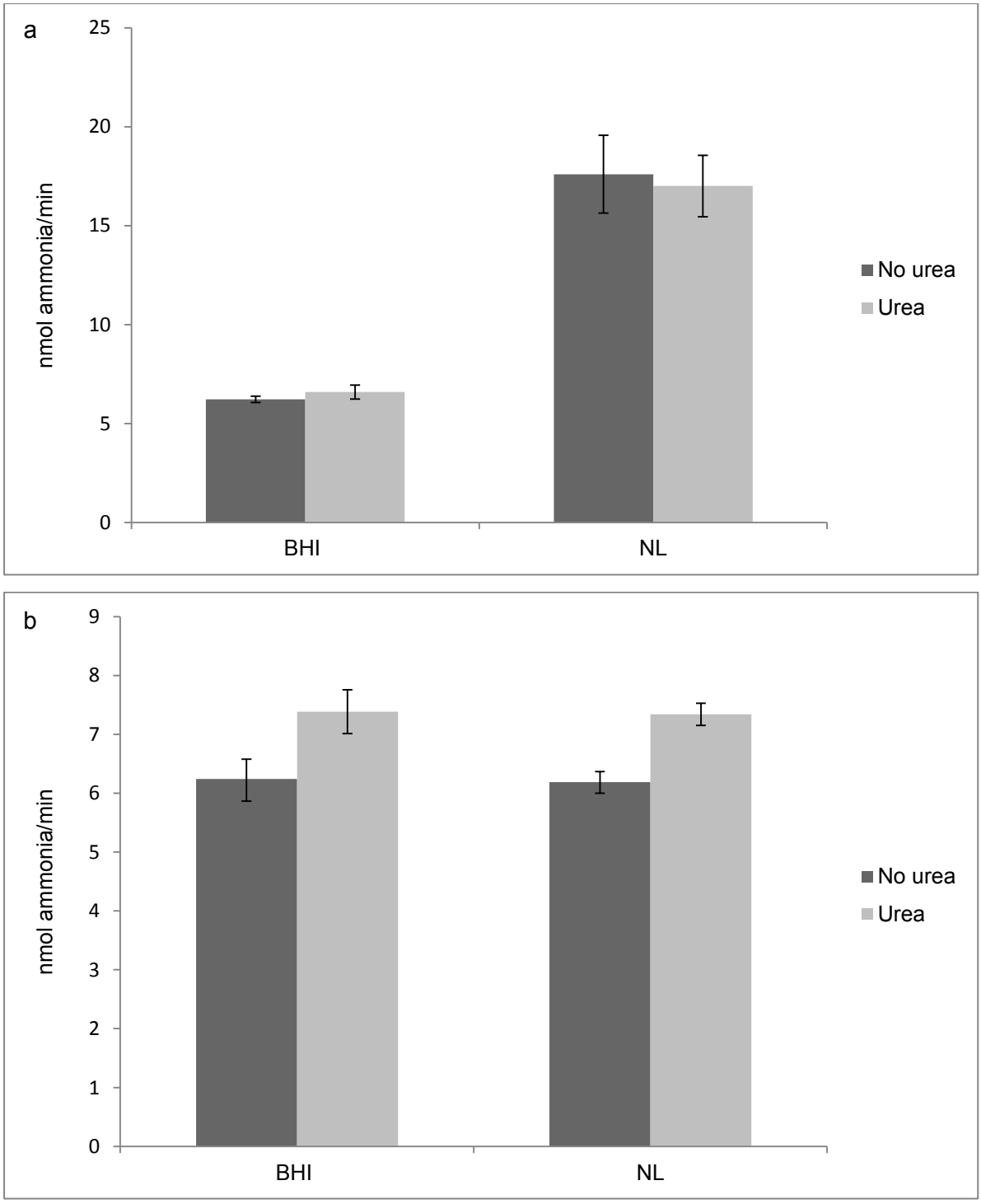

Figure 2.2 Urease activity of *C. neoformans* CAB 1055 (a) and *E. africanus* JX398293 (b) in BHI broth (BHI) and the nutrient-limited medium (NL), both in the presence and absence of 2.5 g/l urea. Enzyme activity in 50 µg crude protein extracts was represented as nmol ammonia produced per min (nmol ammonia/min) in a final reaction volume of 1 ml. Bars represent the mean of three repetitions and whiskers indicate standard error. Urease activity of *C. neoformans* CAB 1055 was significantly higher in the nutrient-limited medium ($p < 0.05$) and was not affected by urea supplementation. In contrast, nutrient limitation had no effect on the urease activity of *E. africanus* JX398293 and urea supplementation resulted in significantly higher urease activity levels in both BHI broth and the nutrient-limited medium ($p < 0.05$).

2.4 DISCUSSION

This study provides strong evidence for the presence of a urease enzyme in *E. africanus*. In addition to the positive urease reaction on Christensen's urea agar, we were able to confirm the presence of a urease gene within the genome of strains representing *E. africanus*. By performing enzyme assays and applying Michaelis-Menten enzyme kinetics, the substrate affinity of the urease produced by *E. africanus* JX398293 (K_m of ca. 26.0 mM) was found to be similar to that of *C. neoformans* CAB 1055 (K_m of ca. 20.6 mM). The K_m of other fungal ureases is scarcely reported in literature and the few that are known differ greatly from the values obtained in this study. For example, the urease of the pathogenic fungus *Coccidioides immitis* was found to have a K_m of 4.1 mM (Mirbod, Schaller and Cole 2002). In another study, a K_m of 1.03 mM was reported for the urease of *Schizosaccharomyces pombe* (Lubbers *et al*. 1996). Thus, indications are that the ureases of both *C. immitis* and *S. pombe* have a higher affinity for the substrate urea than that of *C. neoformans* (20.6 mM) and *E. africanus* (26.0 mM). In contrast, the K_m values of various bacterial ureases (Jones and Mobley 1987; Gatermann, John and Marre 1989; Gatermann *et al*. 1989; Dunn *et al*. 1990; Clemens, Lee and Horwitz 1995) have been reported to range from as low as 0.3 mM for *Mycobacterium tuberculosis* (Clemens, Lee and Horwitz 1995) to as high as 60 mM for *Proteus mirabilis* (Jones and Mobley 1987). Therefore, it is evident that the K_m values we obtained in this study are within the range reported for bacterial ureases.

Further experimentation revealed significant changes in the urease activity of *C. neoformans* CAB 1055 and *E. africanus* JX398293 when the cells were subjected to different nutrient conditions. Unlike *C. neoformans* CAB 1055, urea supplementation of either the BHI broth or the nutrient-limited medium was found to significantly increase urease activity in protein extracts of *E. africanus*. Thus, similar to the pathogenic bacterial species *P. mirabilis* (Jones and Mobley 1988) and *Providencia stuartii* (Mulrooney *et al*. 1988), indications are that the urease of *E. africanus* JX398293 is regulated by the presence of urea in different environments.

Microorganisms exposed to high urea concentrations, such as within the urinary tract of animals and the soil environment, commonly possess ureases with large K_m values ranging from 13 to 130 mM (Mobley *et al*. 1995). The urease K_m value of

E. africanus (26.0 mM) thus points to a natural habitat associated with either soil or animals. *Emergomyces africanus* is a member of the ascomycetous fungal order Onygenales (Dukik *et al*. 2017) that contains many soil-dwelling keratinolytic species, the latter indicating association with animals (de Hoog *et al*. 2000). In addition, it is well known that many of the dimorphic Onygenales able to infect animals show urease activity, a rare trait among ascomycetous fungi (Summerbell, Kane and Pincus 1990; Paré *et al*. 1997; Bagagli *et al*. 1998; Roilides *et al*. 1999; de Hoog *et al*. 2000; Brandt *et al*. 2005; Mirbod-Donovan *et al*. 2006; Marin-Felix *et al*. 2015; Muñoz *et al*. 2015).

Unlike the results observed with urea supplementation, nutrient limitation did not have a significant effect on the urease activity of *E. africanus*. This finding was supported by the results of RT-qPCR analysis, which revealed that urease gene expression levels of *E. africanus* remained constant when yeast-like cells were transferred from BHI broth to the nutrient-limited medium. In contrast, cryptococcal urease activity increased significantly when the yeast was transferred from BHI broth to the nutrient-limited medium. This observed up-regulation was maintained regardless of supplementation with excess urea or ammonium chloride. In addition to enhanced urease activity, analysis of RT-qPCR data revealed that nutrient limitation caused significant up-regulation of urease gene expression in *C. neoformans*. Thus, unlike the generally accepted contention that urease expression is regulated by environmental changes in urea concentration, and/or the availability of nitrogen sources such as ammonia (Mobley, Island and Hausinger 1995), neither of these factors seem to impact cryptococcal urease activity. Future RT-qPCR work should, however, include more than one reference gene for data normalization to ensure the reliability of the results (Derveaux, Vandesompele and Hellemans 2010).

Although not addressed in this study, it is well-known that other microbial ureases, such as that of *Helicobacter pylori*, may also be regulated in response to changes in environmental pH (Li *et al*. 2000; Scott *et al*. 2000; Merrell *et al*. 2003). However, the abovementioned changes in urease activity for both fungi seem not to have been the result of changes in the pH of the media. Both the BHI broth and nutrient-limited medium used in our study had a pH of ca. 7, rendering pH-induced changes in urease activity unlikely.

In conclusion, this study is the first to confirm urease activity in a representative strain of *E. africanus*. In addition, our results provide evidence for the differential regulation of urease activity between *E. africanus* JX398293 and *C. neoformans* CAB 1055. Similar to that observed for pathogenic bacterial species, urease activity in *E. africanus* was enhanced by the presence of the substrate urea. Unlike *E. africanus* JX398293, urease activity and gene expression of *C. neoformans* CAB 1055 was found to increase under conditions of nutrient limitation. Since the macrophage is believed to be a nutrient-poor environment (Lucas and Lee 2000; Roetzer *et al*. 2010; Seider *et al.* 2014), it is possible that phagocytosis may enhance cryptococcal urease activity during infection of the human host. This, however, may not be the case for *E. africanus*, which has also been observed in blood phagocytes *(*Schwartz *et al*. 2015b). Future investigations should therefore include *in vitro* phagocytic studies in combination with RT-qPCR to analyse the transcription profile of fungal urease genes within the macrophage.

AUTHORS' CONTRIBUTIONS

BL conceived and designed the experiments in this study. AB oversaw all aspects of the study. All experiments were performed by BL except for the RNA extractions, cDNA synthesis and RT-qPCR analyses. BL analysed the data and wrote the manuscript. AB and the other authors edited and approved the initial drafts of the manuscript.

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CHAPTER 3

Preliminary study on the effect of pH on the urease activity of *Emergomyces africanus* and *Cryptococcus neoformans*

3.1 INTRODUCTION

The opportunistic pathogens *Cryptococcus neoformans* (Jarvis *et al*., 2014) and the novel species *Emergomyces africanus* (Kenyon *et al*., 2013; Schwartz *et al*., 2015; Dukik *et al*., 2017) are known for their ability to cause fatal disseminated mycoses in HIV/AIDS patients. In contrast to *E. africanus*, cryptococcal pathogenicity is well-studied and is attributed to multiple virulence factors, one of which is urease activity (Casadevall *et al*., 2003). Similar to *C. neoformans*, urease is produced by *E. africanus* (Chapter 2), as well as by a wide diversity of other fungal species, bacteria and plants that rely on the enzyme's catalytic ability to hydrolyse urea into ammonia and carbon dioxide (Mobley and Hausinger, 1989). This reaction is common to all ureases, but the mechanisms involved in its regulation were found to differ between species. Urease activity may be regulated in response to environmental changes in nutrient and/or nitrogen availability, urea concentration as well as pH (Chapter 2; Mobley and Hausinger, 1989). The effect of pH on microbial urease activity has been well-studied for the gastric pathogen *Helicobacter pylori*. Initial studies conducted on this bacterium found that urease-negative mutants were unable to colonize the acidic environment of the murine stomach (Tsuda *et al*., 1994). It was later observed that under acidic conditions, the intracellular urease produced by *H. pylori* is essential for maintaining a periplasmic pH close to neutrality (Rektorschek *et al*., 1998).

Part of the mechanism behind the above-mentioned urease-associated pH increase was subsequently revealed in a study where the *ureI* gene of *H. pylori* was found to encode an acid-regulated urea channel in the bacterial cell membrane (Weeks *et al*., 2000). This channel was found to transport gastric urea to the bacterial cytoplasm in response to gastric acidity. As a result, cytoplasmic urease was activated, leading to an increase in urease-derived ammonia, thereby maintaining pH homeostasis. Further evidence for the pH-dependent urease activity of *H. pylori* was obtained in another study where it was found that an acidic environment resulted in the up-regulation of six genes within the urease operon, which encode both structural and accessory proteins (Merrell *et al*., 2003). In addition to *H. pylori*, pH-regulation of urease activity was found to be characteristic of the oral bacterium *Streptococcus salivarius* (Chen and Bume, 1996). It was demonstrated that the urease of *S. salivarius* contributes to ureolysis within the oral cavity and leads to an increase in

the levels of urease-derived ammonia. Urease activity of cells grown at pH 6 was found to be 160-fold greater than that obtained when cells were grown at pH 7. Furthermore, the level of mRNA transcripts of urease genes was greater in cultures grown under acidic conditions.

In contrast to the abovementioned studies, little knowledge is available on the effect of pH on *C. neoformans* urease activity. In addition, this form of enzyme regulation is yet to be explored for the urease of the novel species *E. africanus*. With the above as background, the aim of this preliminary study was to elucidate the effect of pH on the urease activity of both *E. africanus* and *C. neoformans*. In order to achieve this, clinical strains of the two species were cultured and transferred to media with a pH of 5 and 8, respectively. Subsequently, crude protein extracts were prepared and colorimetrically assayed for urease activity.

3.2 MATERIALS AND METHODS

3.2.1 Strains and inoculum preparation

The strains *C. neoformans* CAB 1055 and *E. africanus* JX398293 were used in this study. Detailed information regarding these strains can be found in Table 2.1 of Chapter 2. In addition, culture maintenance and inoculum preparation were carried out in an identical manner to that described in Sections 2.2.1 and 2.2.5 of Chapter 2.

3.2.2 Urease activity in media buffered at pH 5 and 8, respectively

Due to its high buffering capacity, the phosphate buffer components of Stuart's urea broth (Rustigian and Stuart, 1941) were used to determine the effect of pH on the urease activity of *C. neoformans* CAB 1055 and *E. africanus* JX398293. Experimentation was conducted in triplicate for *C. neoformans* and in duplicate for *E. africanus*.

3.2.3 Media preparation and inoculation

In brief, each strain was inoculated into two 1 L conical flasks, each containing 100 ml medium (0.91 % [w/v] monopotassium phosphate and 0.95 % [w/v] dipotassium phosphate) adjusted to pH 5 using HCl. The same inoculum of each strain was then used to inoculate an additional two flasks (1 L) containing the same phosphate medium as above, but with a pH of 8 adjusted with NaOH. Following

inoculation, all flasks were incubated for three hours at 37 ºC with shaking at 200 revolutions per minute (rpm) on an orbital shaker (Model G53, New Brunswick Scientific Co. Inc., Edison, NJ, USA).

3.2.4 Protein extract preparation and urease activity assay

After incubating the cells suspended in the phosphate medium at pH 5 and 8 respectively, the biomass was washed using centrifugation (Chapter 2) and crude protein extracts were prepared. For each strain, the crude extract resulting from biomass in two 1 L flasks, at the respective pH, was pooled before further experimentation. Protein quantification was then performed, after which 50 µg of crude protein extract was assayed for urease activity. All methods were conducted in the same manner as described in Sections 2.2.6 and 2.2.7 found in Chapter 2. Enzyme activity was recorded as nmol ammonia produced per minute in a reaction volume of 1 ml.

3.2.5 Statistical analyses

All data obtained for *C. neoformans* CAB 1055 are expressed as the mean ± 1 standard error of the mean and were analysed with one-way ANOVA. Thereafter, Fisher's LSD post hoc test was used to determine differences in the mean values of enzyme activities at pH 5 and 8, respectively. A significance level was set to $p < 0.05$ and all statistical analyses were performed using the Statistica software package (Version 13, Dell, Round Rock, TX, USA).

3.3 RESULTS

3.3.1 Urease activity in media buffered at pH 5 and 8, respectively

Incubation of *C. neoformans* CAB 1055 in a medium with a pH of 5 for three hours resulted in crude protein extracts with a urease activity of 17.18 ± 1.52 nmol ammonia/min (Fig. 3.1a). A slight but insignificant decrease in urease activity to 13.53 ± 1.15 nmol ammonia/min was observed in crude protein extracts of cryptococcal cells exposed to a pH of 8 (Fig. 3.1a). For *E. africanus* JX398293, enzyme activities of 5.01 ± 0.18 and 5.24 ± 0.66 nanomole (nmol) ammonia/min were obtained at pH 5 and 8, respectively (Fig. 3.1b).

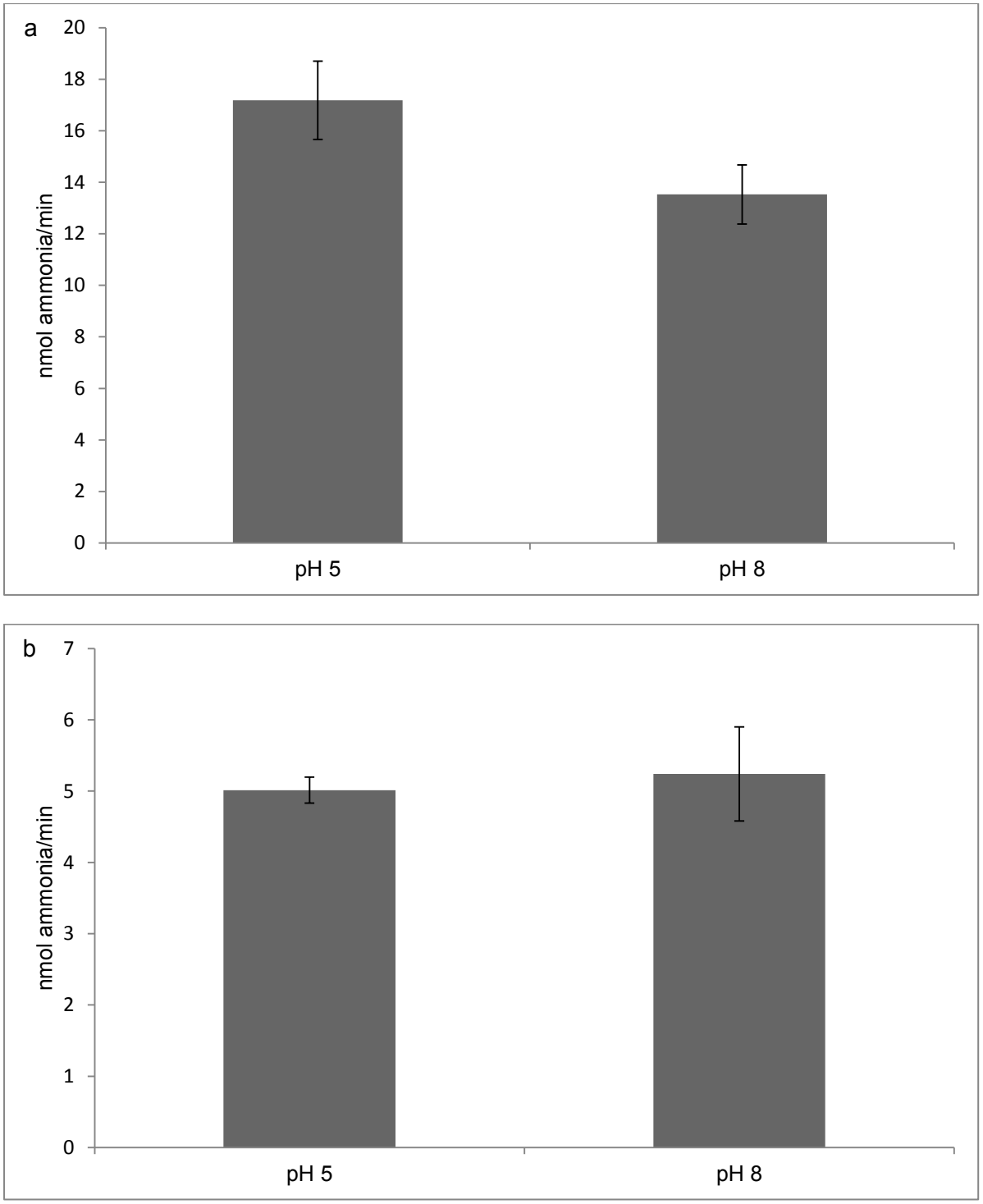

Figure 3.1 Urease activity in crude protein extracts of *C. neoformans* CAB 1055 (a) and *E. africanus* JX398293 (b) exposed to media with a pH of 5 and 8, respectively. Enzyme activity of urease in 50 µg crude protein extract was assayed in a reaction volume of 1 ml and represented as nmol ammonia produced per min (nmol ammonia/min). For both *C. neoformans* CAB 1055 and *E. africanus* JX398293, there was no significant difference in urease activity between the different pH conditions. In (a), bars represent the mean of three repeats and whiskers indicate standard error. The bars in (b) depict the mean of two repeats and whiskers represent data variation.

3.4 DISCUSSION

Cryptococcal urease activity at pH 5 seemed to be slightly higher than that obtained at pH 8, but the difference was found to be insignificant ($p = 0.13$). Thus, although the experimentation should be repeated with more cryptococcal strains, these results suggest that the urease activity of *C. neoformans* CAB 1055 is not regulated in response to environmental pH. This preliminary finding supports the hypothesis that unlike the ureases of bacterial pathogens, such as *H. pylori*, the urease of *C. neoformans* does not serve to increase the pH of acidic microenvironments encountered within the human body (Cox *et al*., 2000). This hypothesis was formulated based on a study by Levitz *et al*. (1999), who found that the acidic phagosomal pH remains relatively constant after phagocytosis of cryptococci. In contrast, the urease of *H. pylori* functions to maintain a periplasmic pH close to neutrality within the acidic conditions of the gastric mucosa (Rektorschek *et al*., 1998).

Similar to the results obtained for *C. neoformans* CAB 1055, the urease activity of *E. africanus* JX398293 did not noticeably differ between the different pH conditions. Thus, indications are that the urease activity of *E. africanus* JX398293 is not subject to pH regulation. However, since only duplicate experiments were performed, this preliminary finding should be confirmed with additional experimentation that includes more repeats. In addition, only two pH conditions were tested in this study, namely pH 5 and 8, and thus future experimentation should include a range of pH values before pH-regulation of urease activity can be excluded for both *C. neoformans* and *E. africanus*.

In conclusion, the results from this preliminary study indicate that the urease activity of *C. neoformans* CAB 1055 and *E. africanus* JX398293 is not regulated in response to environmental pH. The experimentation should, however, be repeated with more fungal strains representing these two species. Furthermore, urease regulation at different pH conditions should be studied at gene expression level using methodology such as real-time quantitative PCR (RT-qPCR).

AUTHORS' CONTRIBUTIONS

BL conceived and designed the experiments in this study. AB oversaw all aspects of the study. BL conducted the experiments, analysed the data and wrote the manuscript. AB edited and approved the initial drafts of the manuscript.

3.5 REFERENCES

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CHAPTER 4

General conclusions and future research

4.1 GENERAL CONCLUSIONS AND FUTURE RESEARCH

The novel species *Emergomyces africanus* was found to produce a urease enzyme with a similar substrate affinity to the well-known urease of *Cryptococcus neoformans*. According to literature, the relatively low substrate affinities we observed for both fungi (K_m ca. 26.0 mM and ca. 20.6 mM, respectively) points to natural habitats associated with an abundance of urea, such as animal habitats and soil. In addition, we found that the urease activities of the two fungi may be affected differently by certain changes in environmental nutrient conditions. Unlike *C. neoformans*, urease activity of *E. africanus* JX398293 was enhanced in the presence of urea in the culture medium. In contrast, while urease activity of *E. africanus* JX398293 remained unchanged, the activity of this enzyme was notably elevated in *C. neoformans* CAB 1055 under conditions of nutrient limitation. This phenomenon was confirmed using both colorimetric enzyme assays and real-time quantitative PCR (RT-qPCR) technology. Colorimetric enzyme assays also revealed that the urease activity of *C. neoformans* CAB 1055 in a nutrient-limited medium remained elevated despite supplementation of the medium with exogenous nitrogen in the form of urea and ammonia, respectively. Thus, the observed increase in cryptococcal urease activity under conditions of nutrient limitation may either be attributed to multiple nutrient starvation and/or limitation of an as yet unknown nutrient. To determine which nutrients are responsible for the phenomenon, colorimetric enzyme assays and RT-qPCR methodology could be used to screen different combinations and concentrations of nutrients for their inhibitory effect on cryptococcal urease activity under nutrient-limited conditions. On the other hand, RT-qPCR could also be employed to study the potential positive effect of different concentrations of nutrients, such as urea, on urease gene expression in both *C. neoformans* and *E. africanus.*

In addition to urea, it is well-known that urease expression may be regulated in response to pH (see Chapter 3). Based on the findings from our preliminary study, different pH environments do not seem to have an effect on the urease activity of either *C. neoformans* CAB 1055 or *E. africanus* JX398293. As noted in Chapter 3, only duplicate experiments were performed for *E. africanus* and therefore this finding needs to be confirmed with additional repeats. Furthermore, only pH values of 5 and 8 were included in the experimentation for both organisms and thus future research

should evaluate urease activity over a range of pH values. Real-time qPCR can also be used to determine the effect of pH on urease gene expression in both *C. neoformans* and *E. africanus.*

Pathogenic fungi may encounter a pH of as low as 4.3 (Chen, 2002) within the acidic microenvironment of the macrophage. It is well-known from literature that acidic conditions within the human body can result in the up-regulation of urease activity in a variety of pathogens (Chen *et al*., 1996; Young and Amid, 1996; Merrell *et al*., 2003). To date, investigations into cryptococcal urease activity within macrophages have focussed only on the inability of this fungus to raise phagosomal pH (Levitz *et al*., 1999; Cox *et al*., 2000). Furthermore, despite available evidence for its presence within human macrophages (Schwartz *et al*., 2015), research is yet to be conducted on the interaction of *E. africanus* with macrophages. Future studies should therefore use RT-qPCR to investigate urease gene expression by *C. neoformans* and *E. africanus* during *in vitro* macrophage infection.

In addition to macrophages, urease activity is known to be required for pathogenesis at other sites of infection. For example, the generation of urease knock-out mutants in *C. neoformans* revealed the role of its urease enzyme in the passage of cryptococcal cells across the blood brain barrier (Olszewski *et al*., 2004). To date, the role of the urease of *E. africanus* in the natural environment and during pathogenesis is yet to be determined. Thus, future research should aim at creating a urease knock-out mutant of an *E. africanus* strain. If successful, this urease-negative mutant can be used in future *in vivo* murine infection models to ultimately provide insight into the role of this enzyme during infection of the human host. Also, to obtain an indication whether urease plays an important role in the survival of this species in the natural environment, the fate of urease knock-out mutants should be studied in soil microcosms.

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APPENDIX A

Supplementary figures

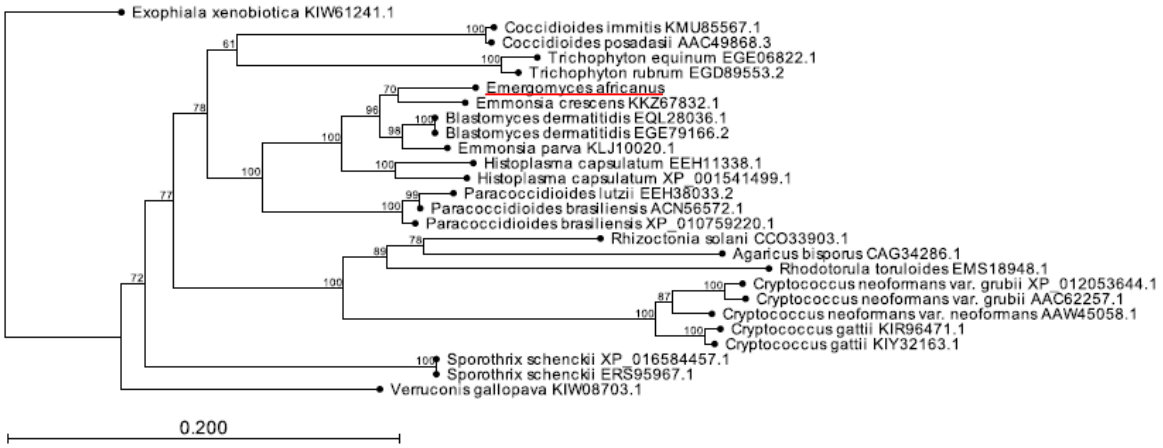

Figure A1. Phylogenetic tree of fungal urease protein sequences. The putative urease of *Emergomyces africanus* (underlined in red) clustered together with other known fungal ureases. The phylogenetic analysis was conducted using the CLC Main Workbench 7.6.4 (CLC Bio) based on the maximum likelihood approach with the JTT matrix and 100 bootstrap replicates. Percentage bootstrap frequency is indicated next to the nodes and branch length is represented by the scale. Fungal urease protein sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the putative urease protein sequence of *E. africanus* was obtained from Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands). The urease of *Exophiala xenobiotica* (GenBank accession number KIW61241.1) was selected as an outgroup.

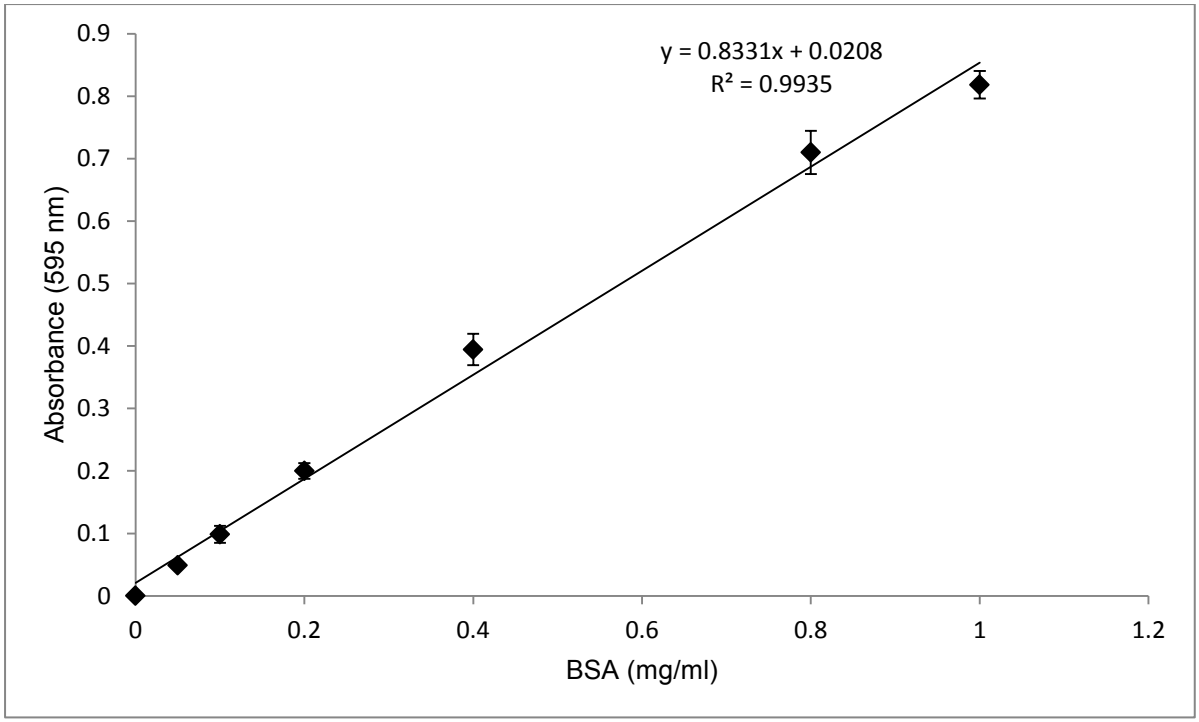

Figure A2. Bovine serum albumin (BSA) standard curve constructed using different concentrations of BSA (0 to 1 mg/ml). Absorbance readings of the standard concentrations were measured at 595 nm. Each data point represents the mean of three repetitions and whiskers represent the standard error.

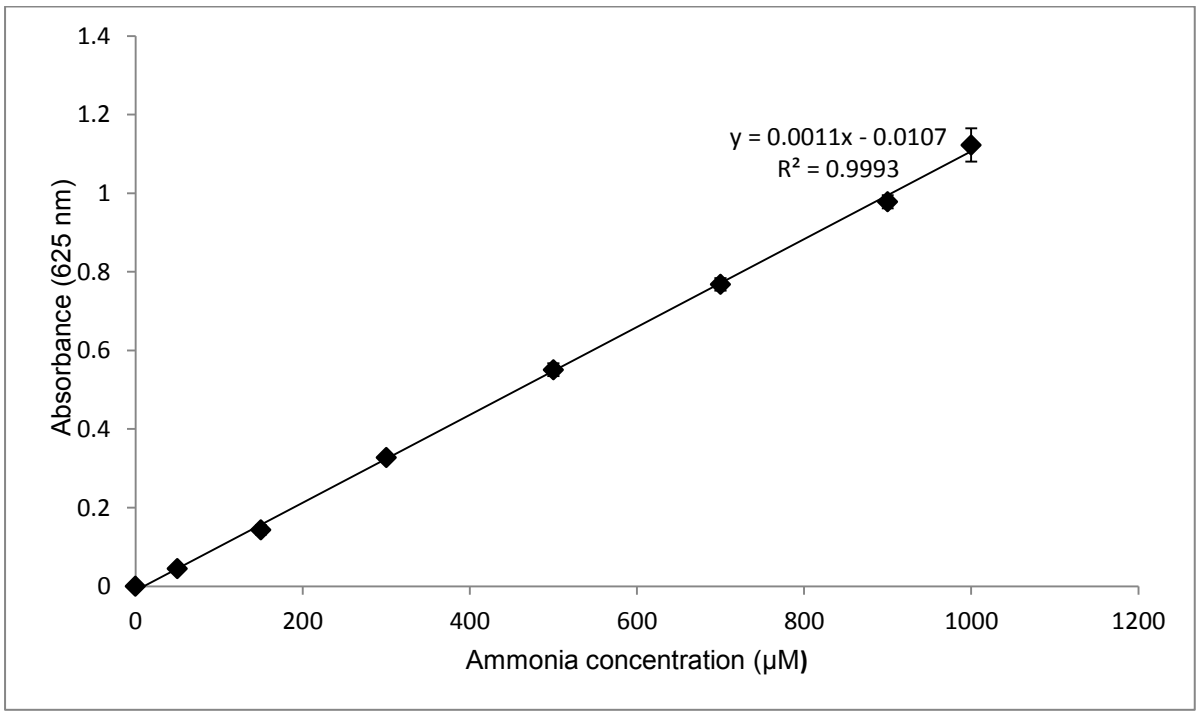

Figure A3. Ammonium chloride standard curve prepared with concentrations ranging from 0 to 1000 µM. Absorbance readings were recorded at 625 nm. Each data point represents the mean of three repetitions and whiskers indicate standard error.

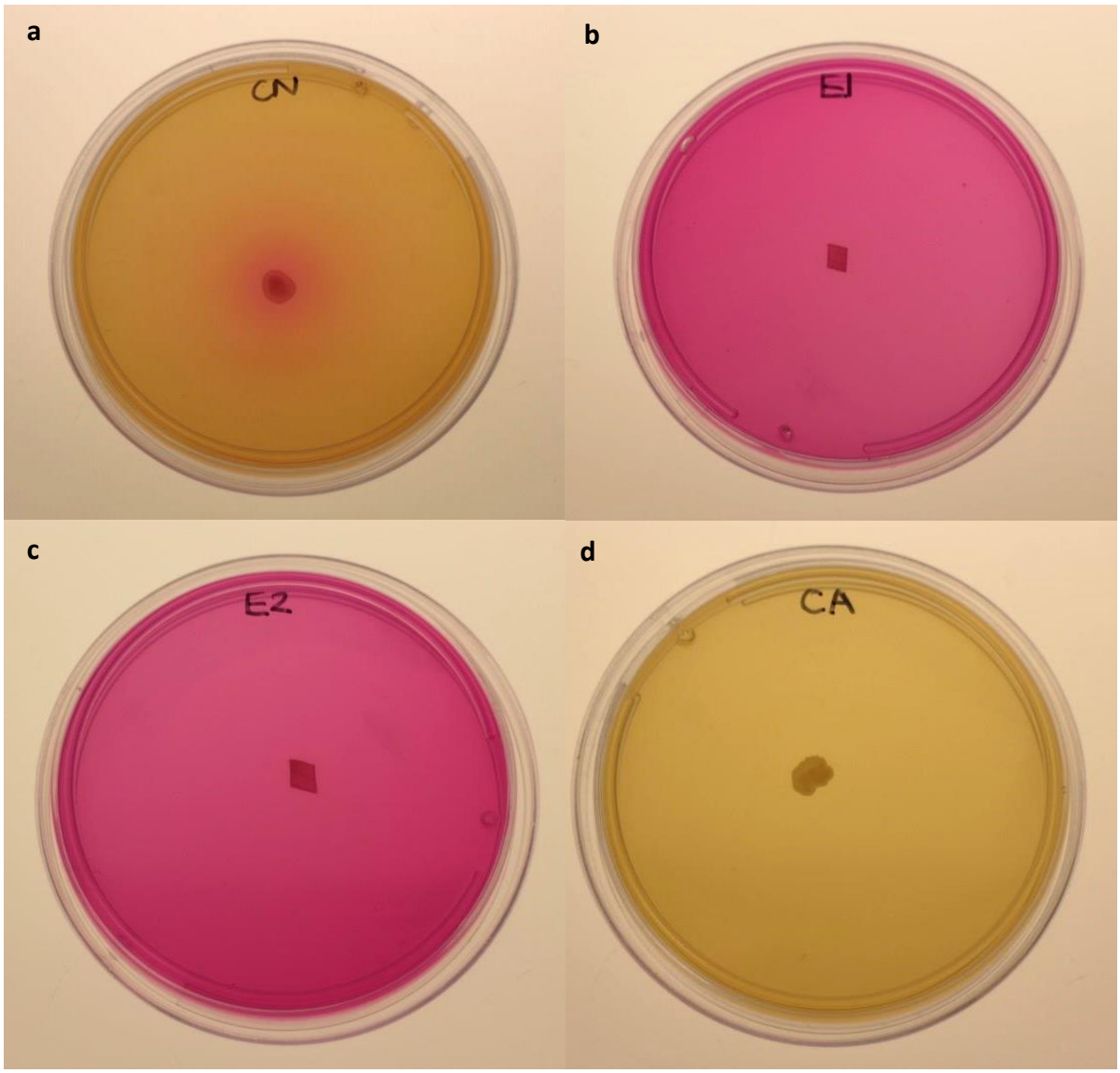

Figure A4. A spot assay for the detection of urease activity on Christensen's urea agar after five days of incubation at 26 °C. A positive reaction, indicated by a pink colour, was evident for *Cryptococcus neoformans* CAB 1055 (a), *Emergomyces africanus* JX398291 (b) and *Emergomyces africanus* JX398293 (c). All positive reactions were compared to the reaction obtained by the negative control *Candida albicans* CAB 397 (d).

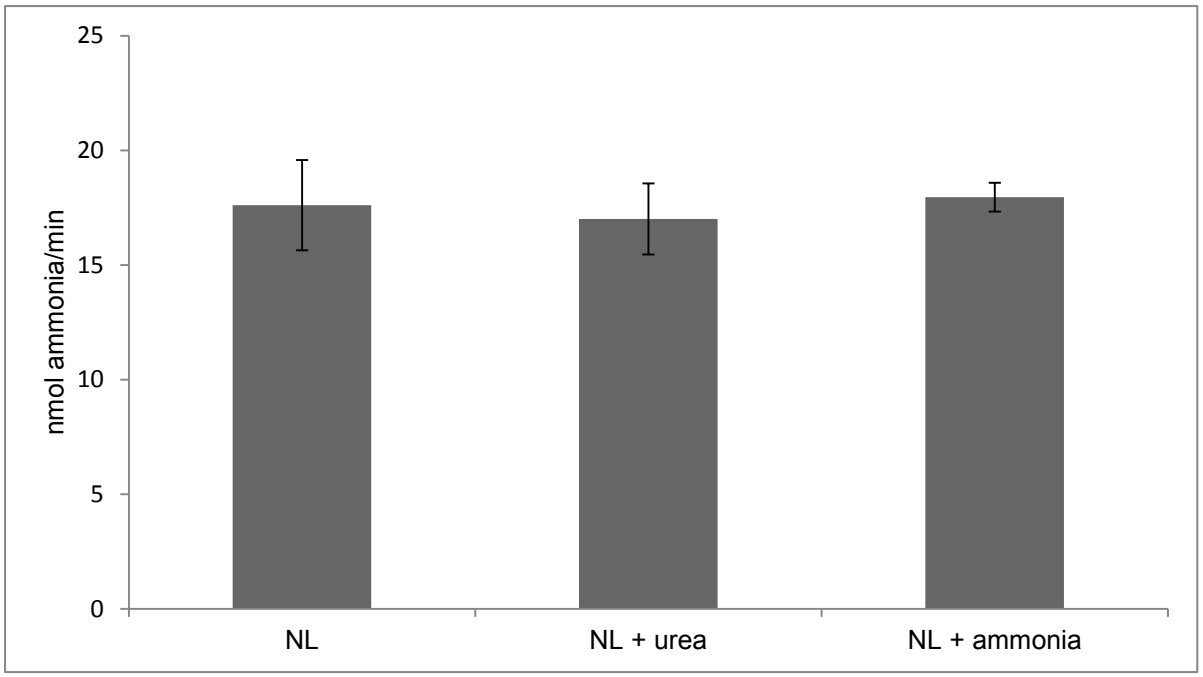

Figure A5. Urease activity of *Cryptococcus neoformans* CAB 1055 in a nutrient-limited medium (NL) supplemented with 2.5 g/l urea (NL + urea) and 4.086 g/l ammonium chloride (NL + ammonia). Enzyme activity in 50 µg crude enzyme extract was assayed in a reaction volume of 1 ml and represented as nmol ammonia produced per min (nmol ammonia/min). Supplementation with urea and ammonia respectively did not have a significant effect on *C. neoformans* urease activity in a nutrient-limited medium ($p > 0.05$). Bars depict the mean of three repetitions and whiskers indicate standard error.

APPENDIX B

Partial urease gene sequences of

Emergomyces africanus

Partial urease gene sequence of *E. africanus* JX398291 – GenBank accession number KY241790

TGCTATCCGGTTCGAGCCGGGGGACACCAAAACGGTTACTCTCGTTGAAATTGGTGGCAAAAAGGAGA
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TTGTCGGTATCGGAAAAGCTGGGAACCCGGACATTATGGAGGGTGTCTCTCCGAATATGATTGTTGGG
GCTGGTACCGATGTGATTTCTGGGGAAAGGAACATAATCACCGCCGGTGGTGTGGATACTCATATCCA
TTTTATTGCCCCGGAGCAGGTGGATGAGGCTTTGGCATCTGGTATCACCACGATGTTGGGGGGTGGTA
CGGGCCCCAGTACGGGAACA

Partial urease gene sequence of *E. africanus* JX398293 – GenBank accession number KY241791

CTATCCGGTTCGAGCCGGGGGACACCAAAACGGTTACTCTCGTTGAAATTGGTGGCAAAAAGGAGATT
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