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Physiology, Enzyme Production, and Zoospore Behavior of Balrachochytrium dendrobatidis, a Chytrid Pathogenic to Amphibians

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PHYSIOLOGY, ENZYME PRODUCTION, AND ZOOSPORE BEHAVIOR OF *BATRACHOCHYTRIUM DENDROBATIDIS*, A CHYTRID PATHOGENIC TO AMPHIBIANS

By

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An Abstract of the Thesis Presented
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Batrachochytrium dendrobatidis is a pathogen of amphibians that has caused severe population declines on several continents, and little is known about the conditions that favor epidemics. The zoospore activity, temperature, and pH requirements of *B. dendrobatidis* were investigated to help understand the ecology and transmission of this pathogen. Over 95% of the chytrid's zoospores stop moving in less than 24 hours, and the zoospores swam less than 2 cm before encysting on tryptone agar. *B. dendrobatidis* zoospores were not attracted to tryptone, gelatin hydrolysate, casamino acids, keratin, gelatin, glucose, or lactose. The chytrid grew and reproduced at temperatures ranging from 4 to 25 °C, and grew best from 17 to 25 °C; it survived and reproduced for more than 6 months at 4 °C. Exposure of cultures to 30 °C for 8 days killed 50% of the cultures. Different isolates of *B. dendrobatidis* did not differ in their temperature optima. The chytrid grew best at a pHs ranging from 6 to 7, but live zoospores were present after two weeks of incubation at pHs ranging from 4 to 8. The zoospore activity and physiological

parameters could determine the transmission and persistence of *B. dendrobatidis* in the environment.

The nutritional requirements of *Batrachochytrium dendrobatidis* were studied to help determine if the chytrid could live on saprophytic substrates outside its host, and to aid in designing an optimal culture medium for the fungus. No synthetic medium tested supported growth. *B. dendrobatidis* cultures grew densest with tryptone or peptonized milk as a nitrogen source. The chytrid did not require additional sugars when grown in tryptone; and grew densest in a liquid medium with 0.5 % tryptone alone. Liquid media with glucose concentrations greater than 1.8% or tryptone concentrations greater than 2% hindered growth. The chytrid grew on autoclaved snakeskin in water or on 1 % keratin agar. *B. dendrobatidis* produced extracellular proteases that degraded casein and gelatin, but had no measurable activity against keratin azure. The proteases were most active against casein at temperatures from 23 to 30 °C at pH 8.0; however, they were active at temperatures from 6 to 37 °C, and in a pH range from 6 to 8. SDS-PAGE analysis of the culture supernatant yielded no visible protein bands when stained with Coomassie blue or copper chloride; but activity gels with 0.5% skim milk revealed two distinct clearings.

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CHAPTER 1- LITERATURE REVIEW

Introduction

Many amphibian populations around the world have suffered precipitous declines that have been larger than normal population fluctuations, and some species have declined to extinction (Blaustein and Wake 1990, Blaustein 1994, Lips 1998). Anthropogenic effects are responsible in part, but many affected populations are dying in remote or protected ecosystems (Blaustein and Wake 1995, Lips 1998, Lips 1999). Recently a fungal pathogen of amphibians was described and implicated as a causal agent of declines of many species and the extinction of some populations (Berger et al. 1998, Pessier et al. 1999).

Batrachochytrium dendrobatidis (Chytridiales) is a species of chytrid fungus that infects the skin of amphibians and can cause death (Berger et al. 1998, Longcore et al. 1999, Nichols et al. 2001). The chytrid has been found in dead or dying anurans in North America, Australia, Central America, and Europe (Berger et al. 1998, Lips 1999, Pessier et al. 1999, Bosch et al. 2000). Many of the affected species inhabit cooler, high-altitude regions, which suggests that environmental conditions may play a role in disease development (Berger et al. 1998, Lips 1998, Lips 1999, Bosch 2000). Many questions surround *B. dendrobatidis* epidemics, and the lack of information on the biology of the fungus necessitates its investigation. This review chronicles the amphibian epidemic as it relates to *B. dendrobatidis*, describes the biology of other chytrids, and briefly reviews the nature of other chytrid epidemics.

Amphibian Declines and *Batrachochytrium dendrobatidis*

Amphibian declines have resulted from habitat destruction and pollutants (Barinaga 1990, Blaustein 1994, Blaustein and Wake 1995, Means et al. 1996); however, other populations decreased from unknown causes (Barinaga 1990, Laurence et al. 1996, Lips 1998). Entire amphibian communities declined in pristine areas of several continents, and acid rain, increased UV-B radiation, and climate changes were implicated in some of these declines (Blaustein and Wake 1995, Houlhan et al. 2000). Some of the affected species had commonalties: they lived in high-elevation regions and were mostly stream dwelling, niche-specific species (Lips 1998, Berger et al. 1998, Williams and Hero 1998, Bosch et al. 2000).

Lips (1998) provided a graphic description of an unexplained, abnormal decline in the protected area of Las Tablas, Costa Rica during 1991 to 1996. Stream dwelling populations of *Atelopus*, *Rana*, and *Bufo* species, abundant in 1991, all but disappeared by 1996 (Lips 1998); tree denizens suffered less. None of the amphibians displayed evidence of systemic viral or bacterial infection, and only a few showed skin necrosis (Lips 1998). Interestingly, many tadpoles were lacking their keratinized mouthparts. Lips (1998) speculated that pathogenic fungi or bacteria were attacking and degrading the tadpole mouths. Lips (1998) ruled out habitat destruction, introduced predators, researcher disruption, increased UV-B radiation, acid rain, and drought as possible causes of the declines. She suggested pollution could be a cause, but believed a pathogen was the most likely agent because the population crashes fit the profile of an epidemic: rapid, precipitous deaths and a wave-like spread across the montane areas (Laurence et al. 1996, Lips 1998).

Unexplained mass-mortalities of anurans were also recorded in Queensland, Australia (1993-1994) and western Panama (1996-1997) (Berger et al. 1998, Lips 1999). Many of these die-offs were found in pristine, high-elevation forests, and fit a similar pattern as the Costa Rican die-offs (Berger et al. 1998). Many tadpoles were found with degraded or missing mouthparts; additionally, over 44% of the dead amphibians had no evidence of bacterial or viral infection, and no protozoa or myxozoa were found (Berger et al. 1998, Lips 1999). These unexplained deaths were not limited to tropical mountains. Between September 1996 and October 1997, 24 blue poison dart frogs (*Dendrobatidis azureus*), four *D. auratus*, and three *Litoria caerula* died unexpectedly at the National Zoological Park (NZP) in Washington, D.C. (Pessier et al. 1999). No amphibian disease outbreaks had been noted at the NZP from 1991 until these deaths (Pessier et al. 1999). Anurans in all three die-offs had gross shedding of skin, and histology revealed numerous spherical cells in the epidermis (Berger et al. 1998, Lips 1999, Pessier et al. 1999). None of these cells were found in preserved specimens collected before the Australian and Panamanian die-offs (Berger et al. 1998). Analyses of ribosomal DNA and zoospore-ultrastructure placed the organism found in the skin in the Chytridiomycota, specifically in the Chytridiales (Berger et al. 1998, Longcore et al. 1999). Additionally, a pure culture of the chytrid was isolated from a NZP frog and used to formally describe the organism, which was named *Batrachochytrium dendrobatidis* (Longcore et al. 1999). Longcore et al. (1999) suggested the "frog chytrid" might be closely related to *Rhizophydium*, another Chytridialean species. The infection was called cutaneous chytridiomycosis, and was determined to be the cause of death for both the caged and wild frogs (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999).

Lips (1999) further documented the presence of the chytrid in Panama. Populations of stream anurans, abundant in the Reserva Forestal Fortuna in 1993-1995 were almost gone in 1997. Of 18 dead specimens, all had chytridiomycosis. Eleven percent of the tadpoles lacked mouthparts, and many infected adults had shedding skin (Lips 1999). Bosch et al. (2000) described a chytrid epidemic in *Alytes obstetricans* at an upland site in central Spain that caused an 86% decrease in toad populations during the summers of 1997-1999. Prior to 1999, tadpoles of *A. obstetricans* were found in 35 ponds in the study site, but in only 5 ponds after 1999 (Bosch et al. 2000). Interestingly, a rare increase in the pH of the ponds (up to pH 9) was recorded in 1999, and the authors suggested this could have stressed the toads or favored chytrid growth (Bosch et al. 2000).

Nichols et al. (2001) showed that *B. dendrobatidis* alone could kill poison dart frogs. The researchers exposed metamorphs of two dendrobatid species to pure cultures of the chytrid. All exposed frogs died of chytridiomycosis and no other infections, whereas the unexposed controls survived (Nichols et al. 2001). Pure cultures of *B. dendrobatidis* were re-isolated from skin of the dead frogs. Clinical signs of chytridiomycosis included: anorexia, lethargy, excessive shedding of skin, hyperkeratosis, and some skin lesions (Nichols et al. 2001). The researchers fulfilled Koch's postulates with *B. dendrobatidis*.

It is still unknown how the pathogen kills its host. Berger et al. (1998) suggested that thickening of the epidermis might hinder respiration and osmoregulation, or that the chytrid might produce a toxin that kills the amphibian. Conventional screening for toxins

produced negative results (Longcore pers. comm.). Berger et al. (1999) speculated that a proteolytic enzyme might be absorbed through the amphibian's skin and cause death.

Chytrid Biology and Ecology

Basic biology

The Chytridiomycota (Chytrids) is the earliest diverging phylum of the kingdom Fungi, and is divided into five orders: Chytridiales, Spizellomycetales, Blastocladiales, Monoblepharidales, and Neocallimastigales (Barr 2001). Chytrids produce motile zoospores, and are common in soil and water, where they function as primary decomposers of an array of substrates (Barr 1990). Most species are saprophytic, but a few are parasites of plants, algae, fungi, protists and invertebrates (Alexopoulos et al. 1996).

The vegetative body of a chytrid is called a thallus, which may produce a single zoosporangium (monocentric) or many zoosporangia (polycentric) (Barr 2001). Zoospores that encyst on a substrate may germinate to form a thallus. The thallus of a monocentric chytrid develops into a zoosporangium and produces asexual zoospores, or it may combine its nuclei with another thallus to form a sexual resting spore (Alexopoulos et al. 1996). Resting spores also may be formed asexually. Zoospores have no cell walls and are short-lived if they do not germinate; conversely, resting spores are thick-walled, and can survive longer (Longcore 2001). A thallus also may divide asexually early in development to form multiple vegetative bodies, each of which becomes a sporangium (colonial development).

Batrachochytrium dendrobatidis develops either monocentricaly or colonially and lives inside individual epidermal cells of amphibians (Longcore et al. 1999). Thalli of *B.*

dendrobatidis can colonize the entire epidermis of adult amphibians. In tadpoles this fungus is found only in mouthparts, which are the only keratinized tissues of the larval stage (Berger et al. 1998). As with many other chytrids, only an asexual form of *B. dendrobatidis* has been described. This fungus has not been observed outside a host in the environment (Longcore et al. 1999).

Some chytrid pathogens pose serious threats. The genus *Synchytrium* (over 200 species) can parasitize more than 1300 species of algae, mosses, ferns, and higher plants (Powell 1993). The greatest chytrid epidemics have been reported from freshwater algae. *Rhizophydium fragilariae* may infect up to 90 % of a phytoplankton species given perfect conditions, which involve light, temperature, and pH (Powell 1993). The optimal temperature for a parasitic chytrid's growth may influence infectivity. A study by van Donk and Ringleberg (1983) indicated that at low temperatures (1.5 ± 1 °C) host algae, *Asterionella formosa*, grew free of a parasitic chytrid, *Zygorhizidium planktonicum*; however, at 5, 10, and 18 °C the fungus had a high infection rate and overtook its host.

Zoospore behavior

Posteriorly uniflagellate zoospores are a defining character of the Chytridiales and are their primary method of dispersal (Alexopoulos et al. 1996). Zoospores, released from sporangia, may swim until they find a suitable substrate to encyst upon, or may encyst directly after release. Chytrid zoospores swim several ways: they may move in wide circular orbits, in a straight line, or along a spiral path (Sparrow 1960, Fuller 1996). Chytrid zoospores generally swim erratically, hopping and gliding through the water, and the rate at which zoospores swim is variable (Sparrow 1960). Pommerville (1978) stated that spores of *Allomyces* (Blastocladiales) swim at rates of 60-140 μm/s, and Carlile

(1986) estimated the average zoospore swims at 160 μm/s. Zoospores may be motile from a few minutes to 2-3 days. One extreme example is the report of zoospores of *Rhizophydium deciptens,* which remained motile inside a sporangium for 108 hours (Sparrow 1960). Carlile (1986) estimated the average time of motility to be 10 hours, whereas Sparrow (1960) stated that most zoospores swim a maximum of 3 to 4 hours. With an average rate of 160 μm/s and an average motile life of 10 hours, an "average" zoospore could swim 576 mm in an hour, or 5.76 m in 10 hours (Carlile 1986). These figures cannot be interpreted for linear distances, because most zoospores do not swim for the maximum time possible in a straight line. Carlile (1986) states that most zoospores swim fewer than 6 cm before encysting.

Many factors affect the rate of zoospore encystment, because spores are extremely sensitive to environmental conditions. Ionic concentrations, oxygen, temperature, zoospore concentration, toxins, species, and spore vitality all influence how long a zoospore is active (Sparrow 1960, Fuller 1996). Calcium appears critical to zoospore motility. With 0.2 – 1 μM Ca^{2+} some zoospores swam for several hours; however if the Ca^{2+} concentrations fell below 0.1 μM, the spores began to lyse (Fuller 1996). Amino acid solutions of 0.05- 0.1 M increased the motility of *Olpidium brassicae* zoospores (Fuller 1996). Zoospores need not remain motile for the maximum amount of time if they find a suitable substrate. Many zoospores demonstrate tactic responses to stimuli that help guide them to a host or substrate (Muelhstein et al. 1988, Fuller 1996). Different zoospores have shown tactic responses to light, chemicals, electrical fields, ionic fields, gravity, streams in liquid, and other zoospores (Fuller 1996). The algal parasites *Rhizidium vorax, Polyphagus euglenae,* and *Rhizophydium littoreum* demonstrate

positive phototaxis, which helps them find their host in the photic zone (Sparrow 1960, Muehlstein et al. 1987). Additionally species of *Allomyces* and *Synchytrium* are positively phototactic (Gleason 1976).

Chemotaxis may be important to zoospores in locating a nutrient source or host. Muehlstein et al. (1988) found that *R. littoreum* is positively chemotactic to many substances. Carbohydrates and polysaccharides elicited strong responses, as did extracts of *Bryopsis* and *Codium*, two algae that the chytrid parasitizes (Muehlstein et al. 1988). Interestingly, some chemicals that supported growth were not positive chemoattractants, and some chemoattractants did not act as carbon or nitrogen sources for *R. littoreum* (Muehlstein et al. 1988). *R. littoreum* zoospores are attracted to bovine serum albumin, casin hydrolysate, peptone and some amino acids. Additionally, *R. pollinis-pini* and *R. sphaerotheca* are positively attracted to protein (Sparrow 1960, Muehlstein et al. 1988). Other chytrids for which chemotaxis has been reported include *Olpidium trofolii* and *O. vicae,* which are attracted to extracts of their hosts (Sparrow 1960).

Temperature and pH requirements

Chytrids are found in diverse habitats worldwide, from the Arctic to tropical rainforests, from acidic to basic waters and soils (Longcore 2001). Thus it would be expected that different chytrids would have different growth optima, but this has not been reported to date. Most chytrid species, especially the Chytridiales, grow best at temperatures ranging from 20 to 30 °C (Table 1), and at a pH range from 6 to 8 (Table 2). These results are from relatively few pure culture studies.

Table 1. Temperature requirements of several chytrid species of the orders Chytridiales and Spizellomycetales. Currently accepted names appear in parentheses.

	Growth Range (°C)	Optima (°C)	No growth (°C)	Reference
Chytridales species				
Rhizophydium capillaceum	15-35	30-35	10,40	Barr 1969
Rhizophydium sphaerotheca	15-30	25	5,35	Barr 1969
Nowakowskiella ramosa	5-30	20		Goldstein 1961
N. elegans	5-30	15		Goldstein 1961
Entophlyctis aureus	10-30	20-25 20-30	>30 <10	Hassan and Catapane 2000
Entophlyctis sp.	10-30	20-25 20-30	>30 <10	Hassan and Catapane 2000
Allochytrium expandens	-30		32	Barr 1986
Spizellomycetales species				
Karlingia rosea *(Rhizophlyctis rosea)*	10-34	30	5,35	Haskins and Watson 1955
Phlyctochytrium punctatum *(Spizellomyces punctatus)*	15-35	30	5,40	Goldstein 1960
P. arcticum *(Triparticalcar arcticum)*	5-25	20	>25	Barr 1970
E. confervae-glomeratae *(Powellomyces hirtus)*	10-25	20-25	30	Barr 1971
P. californicum #15 *(Gaertneriomyces semiglobiferus)*	10-30	20	35	Barr 1969
P. californicum #68 *(Gaertneriomyces semiglobiferus)*	10-35	30-35	40	Barr 1969
P. plurigibbosum #33 *(S. plurigiggosus)*	15-30	30	10,35	Barr 1969
P. plurigibbosum #39 *(S. plurigiggosus)*	15-30	30	10,35	Barr 1969
P. acuminatum #7 *(S. acuminatus)*	10-30	20-30	35	Barr 1969
P. acuminatum #62 *(S. acuminatus)*	15-35	30	10,40	Barr 1969
P. reinboldtae	10-30	20-25	35	Barr 1970

Table 2. pH requirements of chytrid species of the orders Chytridiales and Spizellomycetales. Currently accepted names appear in parentheses.

	Growth Range	Optima	Reference
Chytridales species			
Rhizophydium capillaceum	4.95-8.5	4.95-6.2	Barr 1969
Rhizophydium sphaerotheca	4.95-8.5	4.95-6.2	Barr 1969
Nowakowskiella ramosa	5.9-7.3	6.4	Goldstein 1961
N. elegans	5.9-7.3	6.4	Goldstein 1961
Entophlyctis aureus	6.5-8.5		Hassan and Catapane 2000
Entophlyctis sp.	6.5-8.5		Hassan and Catapane 2000
Chytridium sp	4.5-8.5	5.5-7.2	Craseman 1954
Macrochytrium botrydiodes	4.5-8.0	6.5	Craseman 1954
Karlingia astrocysta	5.0-8.5	6.0-7.5	Murray and Lovett, 1966
Spizellomycetales species			
Karlingia rosea *(Rhizophlyctis rosea)*	3.4-8.0	6.8-7.0	Haskins and Watson 1955
Phlyctochytrium punctatum *(Spizellomyces punctatus)*	5.9-7.3		Goldstein 1960
P. arcticum *(Triparticalcar arcticum)*	5.8-8.5	5.8-7.2	Barr 1970
E. confervae-glomeratae *(Powellomyces hirtus)*	6.0-9.1	8.0-9.1	Barr 1971
P. californicum #15 *(Gaertneriomyces semiglobiferus)*	4.95-8.5	8.3-8.5	Barr 1969
P. californicum #68 *(Gaertneriomyces semiglobiferus)*	4.95-8.5	8.3-8.5	Barr 1969
P. plurigibbosum #33 *(S. plurigiggosus)*	4.95-8.5	6.5-7.85	Barr 1969
P. plurigibbosum #39 *(S. plurigiggosus)*	4.95-8.5	5.5-6.2	Barr 1969
P. acuminatum #7 *(S. acuminatus)*	4.95-8.5	6.2-8.5	Barr 1969
P. acuminatum #62 *(S. acuminatus)*	4.95-8.5	6.2-8.5	Barr 1969
P. reinboldtae	5.2-8.55	8.55*	Barr 1970

* Highest pH tested

Chytrid Nutrition

Chytridiomycetes live on a variety of substrata, from plant matter to exoskeletons of invertebrates. Several investigators reported the carbon, nitrogen, and vitamin requirements of some chytrids could be used for taxonomic purposes. Cantino (1950 &

1955) suggested that the order Chytridiales might be separated based on the sugars they could metabolize, or the vitamins they required. These studies have shown most chytrids can grow on ammonium nitrogen, use several sugars, and require exogenous thiamine (Table 3).

Cantino (1955) states that *Karlingia rosea*, which decomposes cellulose, could use glucose or cellobiose as a carbon source. The use of cellobiose is expected, as this disaccharide is a product of cellulose degradation. Cantino (1955) warns that because a chytrid favors a substrate is no reason to presume it uses the same substrate in nature as its carbon source. A chitinophilic *Chytridium* sp. did not grow on unbleached chitin, but grew well on the complex substrate of crab shell; therefore, Cantino (1955) concluded that chitin was not the sole carbon or nitrogen source for this fungus. Some cellulose-degrading chytrids, however, may use cellulose as their sole carbon source. *Nowakowskiella elegans* and *N. ramosa* (Chytridiales), cellulose-degrading chytrids, grew on cellobiose and cellophane as the sole carbon source; however, glucose, xylose, and mannose gave the best growth yields (Goldstein 1961). Of the chytrids tested by Barr (1969, 1970, 1971), none could survive on chitin or cellulose as sole sources of carbon.

Table 3. Nutrition requirements of chytrid species of the orders Chytridiales and Spizellomycetales. Currently accepted names appear in parentheses.

	Carbon Sources Utilized	Nitrogen Sources Utilized	Vitamin Requirement	Reference
Chytridales species				
Rhizophydium capillaceum	G, F, L, St, Mt	NH_4, NO_3, P, YE, AA	thiamine	Barr 1969
Rhizophydium sphaerotheca	G, L, St, Mt, Cb	NH_4, P, YE, AA	thiamine	Barr 1969
Nowakowskiella ramosa	Xy, G, Mn	NH_4, NO_3, AA, T, YE	thiamine	Goldstein 1961
N. elegans	Xy, G, Mn, Cb	NH_4, NO_3, AA, T, YE	thiamine	Goldstein 1961
Entophlyctis aureus	G, F, Mn, Mt, Rf	NH_4, NO_3, AA	none	Hassan and Catapane 2000
Entophlyctis sp.	G, F, Mn, Mt, Rf	NH_4, NO_3, AA	none	Hassan and Catapane 2000
Chytridium sp	G, Mn, Cb, St, Ga, F	NH_4	thaimine	Craseman 1954
Macrochytrium botrydiodes	G, Mn, Cb, St, Mt, Xy	NH_4	thiamine	Craseman 1954
Spizellomycetales species				
Phlyctochytrium punctatum (Spizellomyces punctatus)	G, F, Cb, St, Gy, Mn, Tr	NH_4, NO_3, T, YE, AA	none	Goldstein 1960
P. arcticum (Triparticalcar arcticum)	G, F, Mn, Mt, St, Cb, Gy	NH_4	thiamine	Barr 1970
E. confervae-glomeratae (Powellomyces hirtus)	G, F, Tr, Gy, Su, Cb, Mt, St	NH_4, NO_3, NO_2, P		Barr 1971
P. californicum #15 (Gaertneriomyces semiglobiferus)	G, Mt, Gy, St	NH_4, P	thiamine	Barr 1969
P. californicum #68 (Gaertneriomyces semiglobiferus)	G, Mt, St	NH_4, P	thiamine	Barr 1969
P. plurigibbosum #33 (S. plurigiggosus)	G, F, Mt, Tr, Gy, St	NH_4, NO_3, P	thiamine	Barr 1969
P. plurigibbosum #39 (S. plurigiggosus)	G, F, Mt, Cb, Tr, Gy, St	NH_4, NO_3, P	thiamine	Barr 1969
P. acuminatum #7 (S. acuminatus)	G, Mt, Cb, Gy, St	NH_4, NO_3, P	none	Barr 1969
P. acuminatum #62 (S. acuminatus)	G, Mt, Cb, Gy, St, Xy,	NH_4, NO_3, P	none	Barr 1969
P. reinboldtae	G, F, Tr, Mt, Mn, Cb, Gy, St	NH_4, NO_3, NO_2, P, AA	none	Barr 1970

Key: G= glucose, F= fructose, L= lactose, St= starch, Mt= maltose, Cb= cellobiose, Xy= xylose, Mn= mannose, Rf= raffinose, Ga= galactose, Gy= glycerol, Tr= Trehalose, Su= sucrose, P= peptone, T= tryptone, YE= yeast extract, AA= amino acids, NH_4= ammonium nitrogen, NO_3= nitrate, NO_2= nitrite

Chytrid extracellular enzymes

Fungi have absorptive nutrition; consequently they must produce extracellular enzymes to degrade their substrate. Although members of the Chytridiales and Spizellomycetales have been found growing on complex substrates like chitin and keratin, few enzymes have been described from these orders. Reisert and Fuller (1962) determined that the chitin-degrading fungus *Chytriomyces* sp. produces an extracellular chitinase; this was the first paper to describe a fungal chitinase. Murray and Lovett (1966) reported that *Karlingia asterocysta* is an obligate chitinophile, which cannot grow without chitin or N-acetyl-D-glucosamine, the chitin monomer. Karup et al. (1994) described proteases of *Rhizophlyctis hyalina*, six *Rhizophydium* species, and one *Entophlyctis* species. *Rhizophydium* sp. 4 produces one protease, *Rhizophlyctis hyalina* produces four, and all others produce three proteases. The three proteases together have activity over a wide pH range (pH 5 to 10) (Karup et al. 1994).

***Batrachochytrium dendrobatidis* biology and ecology**

B. dendrobatidis is the only known chytrid pathogen of vertebrates (Berger et al. 1998). Zoospores are essential to the propagation and spread of this fungus and are the only infective propagule that has been found for *B. dendrobatidis*. Consequently, the number and behavior of zoospores may dictate aspects of an outbreak. Berger et al. (1998) states *B. dendrobatidis* spores may be motile for about 24 hours.

Some information is known about the growth requirements of *B. dendrobatidis*. Longcore et al. (1999) indicated that the fungus grows fastest at 23 °C, slower at lower temperatures, and grows very little at 28 °C. Isolate 197 did not grow at 29 °C after two weeks; however, when returned to 23 °C it began to grow. Nothing is known about the

chytrid's pH tolerances, and little is known about its nutritional requirements. Longcore et al. (1999) indicate the chytrid grows well on a solid medium of 1% tryptone, 1% gelatin hydrolysate, 0.2% lactose and 1% agar, or in 1% tryptone plus 0.3% glucose liquid-medium. The fungus grows in keratinized cells, but it is unknown if it uses the keratin as its sole carbon or nitrogen source. *B. dendrobatidis* probably produces an extracellular enzyme(s) both to enter the keratinized cells and to degrade the amphibian epidermis for its nutrients. Many ascomycete skin-pathogens (e.g., *Trichophyton*) produce keratinases and proteases that aid their invasive growth (Singh 1997). *B. dendrobatidis* may produce a keratinase because the chytrid is found only in keratinocytes. *B. dendrobatidis* grows well on autoclaved snakeskin (part keratin), and produces clearing zones beneath the thalli (Longcore pers. comm.).

Research Objectives

Temperature and pH optima of *B. dendrobatidis* may determine the extent of an epidemic, as has been observed with other chytrid pathogens; therefore, I will describe the temperature and pH tolerance of the chytrid. To help determine what role zoospores play in disease spread, I will describe how long they remain motile, how far they swim, and if they have a tactic response. Because *B. dendrobatidis* grows well in pure culture, it may have a saprophytic life outside its host, but this has not been observed. I will describe the nutritional requirements of the chytrid to help elucidate a saprophytic stage and to aid cultivation. The chytrid prefers keratinized epidermal cells of amphibians, but it is not known if it produces enzymes that degrade these cells. I will determine if *B. dendrobatidis* produces an extracellular enzyme(s) that degrades protein and characterize the temperature and pH optima for enzyme activity.

CHAPTER 2- ZOOSPORE ACTIVITY, TEMPERATURE, AND PH REQUIREMENTS OF *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

Batrachochytrium dendrobatidis is a pathogen of amphibians that has caused severe population declines on several continents, and little is known about the conditions that favor epidemics. The zoospore activity, temperature, and pH requirements of *B. dendrobatidis* were investigated to help understand the ecology and transmission of this pathogen. Over 95% of the chytrid's zoospores stopped moving in less than 24 hours, and the zoospores swam less than 2 cm before encysting on tryptone agar. *B. dendrobatidis* zoospores were not attracted to tryptone, gelatin hydrolysate, casamino acids, keratin, gelatin, glucose, or lactose. The chytrid grew and reproduced at temperatures ranging from 4 to 25 °C, and grew best from 17 to 25 °C; it survived and reproduced for more than 6 months at 4 °C. Exposure of cultures to 30 °C for 8 days killed 50% of the cultures. Different isolates of *B. dendrobatidis* did not differ in their temperature optima. The chytrid grew best at pHs ranging from 6 to 7, but live zoospores were present after two weeks of incubation at pHs ranging from 4 to 8. The zoospore activity and physiological parameters could determine the transmission and persistence of *B. dendrobatidis* in the environment.

Introduction

Zoospores are the primary method of dispersal for chytrids (Alexopoulos et al. 1996). The spread of *Batrachochytrium dendrobatidis* through the environment is partly a result of the behavior of its zoospores. The longevity of the chytrid's zoospores can

determine how quickly they must infect a host; and the distance they swim may dictate how far the disease can spread in a body of water. The "average" chytrid or oomycete zoospore swims for 10 hours and travels 5 cm before encysting (Carlile 1986). Berger et al. (1998) stated zoospores of *B. dendrobatidis* can swim for 24 hours, but did not indicate the percentage of zoospores that swim this long. Zoospores of other chytrid parasites are attracted to components of their hosts (Sparrow 1960, Muelhstein et al. 1988). *B. dendrobatidis* may also be attracted to compounds in amphibian skin, which could help it to find a host more efficiently.

Epidemics of chytrid pathogens of fresh-water algae can be triggered by temperature changes, because temperature can influence infectivity (Powell 1993). A difference of as little as 4 °C can separate epidemic conditions from conditions where no infection can occur at all (van Donk and Ringleberg 1983). *Batrachochytrium dendrobatidis* epidemics also appear to be governed in part by temperature. In the tropics, outbreaks occur in the cooler mountains, while lowland areas are unaffected (Daszak et al. 2000). Information on the temperature requirements of this chytrid could help epidemiologists study and model its spread (Berger et al. 1999). Additionally, if *B. dendrobatidis* cannot survive at as high a temperature as amphibians can, simply raising the temperature the animals are living at could be a treatment for captive, infected animals.

As temperature influences chytrid growth, so may pH (Powell 1993). Berger et al. (1999) suggested that outbreaks of chytridiomycosis might be caused by the pH of a water body. Most chytrids grow optimally in a pH range from 6 to 8, which is the range commonly found in fresh water systems. Nevertheless, nothing is known about the pH

requirements of *B. dendrobatidis*, and they may differ from those of other chytrids. Different isolates of the same chytrid species can have different pH optima (Barr 1969), and this may occur with *B. dendrobatidis*. An understanding of the pH requirements of this pathogen could help researchers resolve its ecology.

My experiments will examine how long zoospores of *B. dendrobatidis* can swim, how far they can swim, and if they are attracted to any compounds. I will also determine the temperature and pH tolerances of the chytrid. These data will lead to a better understanding of the chytridiomycosis epidemic.

Materials and Methods

Experiments with zoospores of *Batrachochytrium dendrobatidis*

Zoospore harvests

Zoospore experiments required a large quantity of *B. dendrobatidis* zoospores. The type culture (isolate 197) was used for all zoospore experiments because it grew the fastest at 23 °C and consistently gave the highest zoospore yields. To harvest zoospores, I first inoculated TGhL agar plates (1% tryptone, 1% gelatin hydrolysate, 0.2% lactose, 1% agar) with 1 mL of two-week-old liquid culture, dried the plates for 45 minutes in a laminar flow hood, and then incubated them for four days at 23 °C. After four days of incubation, all plates had visible growth and numerous zoospores. Any plates with contamination were discarded. I flooded each plate with 5 to 10 mL of sterile distilled water or distilled water with antibiotics (0.02 % penicillin G, 0.04% streptomycin sulfate, Sigma St. Louis, MO) and incubated them at room temperature in a laminar flow hood for one hour to promote zoospore discharge. After one hour, the water was pipetted off of each plate and pooled in a 50 mL centrifuge tube. To measure the concentration of

zoospores, I removed a 1.5 mL sub-sample from the harvest and killed them with one drop of Lugol's solution (Sigma). I used a Brightline™ haemocytometer to count the number of zoospores per milliliter. The number of zoospores was calculated as the average of four counts on the haemocytometer. I used the suspension of live zoospores in experiments immediately after they were harvested.

Length of time zoospores are motile

I tested how long *B. dendrobatidis* zoospores could swim to help understand how much time they have to find a suitable host or substrate. With 8 X magnification, I etched grids of 1 mm X 1 mm cells onto the bottom of 5 cm diameter petri dishes. I sterilized the plates prior to use by soaking them in 70 % ethanol overnight, rinsing them in sterile water, and drying them in a laminar flow hood under UV light. I pipetted 3 mL of the zoospore suspension (with antibiotics), approximately 200,000 zoospores, into each plate. I immediately counted with a microscope the number of zoospores that had settled motionless on the bottom. Two cells per grid were counted on three plates for a total of six counts. I averaged the two counts from each plate to give three replicates. I counted the settled cells every six hours for 24 hours, and repeated the experiment once.

Distance that zoospores can swim

I studied the distance zoospores of *B. dendrobatidis* swim to help understand transmission of the disease. Preliminary experiments indicated that 1 % tryptone did not noticeably affect the rate of encystment; this was determined by observing the encystment of zoospores in distilled water, pond water, and 1 % tryptone liquid medium over 24 hours. I flooded four 0.5 % tryptone agar-plates with 3 mL of sterilized pond water inside a laminar flow hood. I then added one drop of a zoospore suspension (~

65,000 zoospores) to one side of the plates. I dried one plate immediately (control), and incubated the other plates covered in the hood for 24 hours. After 24 hours I dried the remaining plates and incubated them at 23 °C for one week. After one week, the plates were photographed and the distances of the growing colonies from the initial drop were measured. The experiment was repeated once.

Chemotaxis of zoospores

I studied the chemotaxis of the zoospores to determine if they might be attracted to a potential substrate. I used two methods to test for chemotaxis of *B. dendrobatidis* zoospores. The agar-plate method I used was adapted from Pommerville (1978). The test plates were 1 % agarose plates with five wells in each made with a sterile cork-borer (Figure 1). The center well received 0.5 mL of an attractant, and the outer wells received 0.5 mL of a zoospore suspension. Attractants tested were: 1 % tryptone, 1 % gelatin hydrolysate, 1 % casamino acids, 0.1 % glucose, and 0.1 % lactose. After the zoospores were added, the plates were incubated at room temperature. I then observed all the plates microscopically at 10, 30 and 60 minutes. If zoospores were attracted to the compounds that diffused out of the center well, they would aggregate on the edge of their well facing the center well.

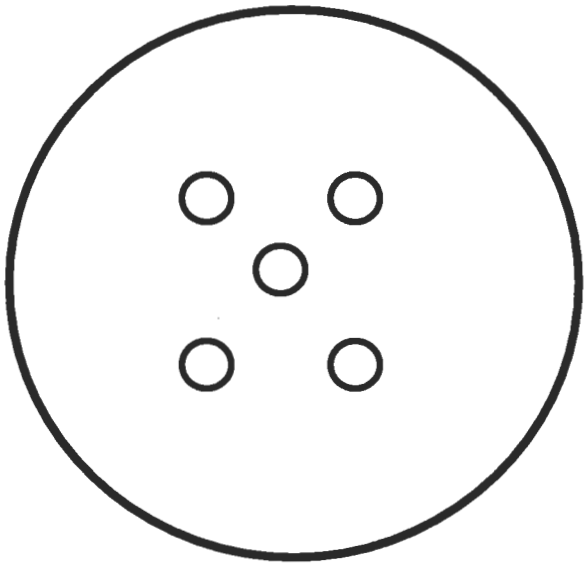

Figure 1. Design of the plate method of testing for chemotaxis (Adapted from Pommerville, 1978). A liquid attractant to be tested is placed in the center well, and zoospore suspensions in the outer wells. If the zoospores are attracted to the compound they will gather on the walls of the well facing the center well, as the attractant diffuses through the agar.

I used another method to test for chemoattraction, which was adapted and modified from Muelhstein and Amon (1987). The tips of capillary tubes were filled with either 1 % keratin or 1 % gelatin in 1 % agarose. I used tubes with 1 % water agarose in the tip as controls. Two tubes (one control and one with attractant) were affixed to the bottom of a Petri dish side-by-side. I filled the dish with 5 mL of distilled water and pipetted 3 drops of a zoospore suspension (~200,000 zoospores) into them. I observed the tubes microscopically after 10, 30, and 60 minutes. If zoospores were attracted to the compounds in the capillaries, a cloud of zoospores would be observable around the tube with attractant and not around the control.

Experiments on the temperature and pH requirements of *B. dendrobatidis*

Description of isolates used in all growth studies

All *Batrachochytrium dendrobatidis* isolates used in the experiments are listed in Table 4, and were provided by Joyce Longcore of the University of Maine, Orono. Isolates from different regions of North America and isolated from different amphibian species were selected to determine if isolates were unique in their physiological requirements. I used isolates 197, 215, and 274 in most experiments. I also tested isolates 230, 231, 275, and 277 in one experiment because genetic data suggest that they are more distantly related to each other (Longcore pers. comm.), which raises the possibility that they might differ in physiology. All isolates are morphologically identical. I subcultured the isolates from the University of Maine's stock collection of *B. dendrobatidis* isolates, which is maintained at 6 °C with each isolate in 30 mL of H-broth (1 % tryptone and 0.3 % glucose) in 75 mL screw-top culture tubes. Each isolate in the collection is transferred into fresh liquid medium every six months. After transfer, the isolates are incubated at 23 °C for 1 to 2 weeks to establish growth and then returned to 6 °C.

Table 4. Isolates of *Batrachochytrium dendrobatidis* and the amphibian and location which they were isolated.

Isolate	Amphibian Source	Location
197	*Dendrobates azureus*, blue poison-dart frog	National Zoological Park, Washington, D.C.
215	*Rana muscosa*, mountain yellow-legged frog	Sierra Nevada, California
230	*Rana yavapiensis*, lowland leopard frog	Santa Catalina Mountains, Arizona
231	*Rana yavapiensis*, lowland leopard frog	Maricopa County, Arizona
274	*Bufo boreas*, boreal toad	Rocky Mountains, Colorado
275	*Bufo boreas*, boreal toad	Rocky Mountains, Colorado
277	*Ambystoma tigrinum*, tiger salamander	Campina Mesa, Arizona

Description of inoculation methods

Inoculum for growth experiments was standardized because growth can be influenced by the amount of inoculum. I tested various methods of inoculation with preliminary experiments. Previous studies on chytrids used a standardized amount of a zoospore harvest, but the number of zoospores was not determined (Barr 1969, 1970). For the first experiments I harvested zoospores, counted them, and standardized the zoospore concentration to 0.5 million to 1 million zoospores per mL for each isolate. This method was not employed in later experiments, because the zoospore harvests varied greatly between isolates. Hassan and Catapane (2000) indicated that growth of monocentric chytrids can be measured by optical density (OD), and I decided to standardize inoculum this way.

To prepare the inocula, I added 1 ml of stock culture, originally subcultured from the University collection to 75 mL of H-broth. I made three flasks per isolate tested. After inoculation, the cultures were incubated 2 weeks at 23 °C, and then the OD of 1 mL of liquid culture from a gently shaken flask was measured at 495 nm. I used uninoculated, two-week-old H-broth as the blank. For each isolate, cultures with similar OD to those cultures of other isolates were used for standardization. To standardize inoculum, I diluted the stocks (if necessary) to an OD of 0.100 or 0.050 absorbance at 495 nm with sterile distilled water. The standardized inocula were immediately used to inoculate experiments.

Methods used to measure growth in liquid culture

Traditionally chytrid growth has been measured by the dry weight of culture filtrate, and I used this method in early experiments (Goldstein 1960, Barr 1969). I grew the cultures in pre-weighed centrifuge tubes; and at the end of the experiment I pelleted the cells at 7000 RPM, poured off the supernatant, dried the tubes overnight at 95 °C, and then reweighed them. The difference in weight should have been the dry weight of the chytrids; however, the weight of the dried tubes was less than the initial weight. I speculated that part of the tubes volatilized in the oven.

Next, I attempted to measure dry weight by filtering the cultures through pre-weighed 0.2 μm glass fiber filters (VWR West Chester, PA), drying the filters at 95 °C overnight, and then reweighing the filters. The difference in filter weight was the dry weight of the fungus. This method worked well; however, it was time consuming and susceptible to error. Most of the dry weights were less than 10 mg, and on humid days the dried filters could absorb moisture and falsely increase the dry weight measurements. Hassan and Catapane (2000) used OD as a measure of growth for two monocentric chytrids in liquid culture, and this method has been employed widely to measure growth of bacteria and single-celled yeasts (White 1995). I tested to see if there was a correlation between the dry weight of cultures and the change in OD at 495 nm and 600 nm during one pH experiment. I measured OD of 1 mL of gently shaken liquid culture along with the dry weight. The growth curves were similar and the dry weight measurements correlated strongly with OD at 495 nm ($R=0.860$). For all future growth experiments I measured growth by OD at 495 nm, which was quick and could detect sparse growth better than the dry weight method. To measure the OD of a culture, I gently shook the

culture to remove thalli from the sides of the tube, and measured 1 mL of the culture in a Spectronic Genesys 2 spectrophotometer. I used uninoculated liquid medium of the same age as a blank. Because contamination by bacteria or other fungi can falsely increase OD, I screened all cultures microscopically and discarded any contaminated cultures.

In addition to screening for contamination, I checked all cultures for *B. dendrobatidis* growth microscopically. I looked for swimming zoospores and living thalli, which would indicate a living culture. For some experiments I inoculated TGhL plates with one mL of liquid culture after the experiments and incubated the plates at 23 °C for one to two weeks to test if the cultures could grow. *B. dendrobatidis* thalli may be alive but not producing zoospores at high temperatures and this plate assay can determine if they are alive (Longcore et al. 1999).

Temperature requirements

These experiments were designed to determine the temperature tolerances of *B. dendrobatidis*. Cultures were incubated in VWR Model 2005 Scientific Low Temperature Incubators calibrated to ± 1 °C prior to use. Inoculum was 1 ml of a two-week-old liquid culture standardized to an OD of 0.100 at 495 nm. I incubated the cultures at 10, 17, 23, 25, or 28 °C. Starting at day 0 (immediately after inoculation), four cultures (replicates) per isolate were removed from the experiment every three days (every two days in earlier experiments) and the cultures were measured by OD at 495nm, screened microscopically, then discarded. The experiments lasted 3 weeks (2 weeks in earlier experiments). I also conducted a low temperature experiment in which cultures were incubated at 4 °C for six months, and samples measured every month. All the temperature experiments were repeated at least once.

Effect of exposure to high temperatures

These experiments tested if prolonged exposure to 30 °C would kill cultures of *B. dendrobatidis*. I used isolates 197, 215, and 274 for the high temperature experiments, and inoculum was standardized to an OD of 0.100 at 495 nm. Each isolate at each treatment (days) had four replicates. After inoculation, all cultures were incubated at 23 °C for 4 days to establish actively growing cultures. After 4 days, I measured the OD of four replicates of each isolate and moved half of the cultures to 30 °C, and the other half remained at 23 °C as controls. At 2, 4, 6, and 8 days after the cultures were placed under different temperature regimes, I measured four replicates per isolate from each temperature treatment. All cultures were screened for contamination and live zoospores. Additionally, I inoculated TGhL plates with 1 mL of culture from each replicate and incubated the plates at 23 °C for 6 to 10 days to determine if the cultures were still viable. This experiment was repeated once.

The effect of pH on growth

The pH experiments were designed to help understand how the pH of the chytrid's environment could affect its growth. I used isolates 197, 215, and 274 in all pH experiments for the following pHs: 4.0, 5.0, 6.0, 7.0, 8.0. Four tubes for each pH were filled with 30 mL of H-broth and adjusted to the required pH using 1 N HCl or 1 M NaOH . Each tube was inoculated with inoculum standardized to an OD of 0.050 at 495 nm. I used unadjusted H-broth (pH 6.8 to 7.0) as the control. I incubated the cultures 2 weeks at 23 °C, and shook them after 1 week to prevent a pH gradient from developing near the colonies. I measured growth as the OD at 495 nm of 1 mL of culture. I examined all of the cultures microscopically. The experiment was repeated once.

Statistical analysis of experimental data

I calculated all statistics using SYSTAT (SPSS Chicago, IL). If the data were normally distributed and had equal variances, ANOVA or PROC GLM with Tukey's multiple comparison was used. If the data did not meet these assumptions, I used the Kruskal-Wallis test to detect significant differences. To compare between certain treatments with the Kruskal-Wallis test, I used the select case option in SYSTAT. Differences in isolates in temperature experiments were tested at two points, during logarithmic growth (approximately at day 9) and during the stationary growth phase (approximately at day 15). Differences are significant if the p-value is less than 0.050. All graphs were created with SigmaPlot (SPSS Chicago, IL).

Results

Experiments with zoospores of *Batrachochytrium dendrobatidis*

Length of time zoospores are motile

After 6 and 12 hours, many zoospores were still motile. After 18 hours, over half of the spores had settled, and after 24 hours only 5% of the zoospores were still moving in the counting grids. I detected no significant increase in the number of zoospores settled between 18 and 24 hours (Figure 2). The repeated experiment had significantly fewer zoospores at each time because the zoospore concentrations were lower in the second experiment, but the rates at which they settled were similar.

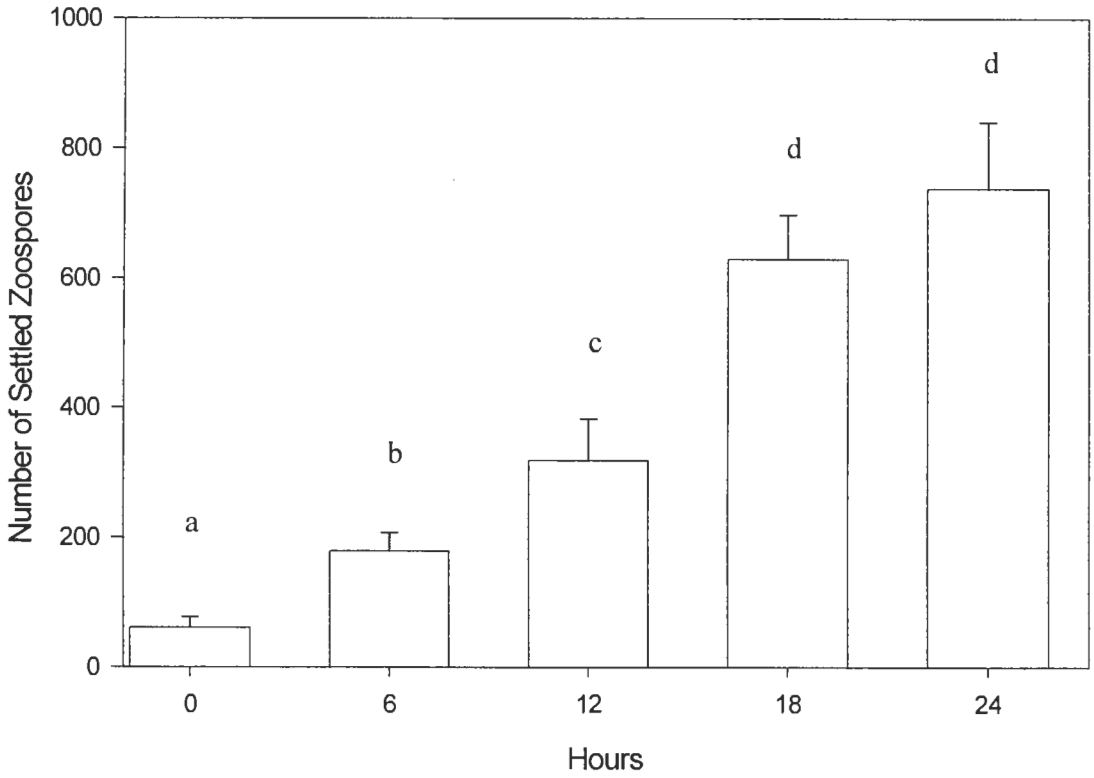

Figure 2. Number of *Batrachochytrium dendrobatidis* zoospores settling over time in a 1 mm^2 area. The mean of three replicates from each time and the standard error of the mean are presented. Zoospores were harvested from 0.5 % tryptone media with pond water plus antibiotics. Bars with the same letter are statistically similar ($p>0.050$). Experiment one presented.

Distance that zoospores can swim

The majority of zoospores swam less than 2.0 cm before encysting (Figure 3). Colonies on the test plates did not appear more dispersed than on the control plate, which was dried immediately after inoculation. All replicate plates displayed similar results.

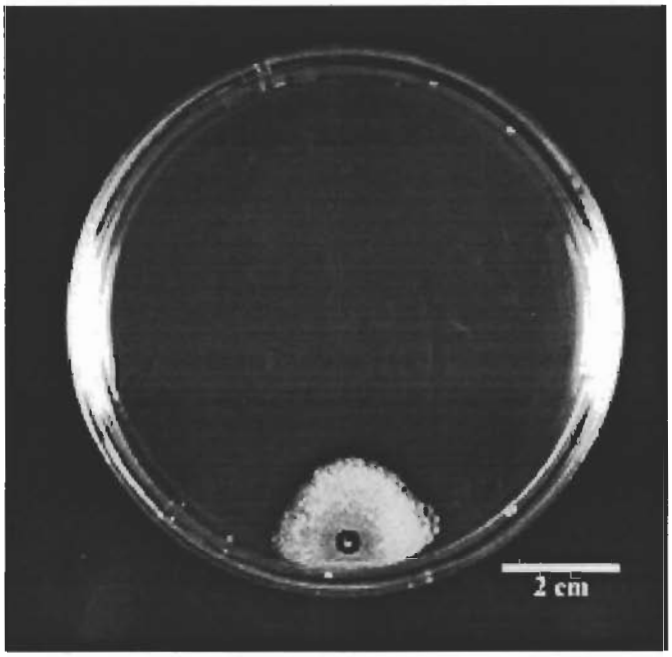

Figure 3. Distance *B. dendrobatidis* zoospores swam in 3 mL of distilled water in 24 hours at room temperature before encysting on 0.5 % tryptone agar. The black circle indicates where 1 drop of a zoospore suspension was introduced, and the white area is chytrid colonies. The scale bar is 2 cm.

Chemotaxis of zoospores

Results from the chemotaxis experiments were uniformly negative. The zoospores had no observable attraction to any of the compounds tested compared to the controls. The zoospores did not appear repelled by the test compounds either.

Experiments on the temperature and pH requirements of *B. dendrobatidis*

Growth at 10 °C

At 23 days of incubation, all isolates were still in logarithmic growth-phase, and had significantly different amounts of growth (Figure 4). In experiment one, isolate 197 had the densest growth, followed by 274. In the second experiment, isolate 274 had the densest growth after 23 days, and isolates 215 and 197 had significantly less growth at 15 and 18 days. At the end of both experiments, all cultures had live zoospores.

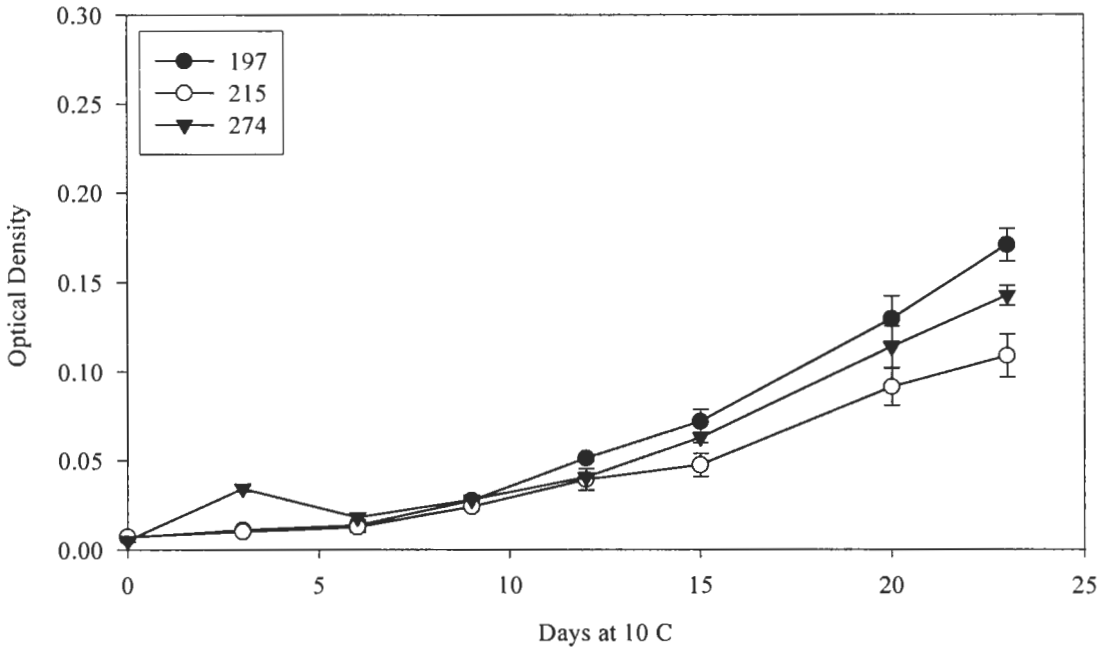

Figure 4. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 10 °C over 23 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment one is shown.

Growth at 17 °C

All cultures grown at 17 °C had entered stationary growth-phase by 12 days (Figure 5). In experiment one, no significant difference in the growth among isolates was detectable after 8 days (logarithmic growth). In experiment two , all isolates had different densities after 9 (log growth) and 15 days (stationary growth); isolate 197 had the densest growth, followed by 274. After 21 days all isolates had equivalent growth in experiment two. At the end of both experiments all cultures had live zoospores.

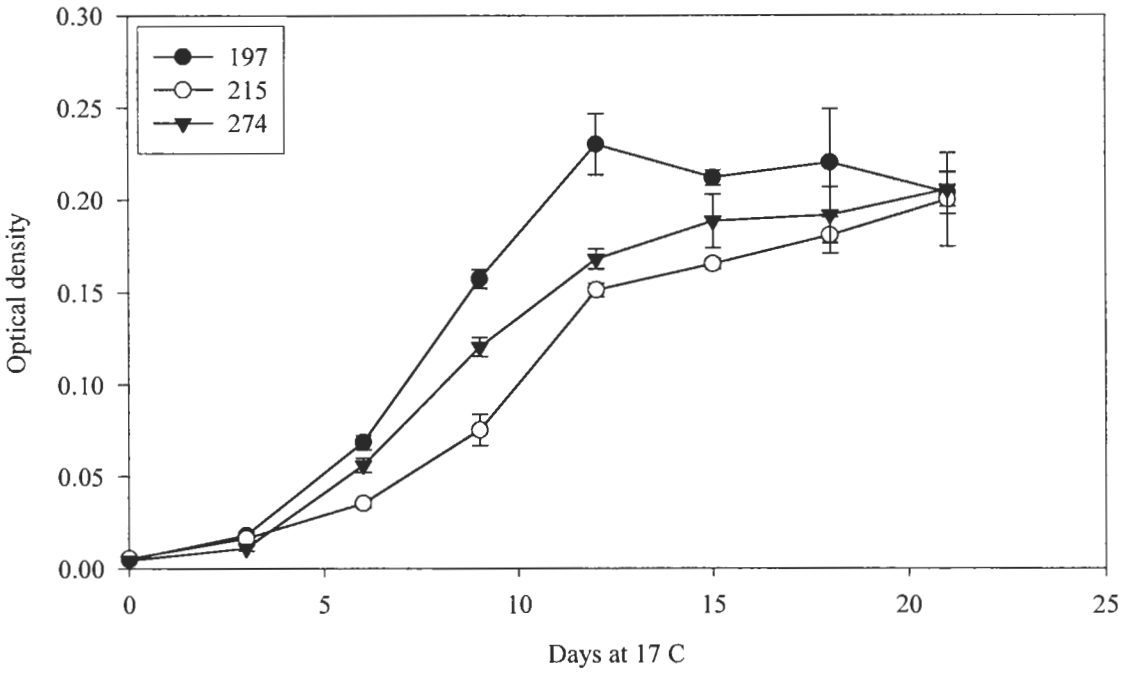

Figure 5. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 17 °C over 21 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment two is shown.

Growth at 23 °C

All cultures had entered stationary growth by 12 days (Figure 6). After 15 days all isolates had equivalent growth in both experiments. During log growth (8 or 9 days), isolates had equivalent growth in experiment one, but isolate 274 had denser growth than 197 and 215 in the second experiment. All cultures had live zoospores at the end of both experiments.

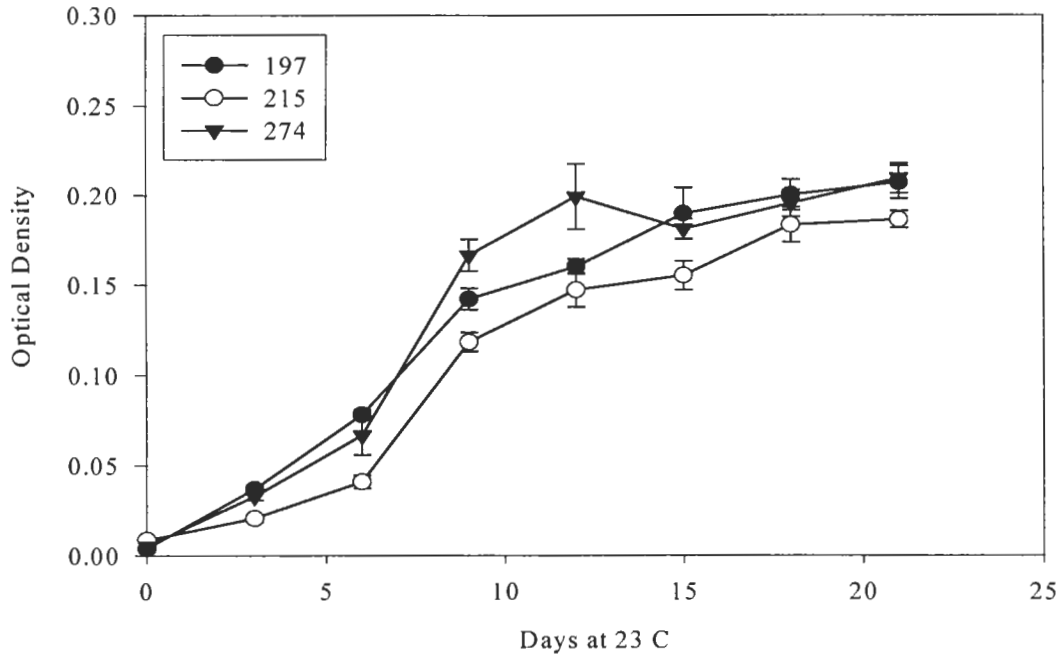

Figure 6. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 23 °C over 21 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment two is shown.

Growth at 25 °C

In experiment one all cultures had entered stationary growth by 9 days, and all but 274 had in experiment two (Figure 7). After 21 days, no difference in growth was detectable among isolates in experiment one; but, in experiment two, isolate 274 had less growth than 197 and 215, which were equivalent. At the end of both experiments all cultures had live zoospores.

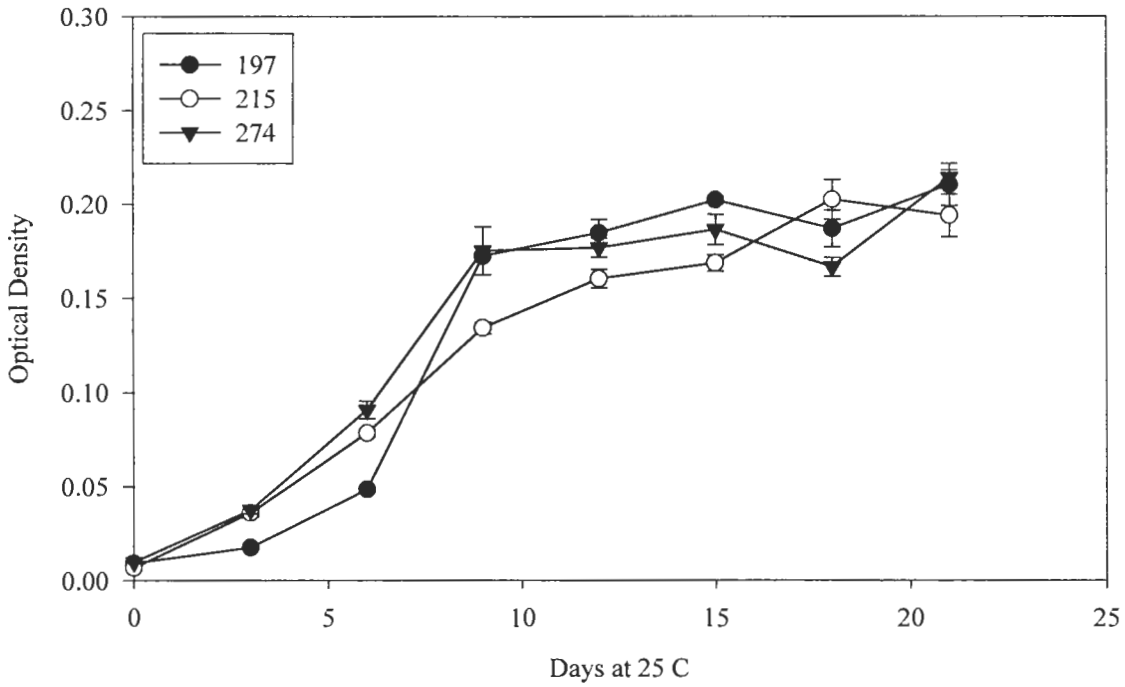

Figure 7. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 25 °C over 21 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment 2 is shown.

Growth at 28 °C

Only a small increase in OD was detectable in the cultures after incubation for 3 days to 21 days (Figure 8). No motile zoospores were present in any cultures at any day past inoculation. The repeated experiment had similar results.

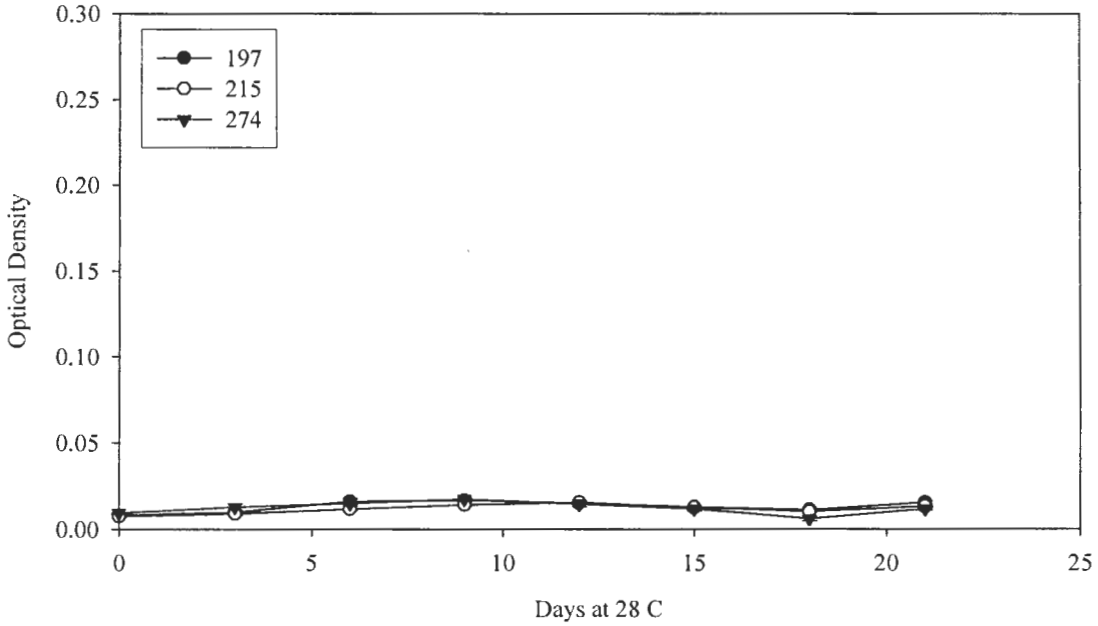

Figure 8. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 28 °C over 21 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment one is shown.

Experiments at 4 °C over six months

After 6 months of incubation at 4 °C, all isolates were still alive and producing zoospores. In experiment one, isolate 197 had the densest growth after 6 months (Figure 9), but had equivalent growth to 274 in experiment two. In both experiments, isolate 215 had significantly less growth after 6 months. In the first experiment, 215 grew less than 197 and 274; but no significant difference was detectable in growth among isolates at 3 months in the second experiment. In experiment two isolate 274 had a drop in OD at 5 months, which was absent in experiment one. Also, isolate 215 had a decrease in OD between 5 and 6 months in the second experiment.

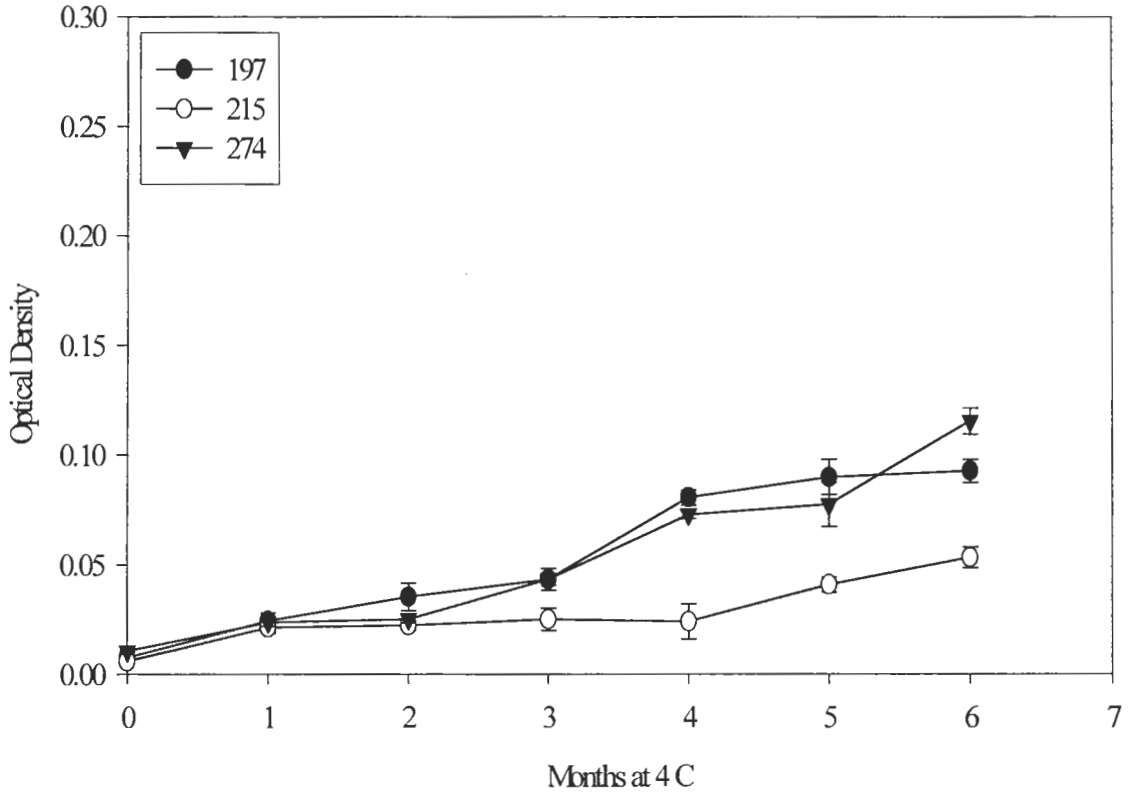

Figure 9. Growth of *Batrachochytrium dendrobatidis* isolates 197, 215, and 274 at 4 °C over six months as measured by optical density at 495 nm. The mean of four replicates from each month and the standard error of the mean are presented. Experiment one is shown.

Comparison of growths at different temperatures

A graph comparing the growth of isolate 197 at temperatures described previously is presented in (Figure 10). Similar curves were obtained by comparing the effect of temperature on isolates 215 and 274. At the end of 21 days, growth at 10 °C was significantly less than at 17, 23, and 25 °C. *B. dendrobatidis* had the lowest growth at 28 °C, followed by 10 °C. No significant difference in growth after 21 days was detected at 17, 23 or 25 °C.

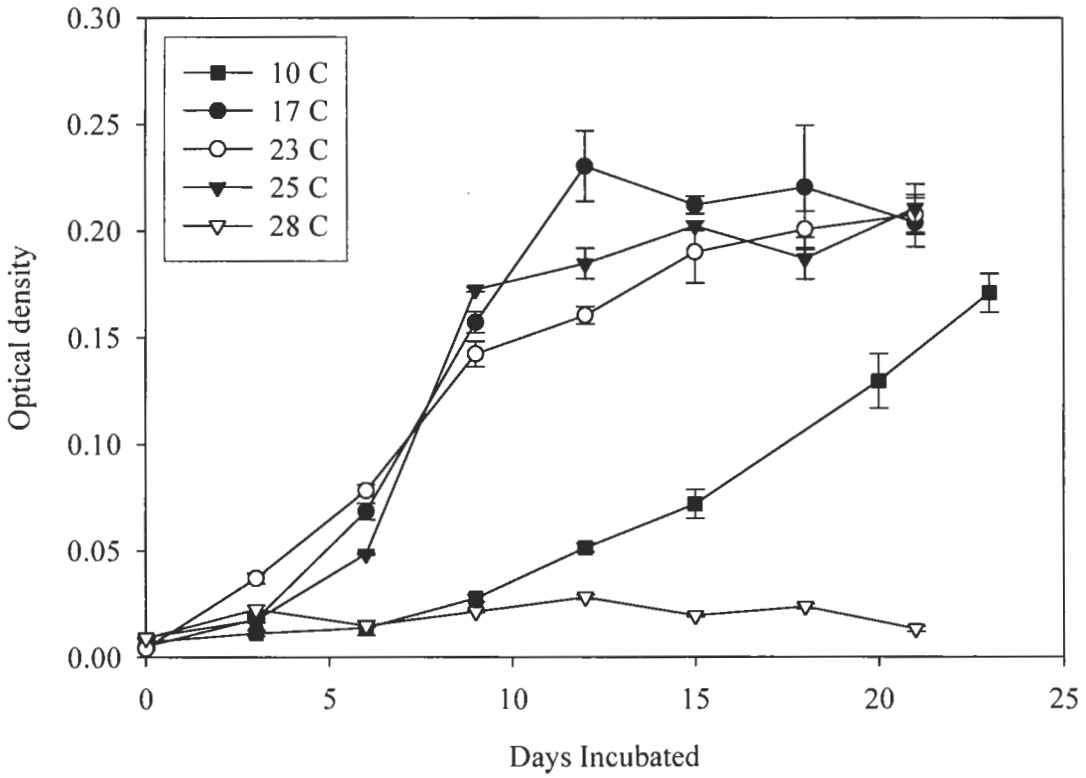

Figure 10. Growth of *Batrachochytrium dendrobatidis* isolate 197 over 21 to 23 days at different temperatures as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented.

Experiments on other isolates tested at 23 °C

All isolates had similar growth curves at 23 °C (Figure 11). In experiment two and experiment three, isolate 197 had the densest growth after 21 days. In experiment one and three isolate 275 had the least growth after 21 days, but had the second densest in experiment two. The cultures of isolate 230 were contaminated in experiment two, and therefore the isolate was not included in the analysis.

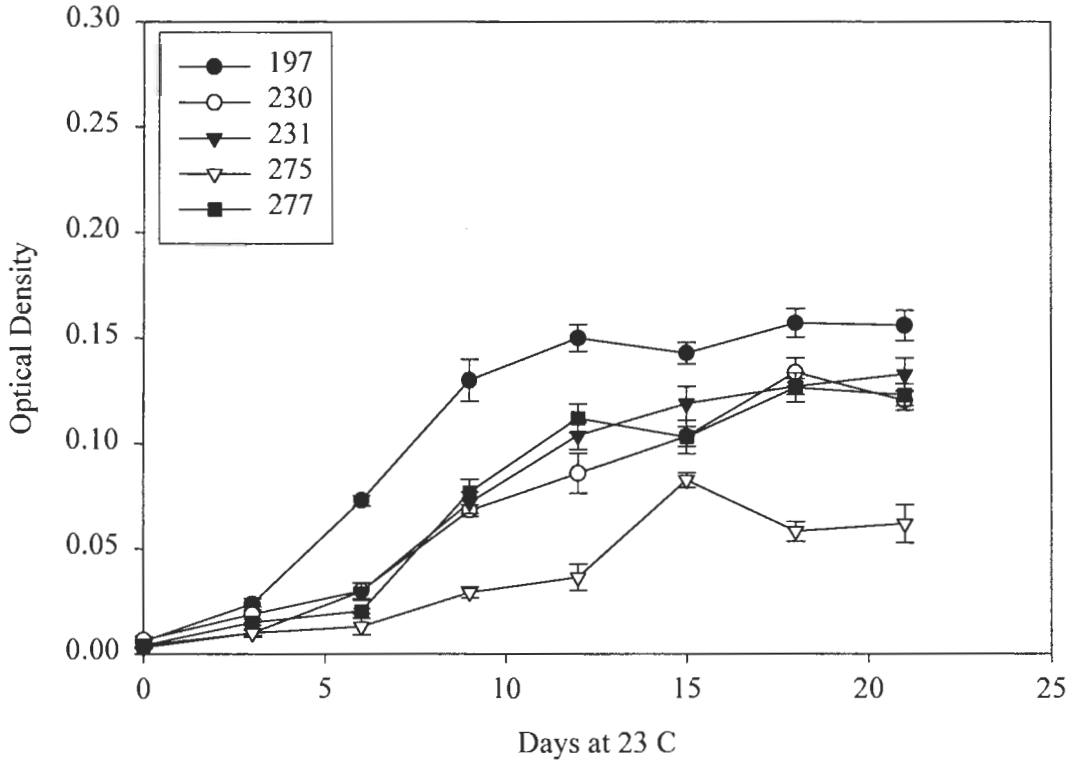

Figure 11. Growth of *Batrachochytrium dendrobatidis* isolates 197, 230, 231, 275, and 277 at 23 °C over 21 days as measured by optical density at 495 nm. The mean of four replicates from each day and the standard error of the mean are presented. Experiment three is presented.

Effect of exposure to high temperatures

After four days at 23 °C, before the cultures were divided between different temperature regimes, there was no significant difference in growth among the isolates (Figure 12, experiment two shown). After 2 days, cultures incubated at 30 °C had significantly less growth than cultures at 23 °C, and the cultures did not increase significantly in OD after being moved to 30 °C. Both experiments had similar results. In each experiment, 50% of the cultures from each isolate were still alive after 8 days at 30 °C.

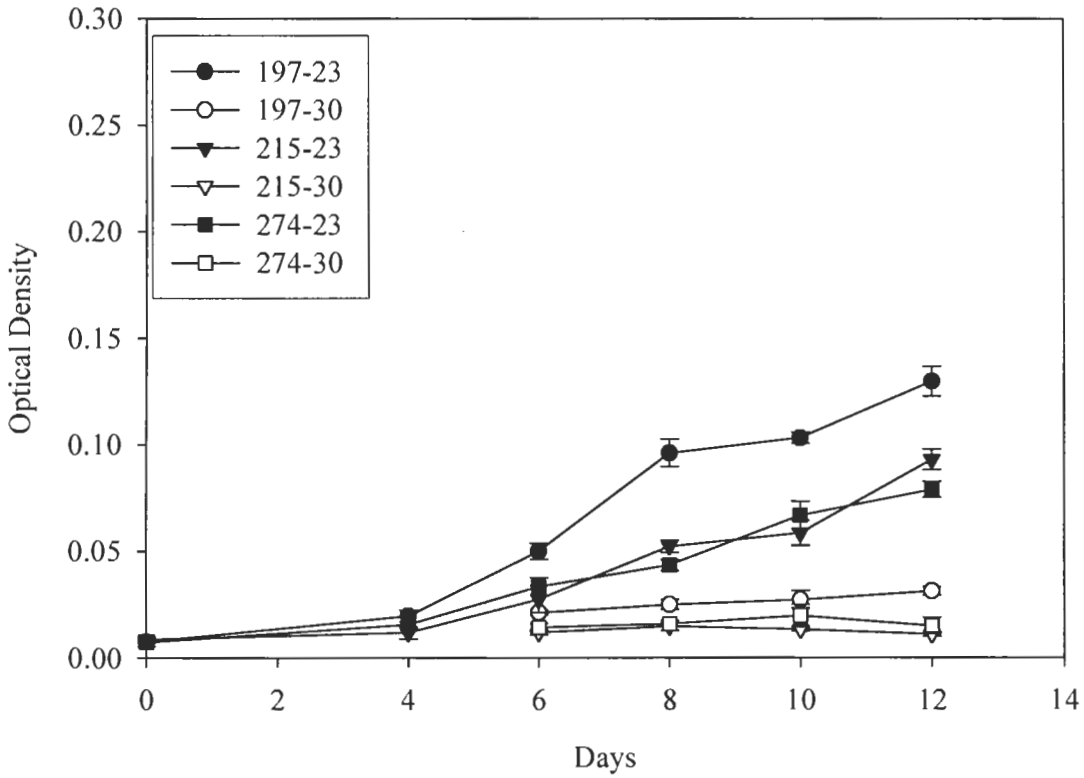

Figure 12. Growth of *Batrachochytrium dendrobatidis* cultures, as measured by optical density at 495 nm, incubated four days at 23 °C, after which, half were transferred to 30 °C. Open symbols are cultures incubated at 30 °C. The mean of four replicates from each day and the standard error of the mean are presented. Experiment two is shown.

The effect of pH on growth

Preliminary experiments indicated several buffers used to adjust the pH of H-broth hindered the chytrid's growth. Growth of the chytrid in unbuffered liquid medium did not greatly change the pH of the media after 2 weeks of incubation; therefore I used unbuffered media in all the pH experiments. In each experiment, all isolates had the densest growth at pH 6 to 7 (Figure 13, experiment two presented). In both experiments, isolates 215 and 274 grew equally dense at pH 6 and 7. Isolate 197 had denser growth at pH 7 than pH 6 in experiment one. All isolates had trace growth at pHs 4 and 5, and more

at pH 8. In the second experiment no significant difference was detectable between isolate 215 at pH 7 and pH 8. Growth at pH 4 and 5 was similar among all isolates.

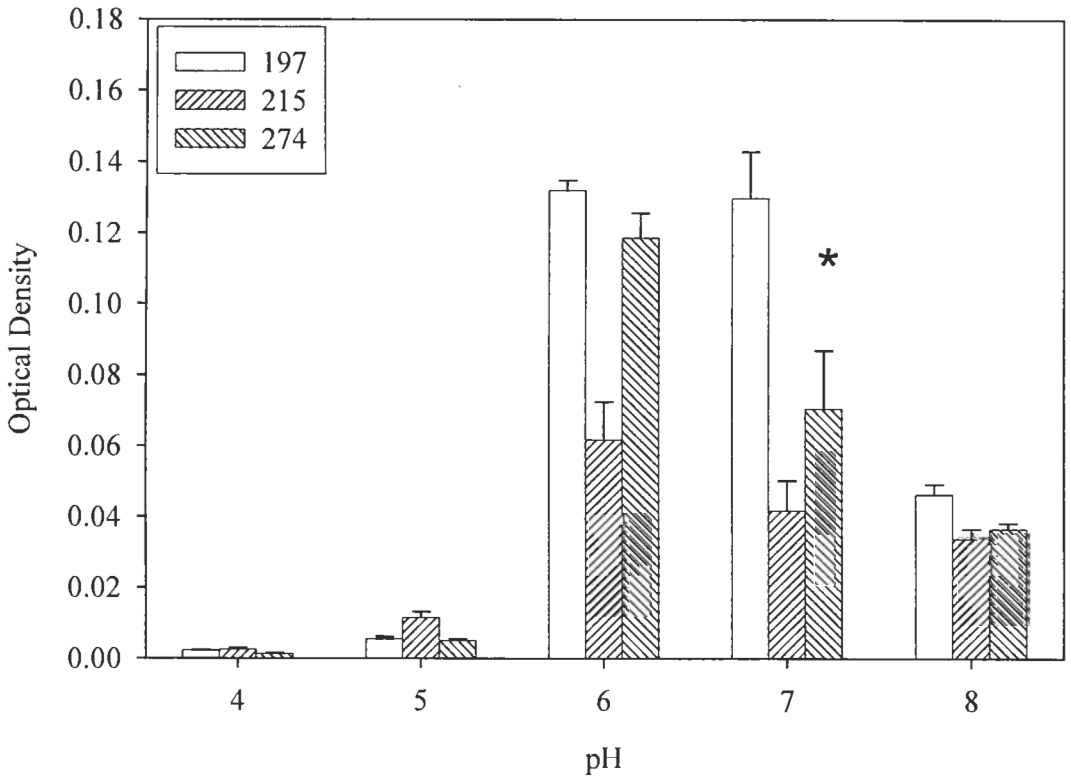

Figure 13. Growth of *Batrachochytrium dendrobatidis* isolates at different pHs at 23 °C as measured by O.D at 495 nm. Growth at pH 7 are the unadjusted control. The mean of four replicates from each day and the standard error of the mean are presented. Experiment 2 is shown.

* = mean of only two replicates

Discussion

Zoospore experiments

Zoospores are the primary method of dispersal for *B. dendrobatidis*, but they have a short time to infect a host and do not swim far. Zoospores of the chytrid may swim for

more than 24 hours, but more than 95% have encysted by this time, which is longer than the "average" chytrid or oomycete zoospore as described by Carlile (1986). Zoospore longevity in this pure-culture experiment may be different from their longevity in the environment. Preliminary experiments indicated that tryptone did not noticeably affect encystment rates, but other compounds may. *B. dendrobatidis* may encyst rapidly in the presence of amphibian skin or other proteins. Zoospores released from sporangia in the host tissue might encyst directly after release, which could explain why infections appear as "clumps" of thalli rather than being more equally distributed (Longcore pers. comm.).

In still water, zoospores of *B. dendrobatidis* swam fewer than 2 cm before encysting. Therefore it's not likely that the zoospores are swimming great distances to find a host. The disease is probably transmitted from amphibian to amphibian by direct contact either during mating or other close encounters. The zoospores could be spread longer distances if carried in water currents, but this would decrease the chances of them finding a host, as they would be diluted to low concentrations. Chances of the zoospore finding an amphibian could be increased if the zoospores were attracted to their host, as in some other parasitic chytrids (Sparrow 1960, Muelhstein et al. 1988). I tested tryptone, gelatin hydrolysate, glucose, and lactose as potential attractants because they are in media the chytrid has been cultured on; keratin and gelatin were tested because they are components of amphibian skin. Experiments in this study did not reveal any evidence of chemotaxis to the proteinaceous and complex nitrogen compounds I tested; however, I did not test amphibian skin as an attractant, which is a complex of many different compounds. If *B. dendrobatidis* were attracted to amphibian skin or compounds released from amphibian skin, the zoospores may swim longer and farther than the results suggest,

because they may swim towards the skin and not encyst until they reach or run out of energy.

Temperature and pH experiments

B. dendrobatidis can grow within a wide range of temperatures, from 4 °C to 25 °C. The chytrid also has a wide range of temperatures where it grows optimally, 17 °C to 25 °C. These temperature tolerances would allow this pathogen to persist in many environments. *B. dendrobatidis* may overwinter in adult amphibians as long as they do not freeze; as temperatures rise, the chytrid may reproduce rapidly, as it did when returned to 23 °C after incubation at 4 °C. *B. dendrobatidis* does not grow well above 25 °C, and higher temperatures do not favor epidemics (Berger et al. 1998, Bosch et al. 2000). Outbreaks of chytridiomycosis will probably be limited to cooler areas or months in the tropics, as has been observed in Australia, Panama, and Spain (Lipps 1998, Berger et al. 1998, Bosch et al. 2000). In temperate zones, outbreaks could occur in montane areas in warmer months or lowlands during the winter.

Prolonged exposure to 30 °C can kill *B. dendrobatidis*. However, even after 8 days at 30 °C half of the cultures were alive, as seen when they grew after being incubated at 23 °C. At temperatures of 28 °C or above, or below 10 °C, chytridiomycosis may not be fatal, because the chytrid may not establish a large enough colony in the epidermis to cause significant damage. If temperatures are optimal for the fungus, the infection could rapidly overtake its host (Nichols et al. 2001). Exposure to temperatures above 30 °C for more than 20 days may be an effective treatment for chytridiomycosis, provided the amphibians could survive the temperature as well, because by this time an estimated 100 % of the thalli might be dead if 8 days kills 50 % of the cultures. It may

take even less time to kill all the chytrids in a host by high temperature treatments, because my experiments used pure cultures with millions of thalli; and this amount of fungus would not be found in a living amphibian.

Even though the inoculum in the experiments was of a standard age and OD, I had significant variation in the growth of isolates between repeats of an experiment. I believe the cause of this variation is a phenomenon that I call "inoculum effect." With an inoculum of both zoospores and thalli, the ratio of these can vary from day to day. When culturing chytrids, one day there could be millions of zoospores because many have recently been released; however, there would be far fewer the day prior to the release. When I inoculate with 1 mL of culture, it could be mostly zoospores, mostly thalli, or various proportions of each. If the inoculum contains mostly thalli, the final optical density could be greater because thalli are closer to zoospore release than zoospores and would ultimately produce more biomass than if the inoculum was mostly zoospores. The extra time would allow more reproduction before growth is measured, and would result in a higher OD. Several of the experiments have such discrepancies between repeats, but the overall growth trends are similar.

Because all isolates tested had a similar optimal temperature range for growth they may be recently spread as suggested by Daszak (2000). As with temperature optimum, all isolates have a similar optimal pH range for growth in unbuffered media. All *B. dendrobatidis* isolates grew best at a pH from 6 to 7. In one experiment, isolate 197 grew significantly denser at pH 7 than 6, but did not in the repeated experiment. This difference could again because of inoculum effect. It is not unusual that all the isolates have similar physiological requirements, different genera of chytrids, even different

orders, have similar temperature and pH tolerances as *B. dendrobatidis* (Barr 1969, 1970).

B. dendrobatidis outbreaks may be affected by pH, but the chytrid's pH optimum is not outside the common pH of freshwater systems. Acidification of streams would likely hinder the pathogen's growth, as it grows poorly below pH 6.0. The fungus may be buffered from the water's pH when inside a host, allowing it to tolerate wider pH ranges. The temperature and pH optima of *B. dendrobatidis* are not unusual compared with other chytrids. Conditions that would favor a chytridiomycosis outbreak would be consistent temperatures below 28 °C but above 10 °C, and a water pH of 6 to 7. The chytridiomycosis epidemic is still prevalent, but these data will aid in the understanding of its spread and persistence.

CHAPTER 3- NUTRITION AND EXTRACELLULAR ENZYME PRODUCTION OF *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

The nutritional requirements of *Batrachochytrium dendrobatidis* were studied to help determine if the chytrid can live on saprophytic substrates in the environment, and to aid in designing an optimal culture medium for the fungus. No synthetic medium tested supported growth. *B. dendrobatidis* cultures grew densest with tryptone or peptonized milk as a nitrogen source. The chytrid did not require additional sugars when grown in tryptone, and grew densest in a liquid medium with 0.5 % tryptone alone. Liquid media with glucose concentrations greater than 1.8% or tryptone concentrations greater than 2% hindered growth. The chytrid grew on autoclaved snakeskin in water or on 1% keratin agar. *B. dendrobatidis* produced extracellular proteases that degraded casein and gelatin, but had no measurable activity against keratin azure. The proteases were most active against casein at temperatures from 23 to 30 °C at pH 8.0; however, they were active at temperatures from 6 to 37 °C, and in a pH range from 6 to 8. SDS-PAGE analysis of the culture supernatant yielded no visible protein bands when stained with Coomassie blue or copper chloride; but activity gels with 0.5% skim milk revealed two distinct clearings.

Introduction

Chytrids are common, primary decomposers of an array of substrates, and many of these fungi live saprophytically in the environment; some are parasitic on plants, protists, fungi, or animals (Barr 1990, Berger et al. 1998). Parasitic fungi also may have a saprophytic life outside their host. Many of the ascomycete skin parasites (e.g.,

Trichophyton spp.) are often found living in soil (Wawrzkiewicz et al. 1991, Singh 1997). These are facultative pathogens that do not require their host for nutrients, but can live independently in the environment.

B. dendrobatidis is a skin pathogen that has not been found outside of amphibians (Berger et al. 1998). Amphibian skin is almost certainly the nutrient source for *B. dendrobatidis* inside its host, and this chytrid probably produces extracellular proteases to degrade components of the epidermis, as other fungal skin pathogens do (Hibino 1985, Wawrzkiewicz et al. 1991, Singh 1997). *B. dendrobatidis* is only found in keratinized cells, and is believed to use the keratin for nutrients, but this has not been proven (Berger et al. 1998). Ascomycete skin-pathogens and bacteria degrade keratin for nutrients, but this has not been documented with certainty in chytrids. Several chytrids have been isolated growing on impure keratin (e.g. snakeskin), but experimental evidence that fungi are degrading the keratin is lacking (Karling 1946).

Cantino (1955) suggested that substrate preference might be inferred from an understanding of the carbon and nitrogen requirements of the chytrid species in pure culture; for instance, *Karlingia rosea* (*Rhizophlyctis rosea*) decomposes cellulose, and in pure culture grows best with cellobiose, a product of cellulose degradation. My experiments were designed to determine the carbon and nitrogen preferences of *Batrachochytrium dendrobatidis*, and these might suggest saprophytic substrates in the environment. This information will also aid in culturing the fungus during future studies on the organism. If *B. dendrobatidis* can live independently of its host under natural conditions, it could change how epidemiologists and ecologists study the disease. I will determine if *B. dendrobatidis* produces extracellular proteases, and what proteins they

can degrade. I will characterize the temperature and pH optima of the enzymes, and will attempt to describe the proteolytic extracellular isozymes of *Batrachochytrium* by their molecular weight.

Material and Methods

Nutritional requirements of *B. dendrobatidis*

Can *B. dendrobatidis* grow on synthetic media?

A synthetic medium is often necessary to determine the carbon and nitrogen sources used by a fungus, because one can control the exact components of the medium. Not all fungi, however, can be cultured in synthetic media. I attempted to grow *B. dendrobatidis* on different synthetic media created for chytrids and other fungi. Formulas for the media tested are listed in Table 5.

Table 5. Composition of synthetic media tested to support *B. dendrobatidis* growth.

Media	Components	Reference
Asparagine Glucose Agar	KH_2PO_4, 0.125 g K_2HPO_4, 0.150 g $MgSO_4$ * 7 H_20, 0.125 g NaCl, 0.025 g $CaCl_2$, 0.025 g Asparagine, 1.25 g Glucose, 5 g Agar, 2.5 g H_2O, 250 ml	Stevens, 1974
Dilute Salts Solution	$(NH_4)_2HPO_4$, 0.067 g/ L KH_2PO_4, 0.068 g/ L K_2HPO_4, 0.087 g/ L $MnCl_2$* 4 H_2O, 0.0018 g/ L $ZnSO_4$* 7 H_2O, 0.0004 g/ L HBO_3, 0.0029 g/ L $CaCl_2$, 0.074 g/ L $MgCl_2$, 0.102 g/ L	Fuller and Jaworski, 1987

Contents of 1 % Yeast Nitrogen base + glucose	KH_2PO_4, 1 g/ L $(NH_4)_2SO_4$, 5 g/ L $MgSO_4$, 0.5 g/ L NaCl, 0.1 g/ L $CaCl_2$, 0.1 g/ L Histidine HCl, 10 mg/ L Methionine, 20 mg/ L Tryptophan, 20 mg/ L Inositol, 2 mg/ L Contains less than 1 mg/L each of vitamins and trace inorganic salts Glucose 0.5 g/ L	Sigma, St. Louis, MO

Preference of nitrogen sources

Whether *B. dendrobatidis* has preferences for certain nitrogen sources was tested by measuring growth in liquid media containing a fixed amount of glucose as a carbon source with the addition of various nitrogen sources. I used isolate 274 (Colorado) in these experiments because it has not been cultured on artificial media (1% tryptone) as long as 197 or 215, and therefore may be the least adapted to tryptone. Chytrids adapted to tryptone could have the densest growth on this medium, which would not accurately reflect the true nitrogen preferences of the fungus in the environment. Inoculum was 1 mL of a two-week-old H-broth culture (see chapter 1) standardized to an optical density (OD) of 0.100 at 495 nm. Each replication for each medium consisted of four 50 mL, screw top culture tubes containing 30 mL of 0.3% glucose and 1% of one of the following nitrogen sources: gelatin hydrolysate (Sigma, St. Louis, MO), yeast extract (Difco, Franklin Lakes, NJ), peptonized milk (Oxoid, Hampshire, England), malt extract (Difco), asparagine (Sigma), peptone (Difco), or tryptone (Difco). The control was 0.3% glucose liquid-medium with no added nitrogen. After inoculation, cultures were incubated for two weeks at 23 °C. To measure growth I took the OD at 495 nm of 1 mL of gently shaken culture. Cultures were checked microscopically for contamination and live zoospores, and the experiment was repeated once.

Preference of carbon sources

These experiments tested what sources of carbon suppotted the best growth of *B. dendrobatidis* with a fixed amount of tryptone. Tryptone has low levels of carbohydrates, which makes it useful in differentiating bacteria by the carbohydrates they ferment, and it may be useful in testing the carbohydrate use of fungi (product information from: BD Franklin Lakes, NJ). I used isolate 274 for the carbon preference experiments. The inoculum was 1 mL of two-week-old H-broth culture standardized to an OD of 0.100 at 495 nm. For each test medium I used four culture tubes with 30 mL of 1% tryptone and 0.3% of one of the following: sucrose, maltose, sorbitol, soluble starch, mannose, glucose, glycerol, or lactose (Sigma). The control was liquid-medium with 1% tryptone and no added sugars. After inoculation, cultures were incubated 2 weeks at 23 °C, and then growth was measured spectrophotometrically. All cultures were screened microscopically for live zoospores and contamination, and the experiment was repeated once.

Carbon/ nitrogen ratio effects on growth

These experiments were to help understand the effect of varying carbon and nitrogen ratios on growth and to determine the optimal carbon and nitrogen ratio. This experimental design was adapted and modified from Emerson and Whisler (1968). I used isolate 274, and the inoculum was 1 mL of a two-week-old liquid culture at an OD of 0.100 at 495 nm. To determine the effect of glucose concentration on growth of the chytrid I used culture tubes with 30 mL of 1 % tryptone and 0, 0.15, 0.3, 0.9, 1.8, or 3.6 % glucose. I used culture tubes with 30 mL of 0.3 % glucose and 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, or 3.0 % tryptone to test the effect of tryptone concentration on the chytrid. Each

treatment had four replicates. After inoculation, cultures were incubated for two weeks at 23 °C, after which I measured the OD of the cultures. All cultures were checked microscopically for live zoospores and contamination, and the experiment was repeated once.

Can *B. dendrobatidis* grow on keratin media?

I tested two different media containing keratin to determine if *B. dendrobatidis* could grow on keratin as the sole carbon and nitrogen source. I prepared a liquid medium with 1g of macerated snakeskin autoclaved in 75 mL of distilled water, and 1 % agar plates with 1 % keratin (From scleroproteins, ICN Costa Mesa, CA). Both the plates and the liquid media were inoculated as in previous nutrition experiments. I incubated the cultures for 1 to 2 weeks at 23 °C and checked them microscopically for live zoospores and contamination.

Extracellular enzyme production by *B. dendrobatidis*

Are proteases produced by *B. dendrobatidis*?

These experiments were to determine whether *B. dendrobatidis* produces an extracellular protease. I used isolate 197 in all enzyme experiments because it grew the fastest and densest of all isolates tested at 23 °C, and denser growth may yield more extracellular enzymes. To detect the presence of proteases I adapted and modified a method from Karaup et al. (1994). First I inoculated TGhL (see chapter 1) agar plates with 1 mL of isolate 197 liquid-culture. The plates were dried in the flow hood for one hour and then incubated at 23 °C for 4 to 6 days, until dense growth and live zoospores were visible. With a sterile cork borer I then removed 5 mm diameter plugs containing colonies from the agar. I inverted the plugs culture-side down on the protease assay

plates, which contained 1 % agarose and either 1 % skim milk or 1 % gelatin. Each assay plate received four plugs: 3 with chytrid colonies and one uninoculated control. I incubated the assay plates at 23 °C for 2 to 4 days. Clearings in the medium surrounding the plugs from the breakdown of proteins indicated protease activity.

Extraction of extracellular enzymes from cultures

To prepare culture supernatants containing protease activity for the enzyme activity experiments, I grew *B. dendrobatidis* on a skim milk medium. I inoculated 75 mL of 1% skim milk in water medium with 1 mL of two-week-old liquid-culture (Isolate 197). I incubated the skim milk cultures for 2 to 4 days at 23 °C, until the skim milk became clear (Figure 14). All cultures were examined microscopically and contaminated cultures were discarded. Cultures were centrifuged at 6000 RPM for 20 minutes to remove cells, and the supernatant was aliquoted into tubes and immediately frozen at –80 °C. I then freeze dried the samples in a Labconco Freezone 4.5 freeze-dryer for 36 to 48 hours at (-40 °C, -40 $*10^{-3}$ mBar). The dried culture supernatant was resuspended in 4 mL of 50 mM Tris-HCl (pH 7.0). I dialyzed the 10 X concentrated supernatant in dialysis tubing with 5 KDa pores for 24 hours at 6 °C in buffer volumes greater than 20 times the volume of the samples. I changed the dialysis buffer three times. After dialysis, the 10 X concentrated supernatant was stored in 1.5 mL aliquots at –20 °C until use.

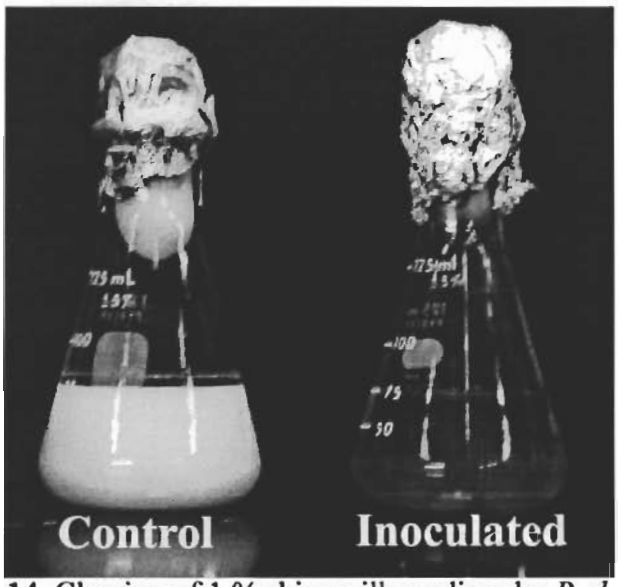

Figure 14. Clearing of 1 % skim milk medium by *B. dendrobatidis* isolate 197 (right flask). The inoculum was 1 mL of two-week-old liquid culture. The cultures were incubated 2 days at 23 °C. The control received no inoculum (left flask).

Temperature range of the extracellular proteases

The temperature range at which the proteases were active was studied in these experiments. The azocasein (Sigma) assays were adapted and modified from Petinate et al. (1999). Azocasein is soluble casein protein bound with a dye; when a protease breaks down the casein the soluble dye is released into solution. For temperature experiments I used the following reaction mixture in 1.7 mL Eppendorf tubes: 500 μL of 0.2 M $CaCl_2$, 500 μL of 5% azocasein in 50 mM Tris HCl (pH 7.5), and 200 μL of 10 X concentrated supernatant or water. Culture supernatants were added to the reactions and incubated for 36 hours at 6, 15, 23, 30, or 37 °C, after which I stopped the reactions with 5 % trichloroacetic acid (TCA). I centrifuged the tubes at 11,000 RPM in a Beckman microcentrifuge for 2 minutes to remove the precipitated proteins. Once the protease

reacted with the azocasein, and the remaining proteins precipitated and pelleted, the absorbance of the supernatant at 440 nm was measured with a spectrophotometer. I used distilled water for the blank, and as a control I boiled the culture supernatant for 1 hour. Each temperature treatment had 3 replicates with unaltered supernatant, 3 with boiled supernatant, and 3 with only water added. The increase in absorbance was calculated as: (the absorbance of the reactions with supernatant)- (the absorbance of the reactions with water). The experiment was repeated once.

pH range of the extracellular proteases

These experiments tested the protease activity from *B. dendrobatidis* in media with pHs ranging from 6 to 8. For these experiments the reaction mixture was: 1 mL of 5% azocasein in Tris-HCl buffer adjusted to pH 6, 6.5, 7, 7.5, or 8, and 200 µL of sample. I incubated the reactions for 36 hours at 23 °C then stopped them with 200 µL of 5% TCA. The reaction mixtures for the pH experiments differed from the temperature experiment because between temperature and pH experiments I found the $CaCl_2$ was unessential, but if removed, more TCA had to be added to precipitate all the proteins. The new reaction mixtures were more sensitive to enzyme activity. Each pH treatment had 3 replications with culture supernatant, 3 with boiled supernatant, and 3 with water only. I calculated the absorbance like in the temperature treatments. The experiment was repeated once.

Assays to detect the breakdown of keratin azure

The keratin azure assay was adapted and modified from Santos et al. (1996). I used keratin azure (Sigma) in an experiment to determine if the 10 X culture supernatant from *B. dendrobatidis* could degrade keratin. Keratin azure is insoluble keratin bound to

a blue dye. When keratin is degraded, dye is released into solution and an increase in absorbance at 495 nm can be measured with a spectrophotometer.

In one experiment I prepared a medium with 1g of keratin azure in 30 mL of distilled water. I inoculated this medium with 1 mL of two-week-old liquid-culture. After two weeks of incubation at 23 °C, I measured the absorbance of the liquid medium's supernatant at 495 nm and checked the cultures microscopically. I also tested if the 10 X concentrated culture-supernatant could degrade keratin azure. For this, 1g of keratin azure, 2 mL of culture supernatant, and 10 mL of 50 mM Tris-HCl (pH 7.5) was used as the reaction mixture. The reaction was incubated for 48 hours at 23 °C, after which I centrifuged the mixtures for 5 minutes to remove the keratin azure and measured the absorbance of the supernatant at 495 nm.

SDS-PAGE analysis of culture supernatant

The 10 X concentrated culture-supernatant was analyzed by SDS-PAGE to determine the number and weight of potential extracellular enzymes produced by *B. dendrobatidis*. Gels were 0.75 mm thick and 10, 15, or 20 % polyacrylamide cast as described by BioRad (Hercules, CA). For each trial, 10 X supernatant was mixed 1:7 with sample buffer (47.5 % distilled water, 12.5 % Tris-HCl (pH6.8), 10 % glycerol, 20 % SDS (10% w/v), and bromophenol blue (0.05% w/v)) with and without the 5 % β-mercaptoethanol. Some of the samples were denatured by incubation at 90 °C for 2 minutes. I ran 20 µL of a mix of sample buffer plus supernatant in the lanes of the gel, and used a wide-range protein standard (Bio Rad) to determine the weight of enzymes. The gels were electrophoresed at 6 °C for 1-3 hours at 50 mV in a Protean II apparatus (Bio Rad) after which they were stained with either 0.25 % Coomassie Brilliant Blue R-

250 for 1 hour or 0.3 M copper chloride for 15 minutes. I destained the gels in 50% MeOH, 10 % acetic acid in distilled water at 6 °C for 3-6 hours and then photographed.

I also cast substrate gels, which were 10% SDS-PAGE gels as above with 0.5% skim milk added. The buffer containing the 10 X supernatant (without β-mercaptoethanol) was not denatured by heat before being loaded on these gels. After electrophoresis, substrate gels were incubated at room temperature overnight, after which they were stained with Coomassie blue and destained as above. Destained gels were then photographed.

Statistical analysis of the experiments

I calculated all statistics using SYSTAT (SPSS Chicago, IL). If the data were normally distributed and had equal variance, ANOVA or PROC GLM with Tukey's multiple comparison was used. If the data did not meet these assumptions, I used the Kruskal-Wallis test to detect significant differences. To compare between certain treatments with the Kruskal-Wallis test, I used the select case option in SYSTAT. Differences are significant if the p-value is less than 0.050. All graphs were created with SigmaPlot (SPSS Chicago, IL).

Results

Nutritional requirements of *B. dendrobatidis*

Can *B. dendrobatidis* grow on synthetic media?

None of the synthetic media tested supported *B. dendrobatidis* growth. No live zoospores were found after incubation on the tested media. Therefore, I employed more complex media to determine the ability of the chytrid to grow on different nitrogen and carbon sources.

Preference of nitrogen sources

Nitrogen source can strongly influence growth of *B. dendrobatidis*. The chytrid grew densest on 1% tryptone in distilled water in both experiments (Figure 15, experiment two shown).

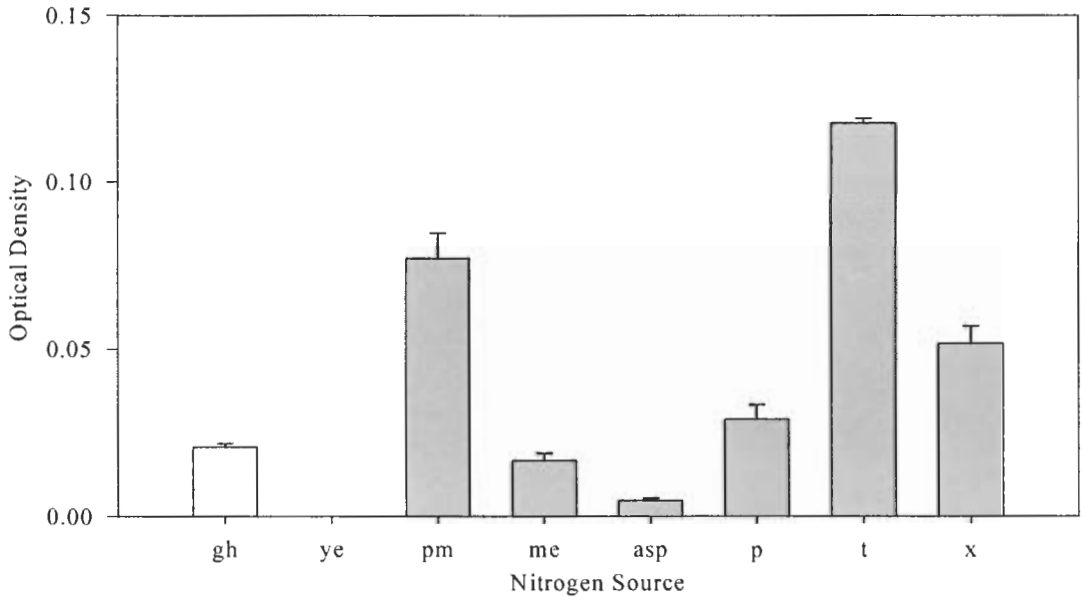

Figure 15. Growth of *B. dendrobatidis* isolate 274 on different nitrogen sources as measured by optical density. Cultures were grown in 30 ml of 0.3% glucose and 1% of the nitrogen source and the optical density was measured at 495 nm. Means of four replicates and the standard error of the mean are presented. All treatments were significantly different from each other ($p<0.050$) Key to nitrogen sources: gh= gelatin hydrolysate, ye= yeast extract, pm= peptonized milk, me= malt extract, asp= aspargine, p= peptone, t= tryptone, x= basal media of glucose with no nitrogen source added.

The chytrid grew second densest on 1% peptonized milk; however, growth was significantly less than growth on tryptone. All the other media supported less growth than the control with 0.3% glucose. Malt extract, yeast extract, and asparagine had only trace growth. All media tested except asparagine had live thalli after two weeks of incubation,

and all except asparagine and gelatin hydrolysate had live zoospores. Thalli in gelatin hydrolysate and peptone were nearly twice as large as thalli in yeast extract, malt extract and peptonized milk. Growth in the repeated experiment was significantly lower at each treatment, but the differences among the nitrogen sources were the same.

Preference of carbon sources

When grown in liquid media with 1 % tryptone, different carbon sources had little effect on *B. dendrobatidis* growth. In experiment 1, *B. dendrobatidis* had the highest OD on soluble starch (Figure 16), but the medium had a small amount of undissolved starch that could have caused an increase in OD.

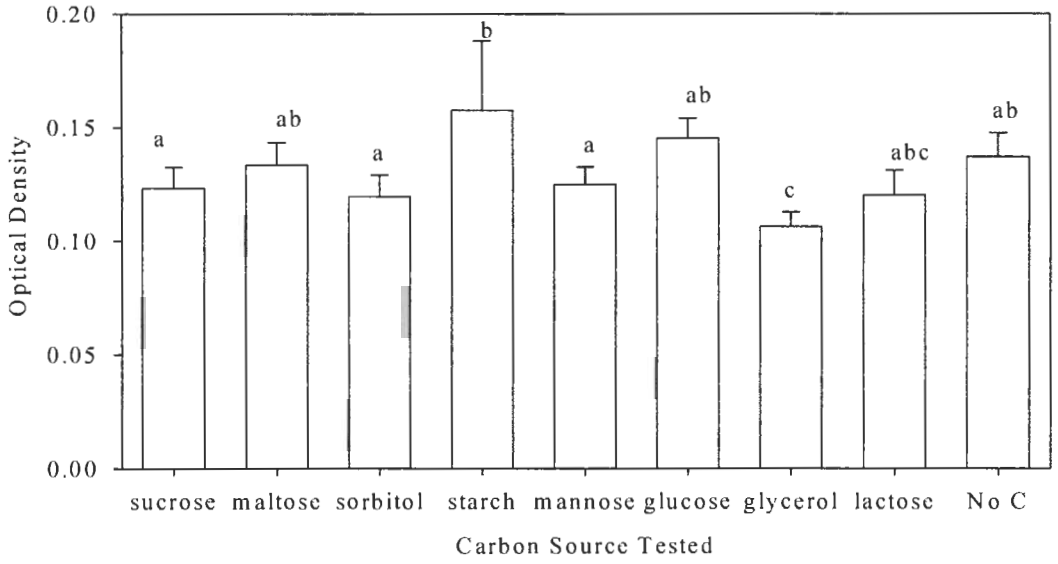

Figure 16. Growth of *B. dendrobatidis* isolate 274 on different carbon sources as measured by optical density. Cultures were grown in 30 ml of 1 % tryptone and 0.3% of a carbon source in the form of a sugar or sugar alcohol, and the optical density was measured at 495 nm. Means of four replicates and the standard error of the mean are presented. The control (No C) has no carbon source added. Treatments with different letters are significantly different ($p<0.050$). Experiment one is shown.

B. dendrobatidis grew significantly less with glycerol than with all other treatments in experiment one. Growth was equally dense on sucrose, maltose, sorbitol, mannose, glucose, lactose, and the control in experiment 1. No significant difference was detectable among the treatments in experiment 2; however, the glycerol medium had the lowest mean OD. All cultures contained live zoospores and thalli at the end of both experiments.

Carbon/ nitrogen ratio effects on growth

Growth of the chytrid varied when glucose and tryptone levels were varied. On 0.3% glucose medium, *B. dendrobatidis* grew equally dense in a range of tryptone from 0.25 to 0.75% in both experiments (Figure 17, experiment one presented). At the highest level of tryptone (3 %) the chytrid grew poorly, less than the control of glucose medium.

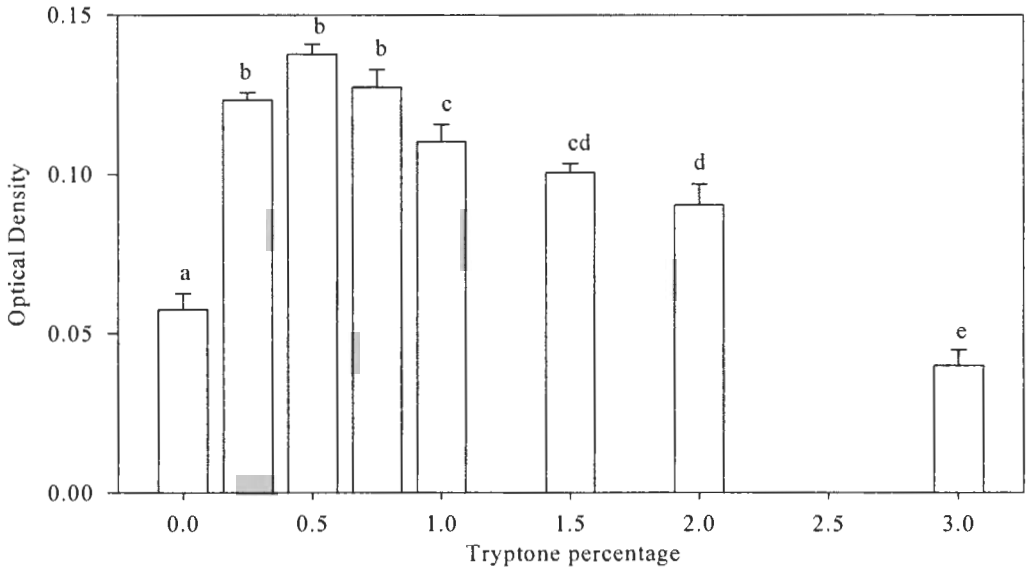

Figure 17. Growth of *B. dendrobatidis* isolate 274 in different tryptone percentages as measured by optical density. Means of four replicates and the standard error of the mean are presented. Cultures were grown in 30 ml of 0.3% glucose and varying tryptone amounts ranging from 0 to 3 %. Treatments with different letters are significantly different ($p<0.050$).

In medium with 1% tryptone and additional glucose (0% to 3.6%), *B. dendrobatidis* grew equally as dense as the control with no glucose (Figure 18, experiment one presented). The chytrid grew poorly with 3.6% glucose, significantly less than in all the other glucose media. All cultures in all experiments had live zoospores and thalli after 2 weeks of incubation.

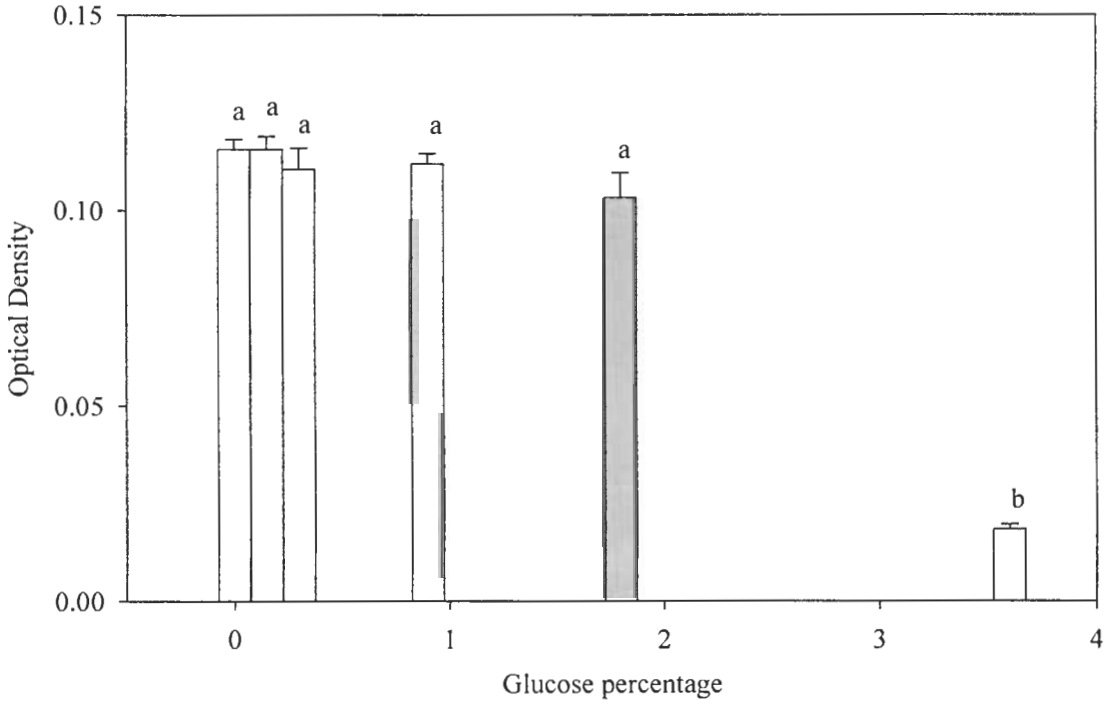

Figure 18. Growth of *Batrachochytrium* isolate 274 in different glucose percentages as measured by optical density. Means of four replicates and the standard error of the mean are presented. Cultures were grown in 30 ml of 1% tryptone and glucose amounts ranging from 0 to 3.6 %. Treatments with different letters are significantly different ($p<0.050$).

Can *B. dendrobatidis* grow on keratin media?

B. dendrobatidis grew and produced live zoospores on both 1% keratin agar and snakeskin liquid medium after 1 week of incubation. The growth on snakeskin medium

was sparse compared to growth in H-broth. Colony morphology was unique on the keratin agar; colonies were greater in diameter and flatter than those on TGhL medium.

Extracellular enzyme production by *B. dendrobatidis*

Are proteases produced by *B. dendrobatidis*?

B. dendrobatidis produces extracellular proteases. After 2 to 4 days of incubation, distinct clearings were visible around each test plug on the skim milk and gelatin assay plates (Figure 19, only skim milk plate shown). The control plugs did not produce clearings. After 2 to 4 days incubation, the skim milk medium beside the colony plug became clear and contained numerous zoospores and thalli.

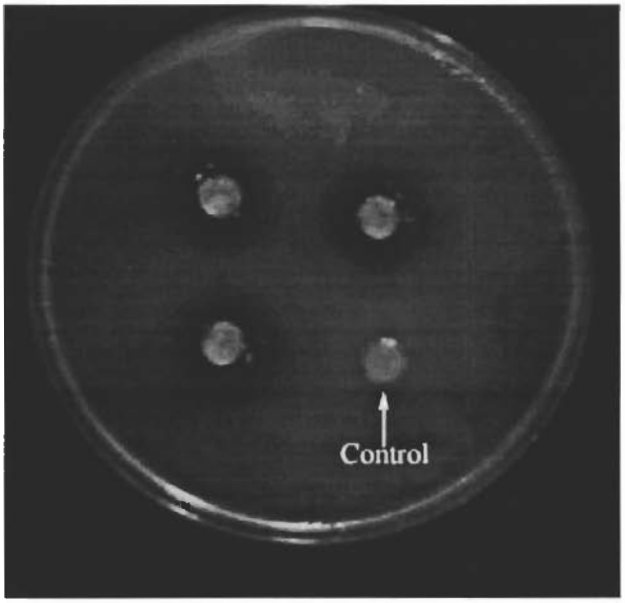

Figure 19. Clearing of skim milk agar by colonies of *B. dendrobatidis* isolate 197 indicating proteolytic activity. The arrow indicates the control, which was an uninoculated plug of agar. The 1% skim-milk agar plate was incubated for 1 week at 23 °C with the agar plugs.

Temperature range of the extracellular enzymes

The 1X culture-supernatant had low activity against azocasein, but the 10X concentrated supernatant had higher activity after 24 hours at 30 °C. The culture-supernatant degraded the most azocasein in a temperature range from 23 to 30 °C, but was active in a temperature range from 6 to 37 °C (Figure 20, experiment one presented). The absorbance of reactions with the boiled culture-supernatant was significantly less than reactions with the unboiled supernatant at all temperatures.

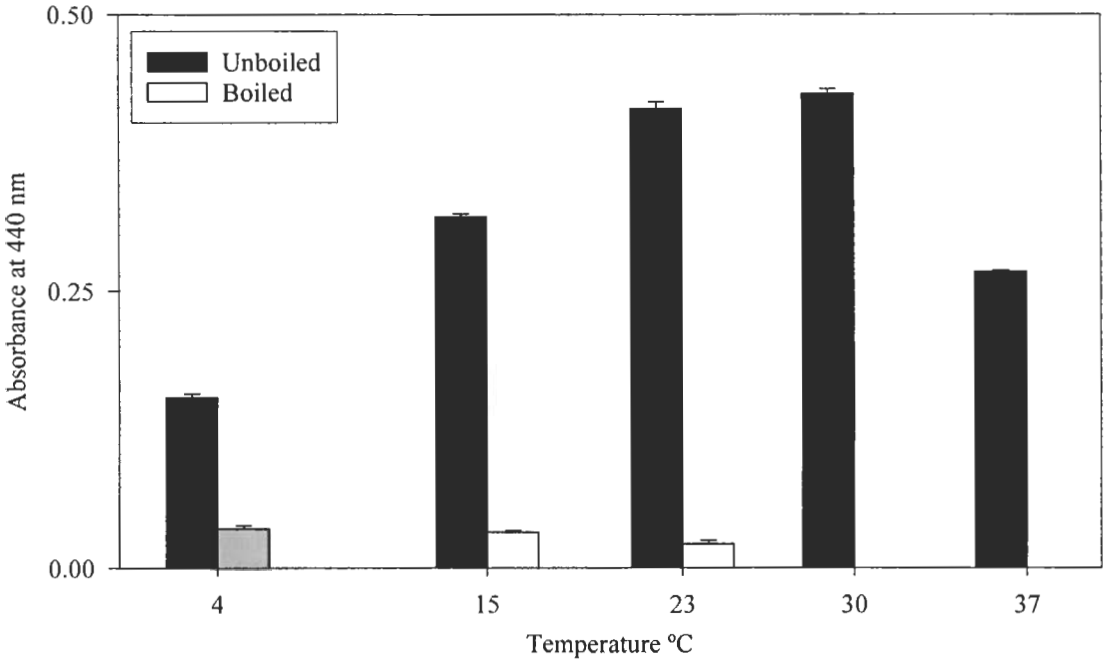

Figure 20. Degradation of azocasein at different temperatures by a 10 X concentrated culture supernatant from *Batrachochytrium dendrobatidis* (isolate 197) as measured by absorbance of dye released at 440 nm. Means of three replicates and the standard error of the mean are presented. Black bars represent reaction mixtures with culture supernatant, and gray bars represent mixtures with culture supernatant that was boiled one hour. The difference in absorbance of the reactions containing the boiled supernatant and reactions containing just water was less than zero at 30 and 37 °C.

pH range of the extracellular enzymes

The protease(s) degraded the most azocasein at pH 8, but was active at a pH range from 6 to 8 (Figure 21). The absorbance of reactions with the boiled culture-supernatant was significantly less than reactions with the unboiled supernatant at all pHs. Both experiments were statistically similar.

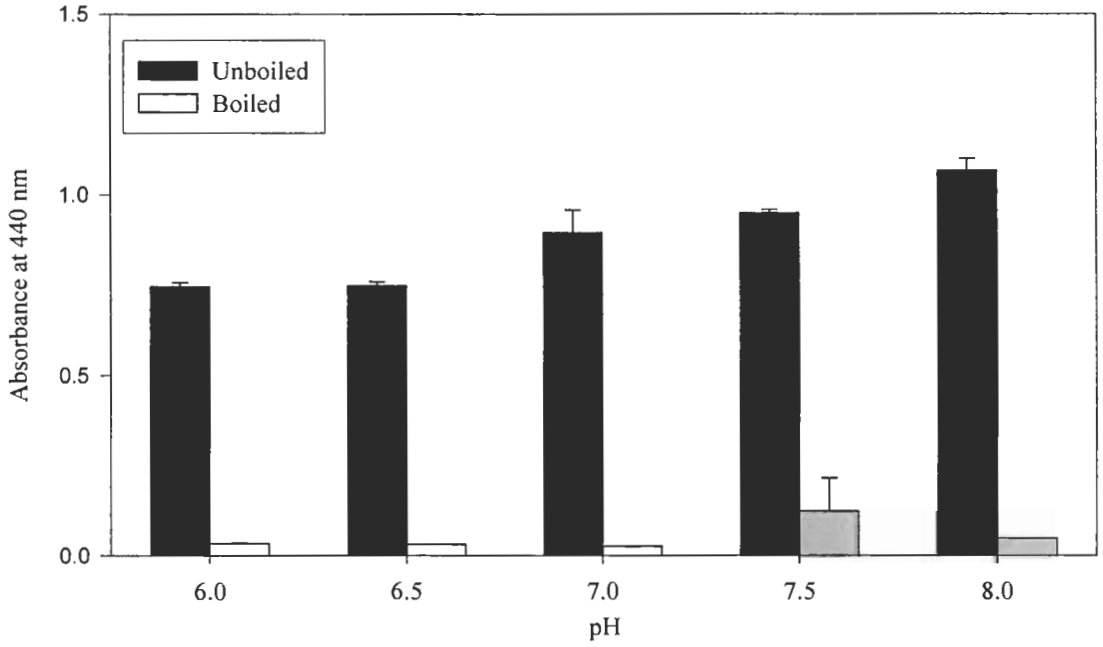

Figure 21. Degradation of azocasein over a range of pH from 6.0-8.0 by a 10 X concentrated culture supernatant from *Batrachochytrium dendrobatidis* (isolate 197) as measured by absorbance of dye released at 440 nm. Means of three replicates and the standard error of the mean are presented. Black bars represent reaction mixtures with culture supernatant, and gray bars represent mixtures with culture supernatant that was boiled for one hour.

Assays to detect the breakdown of keratin azure

The culture supernatant had no measurable activity against keratin azure after 48 hours of incubation. No increase in absorbance at 495 nm was detected in the supernatant of liquid cultures containing the keratin azure. Interestingly, many of the colonies that grew in the keratin azure medium were blue.

SDS-PAGE analysis of culture supernatant

SDS-PAGE analysis of denatured and undenatured 10 X concentrated culture supernatant yielded no visible protein bands when stained with either Coomassie blue or copper chloride. A substrate gel containing 0.5% skim milk showed two distinct layers of clearing in lanes containing the culture supernatant (Figure 22). In the substrate gel, the protein standard did not fully separate, so molecular weights of the clearings were impossible to determine.

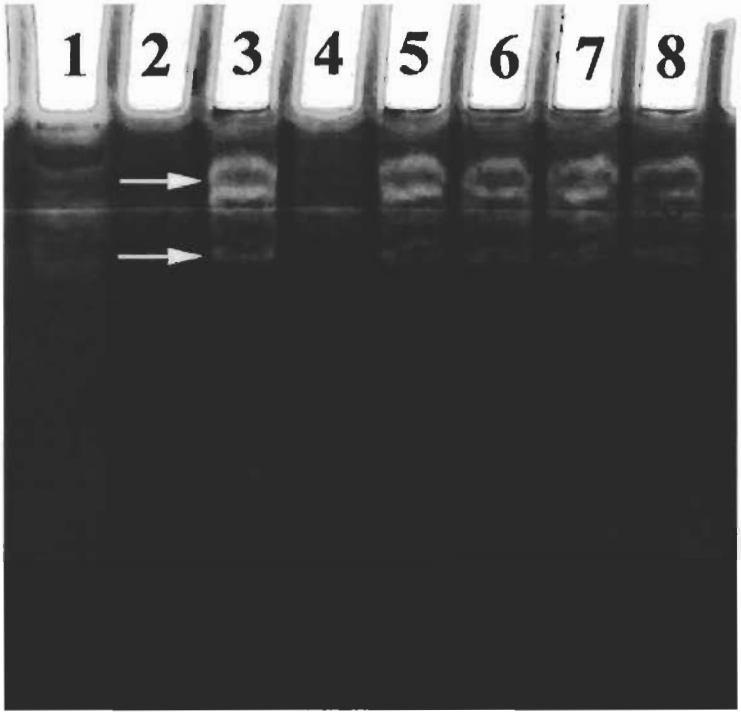

Figure 22. SDS-PAGE analysis of 10 X concentrated culture supernatant from *B. dendrobatidis* (isolate 197) on a 10 % acrylamide substrate gel containing 0.5% skim milk. Lane 1 is the protein standard, lanes 2 and 4 are blank, and lanes 3, 5, 6, 7, and 8 contain the culture supernatant. Arrows indicate clearings of the skim milk possibly by proteases in the culture supernatant.

Discussion

Nutritional requirements of *B. dendrobatidis*

Nitrogen source strongly affects growth of *B. dendrobatidis*, and they may be a result of the pH, micronutrient, carbon, and nitrogen levels of the nitrogen source. *B. dendrobatidis* grew dense on tryptone than on peptone, both of which are digests of casein protein. The two substrates are similar in amino nitrogen content, total nitrogen content, carbohydrate content, and pH (product information from: BD Franklin Lakes, NJ). The difference in growth may be a result of peptone's low thiamine content (less than 0.1 μg/g) compared to that of tryptone (0.4 μg/g), as some other chytrids need

exogenous thiamine (Barr 1969, Barr 1970, product information from: BD Franklin Lakes, NJ). The chytrid grew almost as dense on peptonized milk as on tryptone; both are digests of casein. The difference between the growths on the two media may be because peptonized milk has less than half the total nitrogen of tryptone, and also has a lower pH (product information from Oxoid Ltd. Hampshire, England).

The pH of the different nitrogen sources media may have had the greatest effect on growth. Growth on gelatin hydrolysate could have been hindered by the pH of the liquid medium (pH 5.7), which is slightly below the growth optima for *B. dendrobatidis*. The pH of the malt extract liquid-medium (pH 5.6) may have similarly slowed growth, as could the high sugar content of malt extract (60 to 63% reducing sugars) (product information from: BD Franklin Lakes, NJ,). Yeast extract also has a higher carbohydrate content (17.5%) compared to tryptone (7.7%), which could explain the poor growth in this liquid medium. Yeast extract medium is dark yellow compared to all other media, and has a higher absorbance at 495 nm than the other media tested. Additionally, chytrid colonies are lighter in color than yeast-extract liquid-media, and this could explain why I found growth in the medium but the OD was less than the blank. The poor growth on one-percent asparagine may be explained by the high level of nitrogen in asparagine, the low pH (4.5), or it may not have been a complex enough source of nitrogen. In experiment 2 the control had better growth than all sources other than tryptone and peptonized milk, this could be a result of tryptone being carried over in the inoculum, or the other substrates may inhibit the growth of the chytrid.

B. dendrobatidis may grow best on tryptone because it's the medium all isolates have been cultured on since isolation, and the chytrid has adapted to this artificial

medium. Although sparse growth was measured on media other than tryptone and peptonized milk, live zoospores were present on all media except asparagine and gelatin hydrolysate, suggesting the chytrid can grow, but not thrive, on many different nitrogen sources. The nitrogen-source preferences of *B. dendrobatidis* will be useful in developing culturing techniques for the chytrid. I suggest 0.5 % tryptone liquid medium or solid medium (1 % agar) to culture this organism.

Deducing a non-amphibian substrate from the carbon experiments was not possible because *B. dendrobatidis* grew equally well on all sugars and the control of 1 % tryptone alone in experiment two. In experiment one, the chytrid seemed to have the most growth on soluble starch; however, some starch was not dissolved, which could have increased the OD. The lowest mean OD of a treatment in both experiments was on glycerol, which may be hindering the chytrid's growth. These experiments were not conducted using a synthetic medium, thus it is impossible to determine if the chytrid is using the tested sugars; however, the experiments were useful to developing culture medium for the chytrid. The results from the carbon/ nitrogen ratio experiments suggest that *Batrachochytrium* does not require additional sugars other than those found in tryptone, and high percentages of sugar or tryptone (greater than 2%) hinder growth. Therefore I would expect any natural, non-amphibian substrate would be not plant products, but would be protein rich.

Although *B. dendrobatidis* grows well on snakeskin and keratin agar, I could not determine if it is using the keratin or producing a keratinase. Both media were autoclaved prior to inoculation, and some of the keratin could have degraded during this process. The chytrid may have been growing on the degradation products from autoclaving.

Extracellular enzymes produced by *B. dendrobatidis*

B. dendrobatidis produces extracellular enzymes that degrade casein and gelatin, but cannot degrade the type of keratin tested. Many types of keratin exist, however, and the keratin azure I tested may be more resistant to the chytrid's protease attack than the keratin in amphibian skin. Although the chytrid is found only in the keratinized cells of amphibians, it is uncertain if it actually degrades the keratin, because no experiments revealed evidence of keratin degradation by the fungus. It is possible that *B. dendrobatidis* is found in the keratinized epidermis because these cells are dead and easier to invade. Additionally, chytridiomycosis is characterized by hyperkeratinization of the *stratum corneum* (Pessier et al. 1999). If keratinases were present, one might expect a loss of epidermal keratin. Berger et al. (1999) suggests a protease produced by the chytrid might be absorbed through the amphibian skin and cause death. The proteases produced by *B. dendrobatidis,* if absorbed, might degrade living tissue and disrupt cellular function.

The enzyme production of only one isolate was studied. Preliminary experiments, however, revealed that isolates 215 and 274 produced casein degrading proteases. Different isolates may produce different levels of proteases, and the differences may make some isolates more virulent if the enzymes cause cell damage. The proteases produced by *Batrachochytrium dendrobatidis* may be non-specific, which would allow the chytrid to survive saprophytically in the environment on protein substrates as it did on snake skin and keratin in pure culture. The chytrid can survive on autoclaved snakeskin alone, and this is just one possible saprophytic substrate for the pathogen. Fish scales, amphibian egg masses, feathers are other possibilities.

The temperature and pH ranges of the enzymes are similar to the temperature and pH optima for *B. dendrobatidis* growth on defined media. These parameters of enzyme activity also may be important to disease development. The protease's temperature optimum, not just rate of growth of the chytrid may dictate infectivity and pathogenicity.

SDS-PAGE analysis revealed two distinct clearings, probably from proteases. The protein standard did not fully separate on the gel, so it is not possible to estimate the size of the enzymes. The proteases were not denatured by boiling before electrophoresis on the substrate gel, so in their native conformation they may not have migrated through the gel. The native proteases also may be multimeric, which could prevent them from completely migrating into the gel if not denatured. Additionally, because SDS-PAGE analysis suggests that at least two proteases are present in the supernatant, the activity in the pH range presented may only be that of one enzyme; other enzymes in the supernatant may have optima outside of the range tested.

B. dendrobatidis is best cultured on 0.25 to 0.5 % tryptone with no additional sugars. The chytrid may live and persist saprophytically on protein rich substrates like snake skin or fish scales in the environment. The non-specific proteases produced by *B. dendrobatidis* may aid infectivity and cause mortality in infected amphibians. The information gained by these experiments will aid the study of the ecology and the pathogenicity of this organism.

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