Characteristics of *Achlya bisexualis* Isolated from Eggs of Nile Tilapia (*Oreochromis niloticus* Linn.)

ลักษณะของ *Achlya bisexualis* ที่แยกได้จากไข่ปลานิล (*Oreochromis niloticus* Linn.)

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Abstract

Fungal infected eggs of Nile tilapia (*Oreochromis niloticus* Linn.) were collected from fish hatcheries in Kalasin, Khon Kaen and Sakon Nakhon provinces. The sample collections were conducted in three periods, June-July, October-November 2005 and March-April 2006. Effects of temperature, pH and sodium chloride (NaCl) on mycelial growth were studied. Several fungal species have been isolated and identified from the infected eggs. Among fungal isolates, a species that occurred frequently is *Achlya bisexualis*. Examination of the biological characteristics of the fungal species showed that the optimum temperature and pH for mycelial growth were 15-35 ^{o}C and 4-11, respectively. The vegetative growth of *A. bisexualis* was able to tolerate up to 15 ppt NaCl.

บทคัดย่อ

เก็บตัวอย่างไข่ปลานิล (*Oreochromis niloticus* Linn.) ที่ติดเชื้อราจากโรงเพาะฟักจังหวัดกาฬสินธุ์ ขอนแก่น และสกลนคร ระหว่างเดือนมิถุนายนถึงกรกฎาคม เดือนตุลาคมถึงพฤศจิกายน พ.ศ. 2548 และเดือนมีนาคมถึงเมษายน พ.ศ. 2549 เพื่อศึกษาผลของอุณหภูมิ พีเอช และความเค็มของโซเดียมคลอไรด์ ต่อการเจริญระยะเส้นใยของเชื้อรา ผลการศึกษาสามารถแยกและระบุเชื้อราได้หลายชนิด ชนิดที่พบมากสุดคือ *Achlya bisexualis* จากการศึกษาลักษณะทางชีววิทยาของเชื้อราพบว่าอุณหภูมิ และพีเอชที่เหมาะสมต่อการเจริญระยะเส้นใยอยู่ในช่วง 15-35 องศาเซลเซียส และ 4-11 ตามลำดับ โดยเส้นใยราสามารถเจริญบนอาหารเลี้ยงเชื้อแข็งที่มีโซเดียมคลอไรด์ได้สูงถึง 15 ส่วนในพัน

Keywords: *Achlya bisexualis*, eggs of Nile tilapia (*Oreochromis niloticus* Linn.)
คำสำคัญ: *Achlya bisexualis* ไข่ปลานิล (*Oreochromis niloticus* Linn.)

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Introduction

Nile tilapia (*Oreochromis niloticus* Linn.) is one of the most economically important species of aquaculture fish, more resistant to disease than many other species and has high yield protein potential (Wohlfarth and Hulata, 1983). They have been introduced to many countries worldwide, with a global distribution second only to common carp. The global tilapia aquaculture production is higher than 10 years ago and exceeded 1.5 million tons in the year 2002 (Silva et al., 2004). At present, tilapia culture is very popular in Thailand. In general, infectious diseases due to viruses, bacteria and fungi are still increasing in intensively cultured fish (Woo and Bruno, 1999). Aquatic fungi belonging to class Oomycetes, family Saprolegniaceae, affect fish culture, and fungal disease is one problem in brood stock husbandry. It causes low productivity of fry and low production of fish cultures. The purposes of this research were to isolate and identify fungi from infected tilapia eggs and to study some morphological and biological characteristics of the fungus.

Material and Methods

Egg specimens

All samples of fungal infected eggs were collected from hatching trays at fish farms (Kalasin, Khon Kaen and Sakon Nakhon provinces), Inland Fisheries Station (Kalasin province) and Inland Fisheries Research and Development Center (Khon Kaen province), northeast Thailand during June-July, October-November 2005 and March-April 2006.

Fungal isolation and purification

The fungal infected eggs were washed several times with sterilized tap water (STW) and inoculated onto glucose yeast extract (GY) agar (1% glucose, 0.25% yeast extract and 1.5% agar) (Hatai and Egusa, 1979). Traces of ampicillin (Sigma, Chemical Co., St. Louis, Missouri, USA) and streptomycin sulfate (Meji Seika, Kaisha Ltd., Tokyo, Japan) were added onto the medium to inhibit the growth of bacteria. The plates were incubated at 25 °C for 24-48 h for hyphal growth. The edges of the agar with growing hyphae were cut and put into GY broth (Hatai and Egusa, 1979) and then incubated at 25 °C for 24-48 h. After that, the growing hyphae were cut and washed again with STW, and incubated at 25 °C for 12-20 h for zoospore production. The zoospores ($1x10^3$ spores/mL) were inoculated onto new GY agar to get a pure single colony (Kitancharoen et al., 1995). The purified fungal isolates were maintained at 25 °C on GY agar and sub-cultured to fresh GY agar monthly.

Fungal classification and identification

For morphological observation of fungus at the asexual stage, the isolated fungi were prepared by cutting the edge of the single colony and putting into GY broth, and then incubating at 25 °C for 24-48 h. After that, the edges of young growing mycelia were cut and washed in successive baths of STW and incubated at 25 °C for 12-20 h. The discharge pattern of zoospores from zoosporangia was observed under an inverted microscope (Nikon Phase Contrast-2 ELWD 0.3, Japan) to classify the genus of the fungus (Johnson, 1956). The sexual stage of the fungus was made by preparing zoospores and maintaining them in the same condition as described above. Then, the hemp seeds were transferred to STW containing zoospores. Hemp seed cultures were done at 5, 10, 15, 20 and 25 °C, respectively and sexual organs (oogonial and

antheridial formation) were observed for 1 month. The morphological structures of reproductive organs on hemp seed cultures were identified for the species (Johnson, 1956).

Effect of temperature on mycelial growth

Temperature range and optimum temperature for mycelial growth were determined. The circular blocks of actively growing edge of the pure culture colony on GY agar were cut with a No. 2 cork borer (5.5 mm in diameter) and centrally placed onto Petri dishes (100x22 mm) containing 20 mL GY agar. Plates were incubated at different temperatures of 5, 10, 15, 20, 25, 30, 35 and 40 °C. Colony diameters were measured daily with a vernier caliper for 7 days, and the result shown as the mean of two perpendicular radii. In a case of a negative test, the tested agar plates were incubated again at 25 °C to reconfirm the survival of hyphae.

Effect of pH on mycelial growth

GY broth was adjusted to various pHs of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 by adding 1N HCl or 1N NaOH. The GY agar blocks with young mycelia were cut with a No. 2 cork borer and then put into test tubes containing 10.0 mL GY broth and incubated at 25 °C. The growth of hyphae was observed by the naked eye daily and hyphal length was measured daily by vernier caliper for 5 days.

Effect of sodium chloride (NaCl) on mycelial growth

The actively growing edge of the colony on GY agar was cut with a No.2 cork borer and put onto the center of 20 mL of the GY agar plates containing different concentrations of NaCl at 0, 5, 10, 15, 20, 25, 30, 35 and 40 parts per thousand (ppt). The plates were incubated at 25 °C. The radius of the colonial growth was measured daily with the vernier caliper for 7 days. Three replicates of each experiment were conducted.

Results

Isolation and identification

The infected tilapia eggs were covered with numerous, large white or grey threads of cotton-like mycelia (Figure 1A). Seven strains of fungi were isolated from the infected eggs from fish farms, Inland Fisheries Station and Inland Fisheries Research and Development Center, northeast Thailand. Isolates were re-marked as BKKU 0504, BKKU 0509, BKKU 0510, BKKU 0511, BKKU 0612, BKKU 0613 and BKKU 0616 and all of them were identified as *Achlya bisexualis* and belonging to the order Saprolegniales (Table 1). The isolate BKKU 0613 was selected for identification details.

The morphological characteristics of the fungus are as follows: puffy, whitish, moist colonies on GY agar which reach full plate after 5 days, at 25 °C. Rigid hyphae penetrated into the agar. Hyphae in GY broth were aseptate, stout and had sharp tips. The hyphae were 13.0-27.5 mm in diameter (n=30). At the asexual stage, typical zoosporangia were straight, filiform and occasionally fusiform in shape, 30.0-60.0 x 170.0-520.0 mm in diameter (n=30). Zoospore formation occurred about 8-12 h after mycelia were transferred into STW at 25 °C. Zoospores were elongate and discharged from the end of the discharge tube, while spore clusters usually persisted at exit pores in the manner of the achlyoid type (Figure 1B). Encysted spores were globose and 12.5-17.5 mm in diameter (n=30). At the sexual stage, oogonia were formed on hemp seed cultures after incubation at 25 °C for 3-4 days and then formed at the terminal or lateral parts of

principal vegetative thalii. The oogonia were mainly spherical or pyriform with 45.0-65.0 x 55.0-97.5 mm in diameter (n=30) and oogonial walls were conspicuously pitted under the points of attachment of antheridial cells. Oogonial stalks were stout and straight. The fungus showed a single branch or was sparingly diclinous with antheridial branches. The antheridial cells appresssed the oogonia by lateral, apical and short projections. Mature oospores were spherical and eccentric with diameter of 22.5-27.5 m (n=30). The mature oospores were not abortively filling the oogonium. There were 2-8 oospores per oogonium (n=30) (Figure 1C). Gemmae were very abundant, fusiform, clavate, spherical and single or frequently in chains (Figure 1D). From the morphological characteristics and mode of zoospore release as described above, the isolate was identified as *A. bisexualis* (Johnson, 1956).

The sizes of zoosporangia, oogonia and oospores of BKKU 0504, BKKU 0509, BKKU 0510, BKKU 0511, BKKU 0612 and BKKU 0616 were slightly bigger than that of BKKU 0613. However, all isolates were identified as *A. bisexualis*.

Effect of temperature on vegetative growth

The effect of temperature on vegetative growth of all isolates of *A. bisexualis* is shown in Figure 2. The hyphae could grow in a wide range of temperatures from 10-35 °C. The optimum temperature for vegetative growth was between 25 and 30 °C. The hyphae could not grow at 40 °C but they rarely grew at 5 °C; however, all isolates of 5 °C survived after incubation again at 25 °C.

Effect of pH on mycelial growth

The capabilities of *A. bisexualis* to grow at various pHs is described in Table 2. The isolates could grow in GY broth at a wide range of pH from 4.0-11.0 with optimum pH 7.0-9.0.

Effect of NaCl on mycelial growth

As shown in Figure 3, all isolates of *A. bisexualis* were able to tolerate up to 15 ppt NaCl but no growth of fungus was observed on GY agar containing 20-40 ppt NaCl.

Discussion

Achlya has been commonly reported in aquatic systems or on infected fish and fish eggs (Srivastava, 1980; Post, 1983; Kitancharoen et al., 1995; Kitancharoen et al., 1997; Nejadsattari, 2000; Steciow, 2001; Czeczuga et al., 2002). In the present study, *Achlya* has been isolated from infected tilapia eggs from all hatchery stations and all isolates were identified as *A. bisexualis*. The result was similar to those observed in silver carp (*Hypopthalmichthys molitrix*) and peal gourami (*Trichogaster pectoralis*) from many provinces in northeast Thailand (Yuasa et al., 2000) as well as in eggs of common carp (*Cyprinus carpio* L.) (Rakmanee, 2002).

The biological characteristics from this study showed that the range of optimum temperature for mycelial growth of *A. bisexualis* was 15-35 °C. It was similar to the range of 15-30 °C as reported by Rakmanee (2002). Olah and Farkas (1978) stated that freshwater fungi isolated from the temperate zone could grow at 25-35 °C. The hyphae also grew in a wide range of pH from 4.0-11.0 and the optimum pH for suitable growth was 7.0-9.0. This result was similar to research that reported that *Achlya* could grow over a wide range of pH 4.0-11.0 (Olah and Farkas, 1978). This result suggests that the growth of *A. bisexualis* was not strongly effected by pH in freshwater conditions.

In addition, *A. bisexualis* grows rapidly in GY agar without NaCl and is able to tolerate GY agar containing NaCl of up to 15 ppt. According to Chukanhom and Hatai (2004), the fungi could survive in high salinity freshwater and also in brackish water. In this study, the biological characteristics examination concluded that all isolates of *A. bisexualis* were broadly spread to all freshwater environments and could grow all around the year. Olah and Farkas (1978) stated that strains of *A. bisexualis* are more thermophilic fungi.

According to Post (1983), dead fish eggs are a fertile medium for growth of zoosporic fungi. The mycelial mass extends from the eggs into the water surrounding the eggs. When the fungi occurred over the dead eggs, they spread around healthy eggs, causing the infected eggs to lose oxygen to breathe and thus their death. This effect can be a cause of increasing mass mortality and decreasing production of hatching eggs, which is a primary important step for all fish cultures. However, the possible pathogenic capability of isolated fungi requires further investigation.

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Table 1. Sampling sites and *A. bisexualis* isolated from infected tilapia eggs.

Sites	Duration / Isolated species remarks		
	Jun.-Jul. 05	Oct.-Nov. 05	Mar.-Apr. 06
1. Inland Fisheries Station (Kalasin province)	-	BKKU 0509	BKKU 0612
2. Fish farm (Kalasin province)	-	BKKU 0510	BKKU 0613
3. Inland Fisheries Research and Development Center (Khon Kaen province)	BKKU 0504	-	-
4. Fish farm (Khon Kaen province)	-	BKKU 0511	-
5. Fish farm (Sakon Nakhon province)	-	-	BKKU 0616

- no fungi found

Table 2. Effect of pH on mean of the growth of *A. bisexualis* in GY broth for 3 days incubation at 25 °C.

Isolate	pH									
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
BKKU 0504	-	+	+	+	+++	++	+++	+	+	-
BKKU 0509	-	+	+	++	++	+++	++	+	+	-
BKKU 0510	-	+	+	++	+++	+++	+++	+	+	-
BKKU 0511	-	+	+	+	++	+++	++	+	+	-
BKKU 0612	-	+	+	+	+++	+++	++	+	+	-
BKKU 0613	-	+	+	++	+++	+++	++	+	+	-
BKKU 0616	-	+	+	+	+++	+++	+++	+	+	-

- no growth, + hyphal length less than 2.99 mm,
++ hyphal length between 3.00-3.45 mm, +++ hyphal length more than 3.50 mm

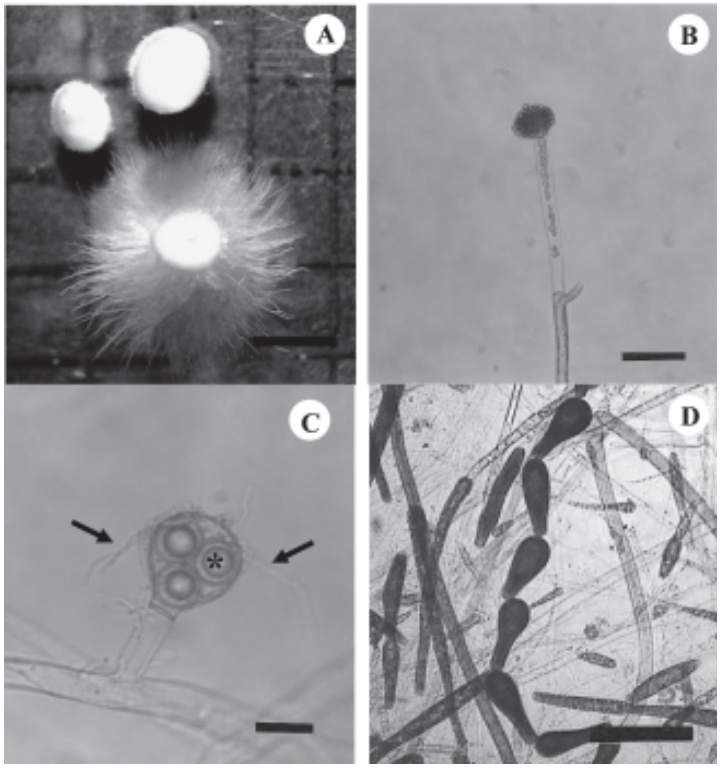

Figure 1. Morphological characteristics of *A. bisexualis* BKKU 0613.

A. Fungal infected tilapia eggs, scale bar 4 mm.

B. Zoospore discharge: achlyoid type, scale bar 20 μm.

C. Oogonia with eccentric oospores (*) and diclinous antheridial cells (⟶), scale bar 5 μm.

D. Chain of fusiform shaped gemmae, scale bar 20 μm.

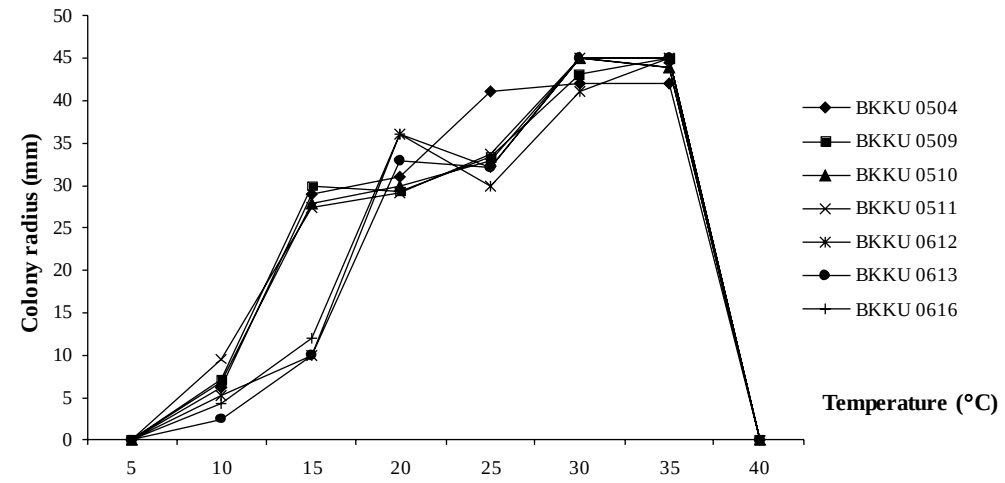

Figure 2. Effect of temperature on mycelial growth of *A. bisexualis* at 4 days after incubation.

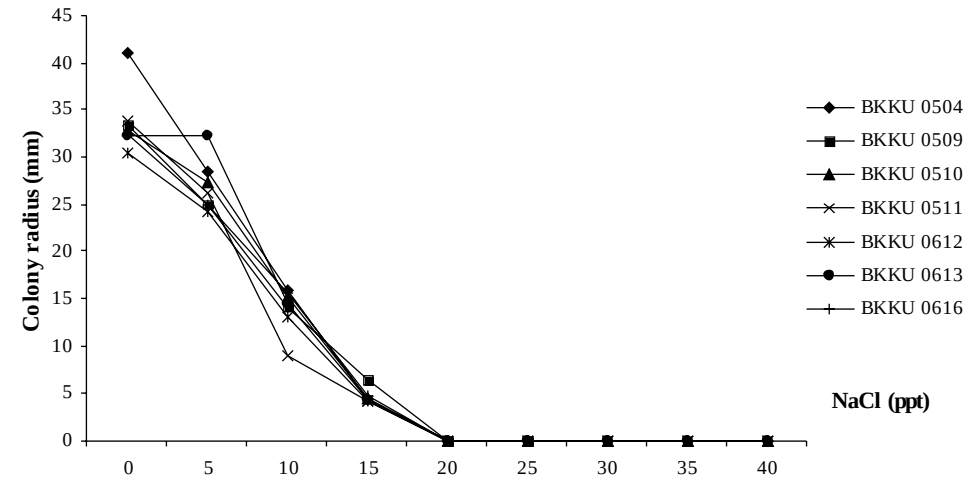

Figure 3. Effect of NaCl on mycelial growth of *A. bisexualis* at 4 days after incubation.