Lagenidium callinectes INFECTION ON ROTIFERS *Brachionus* sp.

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ABSTRACT

Milkfish, *Chanos chanos* and humpback grouper, *Cromileptes altivelis* hatcheries have developed at Gondol, Bali since 1995 and until now still rely on rotifers, the main natural food, supply. Recent problem on mass culture of rotifer, *Brachionus* sp. is harvest failure caused by fungus infection. Under light microscope, infected eggs and bodies of the rotifers was filled with numerous aseptate hyphae. Two isolates of fungi were isolated from rotifer eggs and carcass on June 21st, 2004 and on June 25th, 2004 obtained from milkfish and humpback grouper hatcheries at Gondol. Based on its morphological characteristics, the pathogenic fungus was identified as *Lagenidium callinectes* which grows optimally at 25°C and survives in 1.0%, 2.5%, and 5.0% NaCl as well as in 1.0 and 2.5% KCl. Both of the present isolates utilize only 8 out of 26 carbohydrates and derivatives tested as carbon, nutrition and energy sources. This finding is the first report on rotifer, *Brachionus* sp. infected with *L. callinectes* causing up to 100% mortality.

KEYWORDS: rotifers, milkfish, humpback grouper, *Lagenidium callinectes*

INTRODUCTION

Some fungi belonging to the order Lagenidiales were previously found to be parasites of rotifers. They were identified as *Olpidium Gregarium* Schroet, *Myzocytium Zoophthorum* Sparrow and *Lagenidium oophilum* Sparrow (Sparrow *In* Nakamura & Hatai, 1994) although the scientific names of the rotifers were not given. Karling (1981) has reported *M. microsporum* Sparrow, *L. distylae* Karling, and *L. parthenosporum* Karling as parasites of various rotifers including *Distyla* sp., *Philodina* sp., and *Heterodera* sp. Barron (1989) also found *L. oviparasiticum* Barron on eggs of *Adineta* sp. Recently, Comps *et al*. (1993) isolated *Lagenidium* like fungus from rotifer, *Brachionus plicatilis* Muller. However, Nakamura & Hatai (1994) and Nakamura & Hatai (1995a) have been succeeded to isolated fungi member of the order Lagenidiales belonging to the genus of *Atkinsiella* namely *A. parasitica* from rotifer, *B. plicatilis* Muller.

Rotifers, *Brachionus* sp. is the first food for seed production of milkfish and humpback grouper in hatcheries at Gondol, Bali. Recent rotifers mortalities occurrence is suspected due to fungus infection. This paper reports the isolation and identification of the fungus obtained from milkfish and grouper hatchery, based on its morphological and biological characteristics, including nutritional study.

MATERIALS AND METHODS

Isolation and identification. Infected rotifers, *Brachionus* sp. were examined under light microscope. Rotifers, selected from infected samples with fungus infection as indicated by the presence of hyphae, were inoculated onto peptone-yeast extract-glucose-seawater (PYGS) agar plate (composed of 1.25 g of Bacto peptone, 1.25 g of Bacto yeast extract, 3 g of glucose, 12 g of Bacto agar in 1,000 mL seawater) added with 500 mg mL^{-1} streptomycin sulphate and ampicillin to retard bacterial contamination (Hatai, 1989). The cultures were then incubated at 25°C for 3 days. A small block agar with fungus mycelium was cut out and then inoculated onto

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fresh PYGS agar to make a pure culture and used for all experiments. For morphological observation and identification, the fungus were inoculated into PYGS broth and incubated at 25C for 3 days. The small colonies in PYGS broth were transferred into sterilized seawater and incubated at 25°C to induce zoospore production. Zoospore germination was observed under microscope when were incubated in PYGS broth at 25°C. The isolates were identified according to Karling (1981), Hatai (1989), and Nakamura & Hatai (1995[b]).

Effect of temperature on vegetative growth. Range and optimum temperature for vegetative growth were examined using mycelia. Each isolate was inoculated onto PYGS agar and incubated at 25°C for 7 days to produce a giant colony. Inocula were taken from the edge of each giant colony with a no. 2 cork borer (5.5 mm diameter) and inoculated onto PYGS agar plates. Each medium was prepared with 25 mL of PYGS agar in plastic Petri dish (8.25 cm diameter). Plates were incubated at 7 different temperatures, i.e. 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C. The growth rate was observed by measuring the colony diameter after 1, 3, 5, 7, 10, and 15 days then compared with *L. callinectes* ATCC 200337 isolated from egg of swimming crab, *Portunus pelagicus* Linnaeus (Nakamura & Hatai, 1995[b]).

Mineral requirements for vegetative growth. The fungi were inoculated onto PYG agar containing various concentrations of NaCl or KCl to determine whether those minerals were required for vegetative growth. PYG agar was prepared using distilled water instead of seawater. PYG agar was mixed with NaCl or KCl at concentrations of 1.0%, 2.5%, and 5.0% (w/v). PYGS and PYG agars were used as a control media. Inoculation and measurement of the colony diameter followed method for the experiment on effect of temperature and compared with those of *L. callinectes* ATCC 200337.

Nutritional study. Samples of three-days old cultured on PYGS agar were removed with no. 2 cork borer and inoculated into 20 mL PYS agar (formula as PYGS, with the usual base replaced by yeast extract carbon base) containing 0.5% (w/v) of individual carbohydrates and derivatives. The colony diameters were measured after 1, 3, 5, 7, 10 and 15 days incubation at 25°C in comparison with *L. callinectes* ATCC 200337.

RESULTS

Incidence. In mid year 2004, fungus infection occurred in eggs and bodies of rotifers, *Brachionus* sp. reared in hatchery at Gondol-Bali, and the population could not increase. The rotifers were cultured in a concrete tank as the first food supply for milkfish and humpback grouper seed production. Under microscopic observation, aseptate stout hyphae fill rotifers eggs and bodies (Figure 1-2). Final mortality due to the infection was reaching 100%. From these

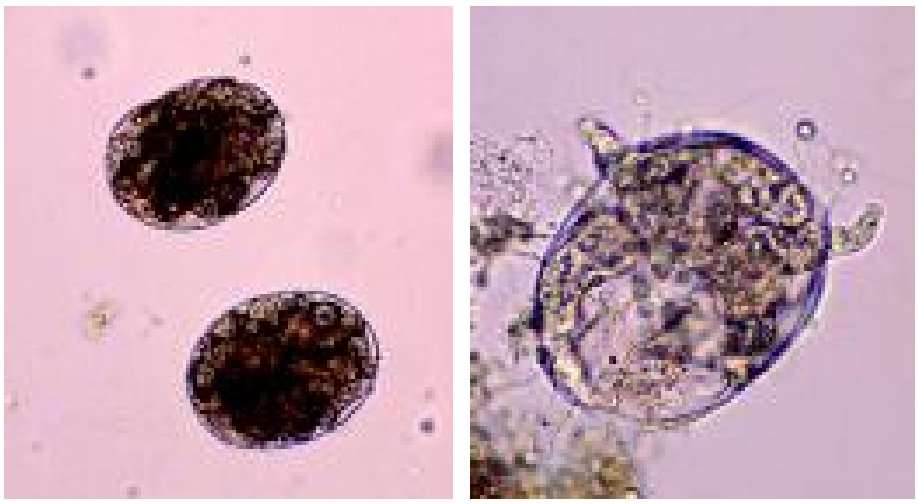

Figure 1. Natural fungal infection in rotifers, *Brachionus* sp. egg

Figure 2. Spontaneously fungal infection in body of rotifers, *Brachionus* sp.

observations the infecting fungus was identified as a species belonging to the order Lagenidiales. Fungus was isolated by inoculating 10 infected rotifers onto PYGS agar and incubated at 25°C.

Isolation and identification. The fungus which was purely isolated from infected bodies and eggs of rotifers, *Brachionus* sp. were belonging to the order Lagenidiales, and identified as a member of the genus *Lagenidium* because vesicles produced at zoospore production. The present isolate were maintained at 25°C and sub cultured onto PYGS agar at approximately monthly intervals. The morphological characteristics of the present isolates are given as follows:

Lagenidium callinectes *Couch*

Colonies on PYGS agar were whitish and reached 15—17 mm in diameter after 5 days of incubation at 25°C. The centre was damp. Hyphae were aseptate, irregularly branched, stout, with numerous shiny rod-shaped granules, 5—35 mm width (Figure 3). Zoospore formation was observed about 12 hours after the mycelia being transferred into sterilized seawater. Masses of protoplasm flowed into the tip of the discharge tubes (Figure 4), where vesicles appeared. Each protoplasmic mass was connected in a chain with a protoplasmic thread. The volume of the vesicles increased with the continuous entrance of protoplasmic masses, division into initial zoospores and

Figure 3. *Lagenidium callinectes*, coiled hyphae in PYGS broth

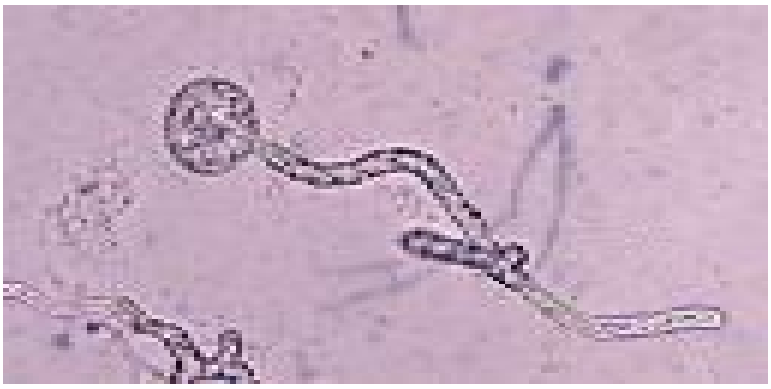

Figure 4. Vesicle formation and flow of protoplasmic masses into vesicle

active movement of the zoospores. After all of the protoplasm entered into the vesicles, flagella appeared around the protoplasm in the first 5 minutes, and individual zoospores not divided completely were recognized in 10—12 minutes, swam freely inside the vesicles in 25—30 minutes and were released in 40—60 minutes.

This morphological process was observed after the time lapses. Zoospore production was successively observed up to 7 days. Mature vesicles (Figure 5) were gelatinous, globose to subglobose, 15—100 mm diameter. The discharged tubes were 5—20 x 30—200 mm, usually broad at the orifice. The way of zoospore liberation varied; sometimes they were released simultaneously by rupture of the vesicle, sometimes singly through a hole in the vesicle wall (Figure 6). When zoospores were discharged singly, vesicles usually persisted for a few minutes. Zoospores were monoplanetic and 5—15 x 10—17 mm, 10 x 15 mm on average. Usually they swim for 20 hours, and stop swimming over 24 hours. When encysted, they were globose to subglobose without flagella, 5—12 mm diameter, 8 mm on average. Germination was observed about 5 hours after spores had encysted. Sexual reproduction was not observed.

Specimen examined BM-2004(1) isolated from egg and BH-2004(2) isolated from bodies of rotifer, *Brachionus* sp. with a fungus infection, were obtained from milkfish hatchery on 21st of June 2004 and from humpback grouper hatchery on 25th of June 2004 at Gondol, Bali.

Effect of temperature on vegetative growth. The results are displayed in Table 1. Isolates BM-2004(1) and BH-2004(2) could grow over a wide temperature ranged from 15°C to 35°C with an optimum range 25°C. The isolate *L. callinectes* ATCC 200337 as a comparison also showed the same effect. These results suggested that the organisms were adapted to tropical environment.

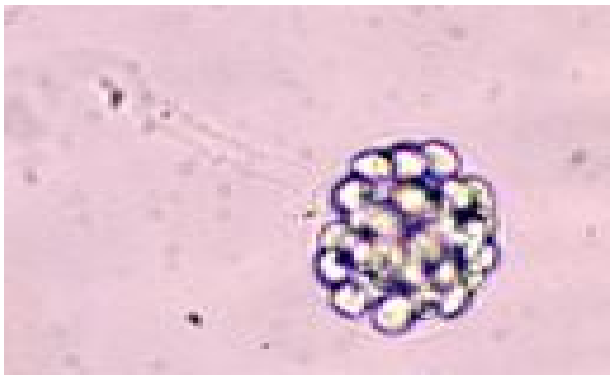

Figure 5. Mature vesicle

Figure 6. Zoospores released from a vesicle

Table 1. Effect of temperature on vegetative growth of isolate BM-2004 (1) and BH-2004(2) in comparison with those *Lagenidium callinectes* ATCC 200337

Temperature (°C)	Colony radius (mm) incubated for 15 days		
	BM-2004(1)	BH-2004(2)	*L. callinectes* ATCC200337[1)]
10	-[2)]	-	-
15	12.5	8.0	10.0
20	30.5	27.0	25.5
25	> 60.0	> 60.0	55.5
30	29.5	28.0	32.5
35	11.0	15.5	14.0
40	-	-	-

Remarks: [1)] Isolated from egg of the swimming crab, *P. pelagicus* Linnaeus (Nakamura & Hatai, 1995[b]); - [2)] = no growth

Mineral requirements for vegetative growth. The results, shown in Table 2, revealed the optimum growth on PYGS agar for all isolates. The isolate BM-2004(1) and BH-2004(2) including *L. callinectes* ATCC 200337 could grow at various concentrations of NaCl and on PYG agar without seawater but not in 5.0% KCl.

Nutritional study for vegetative growth. Carbohydrates and derivatives capable of serving as sole carbon, nutrition and energy sources for the isolate BM-2004(1) and BH-2004(2) are presented in Table 3. Both of the present isolates including *L. callinectes* ATCC 200337 were able to utilize only 8 out of 26 carbohydrates and derivatives tested such as glucose, mannose, fructose, maltose, trehalose, cellobiose, dextrin and starch. Glucose and carbohydrates composed of glucose molecules, such as maltose, starch and dextrin, provided good support as carbon and nutrition sources. However, arabinose, galactose, rhamnose, sorbose and lactose were poor. Sugar alcohols were not utilized as sole carbon and nutrition sources.

DISCUSSION

Based on the above morphological characteristics, isolate BM-2004(1) and BH-2004(2) were classified as a member of the

Table 2. Effect of NaCl or KCl on vegetative growth of isolate BM-2004(1) and BH-2004(2) in comparison with those *Lagenidium callinectes* ATCC 200337

Medium	Colony radius (mm) incubated for 15 days		
	BM-2004(1)	BH-2004(2)	*L. callinectes* ATCC 200337[1)]
PYG agar + 1.0% NaCl	22.5	25.0	20.5
PYG agar + 2.5% NaCl	41.0	50.5	49.5
PYG agar + 5.0% NaCl	19.5	17.0	20.0
PYG agar + 1.0% KCl	6.0	6.0	5.5
PYG agar + 2.5% KCl	7.0	6.5	7.5
PYG agar + 5.0% KCl	-[2)]	-	-
PYGS agar	>60.0	>60.0	55.5
PYG agar	6.0	5.5	4.5

Remarks: [1)] Isolated from egg of the swimming crab, *P. pelagicus* Linnaeus (Nakamura & Hatai, 1995[b]); - [2)] = no growth

genus *Lagenidium* (Oomycetes, Lagenidiales). The fungus was endobiotic and holocarpic and laterally biflagellate zoospores were produced in the vesicles (Hatai, 1989; Karling, 1981; Bland & Amerson *In* Nakamura *et al*., 1994; Couch *In* Nakamura & Hatai, 1995[b]).

Lagenidium callinectes was first described by Couch i*n* Nakamura & Hatai (1995[b]) on eggs of blue crab, *Callinectes sapidus* Rathbun, and later discovered from eggs and larvae of various crabs and shrimps (Crisp *et al*., 1989). *L. callinectes* was also discovered on surface of the marine algae including *Chordaria* sp. and *Ectocarpus* sp. (Fuller *et al.* in Nakamura & Hatai, 1994). Later Nakamura & Hatai (1995) isolated from infected zoea of the swimming crab, *P. pelagicus* Linnaeus. This the first report on *L. callinectes* infected of the rotifer, *Brachionus* sp. However, *Lagenidium* infection on marine crustaceans culture farms, mainly on mangrove crab has been recognized in Indonesia, although the causative agents were not determined. It was considered that *L. callinectes* was involved in some of the infections. When the morphological characteristics of the present isolate BM-2004(1) and BH-2004(2) were compared with *L. callinectes* Couch *in* Nakamura & Hatai (1995[b]) and *L. callinectes* ATCC 200337 (Nakamura & Hatai 1995[b]), no significant differences were found.

In the present isolates, zoospores were released by rupturing of the vesicles or through a small hole in each vesicle. The former seems to be the main way of zoospore liberation for this fungus. Later when zoospores moved relatively slowly in the vesicles occur, and was also observed in *L. skyline* (Bian *et al.* 1979; Hatai, 1989). The present isolate BM-2004 (1) and BH-2004(2) was close to those of *L. callinectes* and *L. scyllae* in the dimensions of hyphae, vesicles, discharged tubes and zoospore (Bian *et al*., 1979; Crisp *et al*., 1989). *L. scyllae* was similar to *L. callinectes* except for its thermo tolerant growth and the method of zoospore liberation (Bian *et al*., 1979; Hatai, 1989; Couch *In* Nakamura & Hatai, 1995[b]). Zoospores of *L. scyllae* were released one by one from the openings of the vesicles, or simultaneously by rapid delinquency of the vesicles (Bian *et al*., 1979). However, these reported both modes of liberation in *L. callinectes* isolated from eggs and bodies of the rotifer, *Brachionus* sp.

The fungi grew at temperature 15°C—35°C and optimum temperature 25°C of the isolate BM-2004(1) and BH-2004(2) also *L. callinectes* ATCC 200337 were similar to those *L. scyllae* (Nakamura & Hatai, 1995). Thermo tolerant growth was observed in *L. scyllae* (Bian *et al*., 1979), *L. thermophilum* (Nakamura *et al*., 1994), *L. callinectes* ATCC 200337 (Nakamura & Hatai, 1995[b]) and both of the present isolates.

Both of the present isolates were not obligatory marine fungi, and NaCl could be replaced by other ions such as KCl. However,

Table 3. Utilization of carbohydrates and derivates of the isolate BM-2004(1) and BH-2004 (2) in comparison with those *L. callinectes* ATCC 200337

Carbohydrates and derivatives	Utilization activity[1)]		
	BM-2004 (1)	BH-2004 (2)	*L. callinectes* ATCC 200337[2)]
Monosaccharide :			
Arabinose	-[3)]	-	-
Galactose	-	-	-
Glucose	+++[4)]	+++	+++
Mannose	++[5)]	++	++
Rhamnose	-	-	-
Sorbose	-	-	-
Xylose	-	-	-
Fructose	+[6)]	+	+
Disaccharides :			
Lactose	-	-	-
Maltose	+++	+++	+++
Trehalose	++	++	++
Cellobiose	++	++	++
Sucrose	-	-	-
Mellibiose	-	-	-
Tri-Polysaccharides :			
Dextrin	+++	+++	+++
Melezitose	-	-	-
Raffinose	-	-	-
Salicin	-	-	-
Starch	+++	+++	+++
Cellulose	-	-	-
Sugar alcohols :			
Dulcitol	-	-	-
Erythriol	-	-	-
Inositol	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
Xylitol	-	-	-

Remarks: [1)] Evaluated by colony radius after incubation at 25°C for 15 days;
[2)] Isolated from egg of the swimming crab, *Portunus pelagicus* Linnaeus (Nakamura & Hatai, 1995[b]);
[3)] Not grow;
[4)] Colony radius ³ 10.0 mm;
[5)] Colony radius 5.0 mm < 10.0 mm;
[6)] Colony radius < 5 mm

Bahnweg & Bland (1980), reported that *L. callinectes* did not require NaCl, but displayed a rather broad range of good growth from 0.1%—3.0% NaCl. The rather broad tolerances to varying salinities of the fungi parasites may be of adaptive value for life in estuarine and coastal habitats (Bahnweg & Gotelli, 1980).

The present isolate BM-2004(1) and BH-2004(2) were able to utilize only 8 out of 26 carbohydrates and derivatives tested as carbon and nutrition sources. Glucose, maltose, dextrin and starch has been known provided good support as carbon and energy for both of them, but salicin, galactose and lactose, albeit poorly. Bahnweg & Gotelli (1980) also reported *L. callinectes* could assimilate glucose as sole source of carbon, energy and supported meagre growth. It might have enzymes that can utilize glucose and carbohydrates composed of glucose molecules. The nutritional study may provide criteria to identify the fungus level in taxa.

CONCLUSIONS

One species of fungus member of the genus *Lagenidium was* isolated from infected eggs and bodies of the rotifer, *Brachionus* sp. cultured in hatcheries of milkfish and humpback grouper at Gondol, Bali on 21st and 25th June 2004. The pathogenic fungus was identified as *Lagenidium callinectes*. Optimum temperature for vegetative growth is 25°C; best growth occurs in 1%, 2.5%, and 5% NaCl and not grows in 5% KCl. However, the isolates were only capable to utilize 8 out of 26 of carbohydrates as carbon, nutrition and energy sources.

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