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Fungicidal Efficacy of Sodium Hypochlorite on a Fish-Pathogen Oomycetes, *Saprolegnia diclina* from Thailand

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Abstract

We examined the fungicidal efficacy of sodium hypochlorite (NaOCl), a food additive, on a pure strain THMK0306 of *Saprolegnia diclina* Humphrey isolated from the eggs of carp *Cyprinus carpio* in Thailand. The efficacy was evaluated by inhibitive activities on mycelial growth and zoospore germination of the fungus after NaOCl treatments at various combinations of residual chlorine concentration and exposure time. The minimum inhibitive concentration (MIC) for the mycelial growth was 20mg/L (60 min exposure), and the MIC for the zoospore germination was 2.5mg/L (5 min exposure). These results indicate that NaOCl has strong fungicidal activities against the fungus from the tropical country.

Key Words: sodium hypochlorite, fungicide, *Saprolegnia diclina*, saprolegniasis

Introduction

Aquatic fungi of the order Saprolegniales often cause serious damage to freshwater fishes if no effective prophylactic or therapeutic treatments are applied to them[1]. Malachite green is a quite effective antifungal agent, but it is teratogenic[2], residual[3] and carcinogenic[4]. The use of it was prohibited in the USA in 1991, and in Japan in 2003. Current research on alternative antifungal agents has focused mainly on three candidates, formalin[5-8], hydrogen peroxide[6-11], and sodium chloride or seawater[6-12]. These are effective, but each of them has some weak points. For example, formalin is potentially harmful to the user's health and remains in the environment. Sodium chloride, in spite of its safety, may be limited in its applicability due to the high cost of acquiring effective concentrations. In hydrogen peroxide, the undiluted solution is strongly corrosive and combustible and the effective concentration of which is as high as 1,000mg/L, has not been permitted in the USA[6].

Recently, we indicated that sodium hypochlorite (NaOCl) had strong fungicidal properties against *Saprolegnia parasitica*[13-15] and related species[16], and suggested that the agent can take the place of malachite green, because it is used widely as a food additive for sanitation, and is superior in both safety and affordability to those candidates. Under these circumstances, we obtained another species, *S.diclina*, isolated from the carp *Cyprinus carpio* which live in relatively warm water in Thailand in where saprolegniasis of the

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fish is the serious problem to be controlled preventively[17].

The purpose of this study is to determine the inhibitive activities of NaOCl on mycelial growth and zoospore germination of the *S. diclina*.

Materials and Methods

The pure strain THMK0306 of *S. diclina* Humphrey used in this study was provided by Dr. N. Areechon, Kasesart University, and Dr. N. Kitancharoen, Khon Kaen University, who isolated it from carp eggs at a hatchery in Mahasarakham Province, northeastern Thailand. In our laboratory, the fungus has been maintained on Glucose-Yeast agar (GY agar)[18] and refreshed every 2 or 3 weeks at 5℃.

The fungicidal activities were evaluated by observing the mycelial growth and zoospore germination of the fungus after treatments with NaOCl at various combinations of exposure time and residual chlorine concentration.

For the mycelial growth inhibition, the agar plugs, containing four-day old fungal mycelia were cut out with a cork borer of 5.5mm diameter from the edge of an active growing colony and exposed to 1,000mL NaOCl at 10, 20, and 30mg/L of residual chlorine concentrations for 30, 45, and 60 min. Each plug was rinsed twice with 50mL sterile tap water and placed on the center of two experimental GY agar plates, then incubated for two days at 15℃. The inhibitive activity was evaluated by comparing the colony diameter between NaOCl and its control treated with sterile distilled water. The data were subjected to one-way analysis of variance followed by Duncan's new multiple range test to identify differences among means at $p<0.05$.

For the zoospore germination inhibition, two agar pieces, 8×8mm in size were cut out from the edge of an active growing colony and incubated in a 500mL GY broth for six days at 15℃. The mass of grown mycelia was then rinsed thrice with sterile tap water and kept in 500mL sterile tap water for three days. As sporangia producing zoospores developed during this period, zoospores in suspensions were obtained by aseptic filtration through gauze. The zoospores were exposed to 1,000mL NaOCl at 2.5, 5.0 and 7.5mg/L of chlorine concentrations for 10 s, 1 min and 5 min. Here, the density of zoospores was adjusted to 2×10^5 spores/1000 mL by the Bürker Türk haemocytometer. After that, one mL from each mixture containing the treated zoospores was put into four Petri-dishes with three hemp seeds and 30mL sterile tap water, then incubated for five days at 15℃. All hemp seeds were checked for germination.

Results

Inhibtiive activity of NaOCl on mycelial growth

The experimental results are shown in Table 1. Colony diameters of the controls were around 50mm in all exposure times. The growth rate was equal to that of *S. parasitica*[13, 14] which was incubated in the same conditions. On the other hand, the diameters of mycelia exposed to NaOCl were decreased with increasing chlorine concentration and exposure time. According to the Duncan's new multiple range test, there were statistically significant differences between the control and NaOCl treatments except for the two combinations of 10 mg/L chlorine concentration with 30 and 45 min exposure ($p<0.05$). In particular, mycelia did not grow with 20mg/L chlorine concentration and 60 min exposure or with 30mg/L and 30, 45

Table 1 Colony diameters (mm)* after NaOCl treatments at various combinations of residual chlorine concentration and exposure time.

Residual chlorine concentration (mg/L)	Exposure time		
	30 min	45 min	60 min
0 (DW)	52.5 ± 1.0^{a}	53.3 ± 4.8^{a}	52.4 ± 3.0^{a}
10	50.1 ± 1.4^{ab}	46.8 ± 0.1^{abc}	41.9 ± 2.1^{bc}
20	40.6 ± 5.4^{c}	7.7 ± 10.9^{d}	0 ± 0^{d}
30	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}

*The different superscript letters of each colony diameter indicate a significant difference between treatments (Duncan's new multiple range test, $p<0.05$)

Table 2 Zoospore germination* after NaOCl treatments at various combinations of residual chlorine concentration and exposure time.

Residual chlorine concentration (mg/L)	Exposure time		
	10 sec	1 min	5 min
0 (DW)	+++ +++	+++ +++	+++ +++
	+++ +++	+++ +++	+++ +++
2.5	+++ +++	+++ − − −	+++ − − −
	− − − − − −	− − − − − −	− − − − − −
5.0	+++ − − −	− − − − − −	− − − − − −
	− − − − − −	− − − − − −	− − − − − −
7.5	− − − − − −	− − − − − −	− − − − − −
	− − − − − −	− − − − − −	− − − − − −

*Symbols (+) and (−) mean positive and negative germinations of zoospores on the hemp seeds respectively. Each symbol indicates one hemp seed, and a group of three symbols shows an Petri-dish with three hemp seeds.

and 60 min. The minimum inhibitive concentration (MIC) for mycelial growth was 20mg/L (60 min).

Inhibitive activity of NaOCl on zoospore germination

The results are shown in Table 2. Germination was observed in all hemp seeds of the controls, but not in those treated with NaOCl, except for three combinations: 2.5mg/L chlorine with 10 sec or 1 min exposure, and 5.0mg/L with 10 sec. The MIC for the zoospore germination was 2.5mg/L (5 min).

Discussion

Formerly, we investigated the fungicidal effects of NaOCl on *S. parasitica* Coker (NJM8604)[14] isolated from the cultured coho salmon *Oncorhynchus kisutch* and *Saprolegnia* sp. (MUI0112)[15] from the eggs of the chum salmon *O. keta*. We indicated that the MICs for mycelial growth was 30mg/L chlorine in both fish

(respective exposure times were 60 min and 45 min) and that for zoospore germination was 2.5mg/L (1 min). Also, we reported[13] that using NaOCl obtained from the electrolysis of a diluted sodium chloride solution, the MIC was 30mg/L (60 min) for mycelial growth and 2.5mg/L (1 min) for zoospore germination, demonstrating that the solution is no less fungicidal than malachite green. Namely, the present results with *S. diclina* accord closely with those of earlier experiments. In conclusion, NaOCl is effective not only on *S. parasitica* from cold water fish, but also on *S. diclina* from warm water fish. These results support the general validity of the fungicidal efficacy of NaOCl against Saprolegniales.

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タイ産ミズカビ病原因菌 *Saprolegnia diclina* に対する次亜塩素酸ナトリウムの殺菌効果

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マラカイトグリーンに代わるミズカビ病防除剤としての次亜塩素酸ナトリウム（NaOCl）の有効性を明らかにするために，タイにおいてコイ卵から分離したミズカビ菌 *Saprolegnia diclina*（THMK0306）に対する NaOCl の菌糸発育阻止効果と遊走子発芽阻止効果を検討した。その結果，NaOCl の菌糸最小発育阻止塩素濃度は 20 mg/L（60 分間暴露），遊走子最小発芽阻止塩素濃度は 2.5 mg／ L（5 分間暴露）であった。