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In Vitro Inhibition of Growth in *Saprolegnia* sp. Isolated from the Eggs of Persian Sturgeon *Acipenser persicus* (Pisces: Acipenseriformes) by *Pseudomonas aeroginosa* (PTCC:1430)

A. Aghaei Moghaddam*[1], A. Hajimoradloo[2], M. Ghiasi[3], R. Ghorbani[2]

1. Inland Waters Aquatics Stocks Research Center. Golestan province, Iran
2. Gorgan University of agriculture sciences and natural resources, Gorgan, Iran
3. Caspian Sea Ecology Research Institute, Sari, Mazandaran Province, Iran
**Corresponding author's E-mail: Aghaeifishery@gmail.com*

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ABSTRACT

Saprolegnia is one of the most important agents decreasing the eggs survival rate in sturgeon hatcheries. There are some chemical substances for controlling the fungal infection of eggs. In this study, an attempt was made to introduce a germ negative bacterium, *Pseudomonas aeroginosa* (PTCC1430)(Persian Type Culture Collection) as a biocontrolling agent of water mold. *Saprolegnia* was isolated from the eggs of some infected Persian sturgeon, *Acipenser persicus* in a sturgeon hatchery and then was purified. *P. aeroginosa* was cultured in Potato dextrose Agar (PDB) media and then was prepared in 5 concentrations (10^3,10^4,10^5,10^6and10^7cfu.ml^{-1}) while challenging with fungi in petri dishes under laboratory conditions. The results showed that by increasing the concentration of the bacteria in plates, hyphal growth of the fungi was reduced. The highest concentration of *P. aeroginosa* concentration (10^7) roughly stopped the -fungi growth and the Minimum Inhibitory Concentration (MIC) was 10^4cfu.m^{-1}. Results in this study implied the potential of *P. aeroginosa* (PTCC1430) as a biological agent in controlling saprolegniosis.

Key words: *Biocontrol, Persian sturgeon eggs, Pseudomonas aeroginosa, Saprolegniasis.*

INTRODUCTION

The main reducing agent of eggs in hatcheries is fungal infection (Hanjavanit *et al*., 2008) which has been reported from many fish species (Gaikowski *et al*., 1993; Jalilpur *et al*., 2005; Gaikowski *et al*., 2003; Rach *et al*., 2005 and Rasowo *et al*., 2007). Typical water mold infection caused by Oomycetes by far is the most common infections in freshwater fish, which is distributed worldwide, and the fungi are increasingly recognized as important pathogens in estuarine fishes. The Class Oomycetes is divided into four orders, three of which can infect fish (Saprolegnials, Leptomitales, and Peronosporales). A majority of fish pathogens are in the Family saprolegniaceae (Saprolegniales) (Noga, 2000). Persian sturgeon, *Acipenser persicus* belonging to the family Acipenseridae is distributed throughout the Caspian watershed and also is most common in the Caspian Sea (Bakhshalizadeh *et al*., 2011; Baradaran Noveiri *et al*., 2005; Pourkazemi et al., 2012). Natural spawning in wild environments has dramatically declined in recent years due to overfishing, environmental degradation and decrease in the brood stock migration into rivers (Barannikova *et al*., 1995).

Artificial propagation is now the main source of sturgeon resources (Barannikova *et al*., 2005). Saprolegniosis is one of the most important factors responsible for reducting the eggs survival rate in sturgeon hatcheries. There are some chemical antifungal agents such as hydrogen peroxide, formalin and malachite green that are being tried to control saprolegniosis in hatcheries.

Scherier *et al*. (1996) tried the formalin, hydrogen peroxide and sodium chloride on fungal–infected rainbow trout eggs. Forneris *et al*. (2003) tried ozone in trout hatcheries to reduce saprolegniosis incidence. Khomvilai *et al*. (2006) applied sodium hypochlorite on *S. parasitica*. Khodabandeh *et al*. (2006) tested the effects of sodium chloride, formalin and iodine on the hatching success of the eggs of common carp (*Cyprinus carpio*) .

The health of agents for human and environment, ecological impacts, and their long term effects on fish physiology, are very important points to choose them as antifungal agents.

According to the World Health Organization (WHO), much more is needed to be done in order to reduce the overuse and inappropriate use of antimicrobials and antifungals. According to Verschere *et al*. (2000), one of the most significant technologies that have evolved in response to disease control problems is the use of probiotics.

MATERIALS AND METHODS

Preparation of fungi for challenge

Fungal infected eggs were selected from the Shahid Marjani Sturgeon Propagation Center in the southern part of the Caspian Sea. Samples were put in the dishes containing sterile distilled waters with 30 drops of 5% chloramphenicol as an antibiotic (Husein *et al*. 2010).They were then transferred to the laboratory of the Caspian Sea Ecology Research Institute in southern part of Caspian Sea. Egg shells were removed and washed three times with sterile distilled water. Five infected eggs were placed in a petri dish containing 20 ml of glucose yeast agar (GYC) media and then incubated at 18°C for 5 days to produce mycelia. For purification of the fungi, edges of 5 day- colonies were cut and placed in new petri dishes containing GYC media and then incubated at 17°C for 5 days. These stages were repeated three times to obtain some more purified fungi (Ghiasi et al., 2010).

Preparation of *P. aeroginosa* (PTCC1430):

The bacteria were obtained from Laboratory of Microbiology of the Caspian Sea Ecology Research Institute, and then were cultured in PDB (Potato Dextrose Broth) media. After centrifugation (6038 g) at 4°C for 10 min, bacteria sediment was separated from the media. For confidence, the sediment was centrifuged three times. Afterwards, PBS (5°C) was added with the latest sediment and then was shaken well with a rotary shaker set. The absorbance of this liquid was read at 600 nm using a spectrophotometer (Gopalakannan & Arul, 2011).

The basic media was inoculated with bacteria at a concentration of 10^7 cfu. ml^{-1}. The next treating concentrations (10^6,10^5,10^4, and 10^3 cfu.ml^{-1}) were prepared from this main bacterial solution.

Challenge trial in Vitro

Each bacterial concentration (1ml) was cultured in petri dishes containing Sabouroud Dextrose Agar (SDA) media. All the treatments were analyzed in triplicate and incubated for 24 hrs at 17°C (Ghiasi, 2009). In order to test bacterial ability in the control of saprolegnia growth in vitro, hyphal tips in SDA petri dishes incubated in petri dishes containing bacteria while inoculation of hyphal tips in the plates without bacteria served as a control. To ensure the presence of live bacteria in the experimental treatments, a bacterial control treatment was prepared in the same concentration in a separate petri dish. The diameter of hyphal growth in both groups was measured and recorded.

Data Analysis

The experiment was performed in a completely random design to investigate the effects of five concentrations, five levels of bacterial solution (10^3,10^4,10^5,10^6 and 10^7 cfu. ml^{-1}). Data from obtained results were subjected to the analysis of variance (ANOVA). Mean comparisons were conducted by a LSD test and paired sample T-test using statistical software package of SPSS17. Drawing of diagrams and regression coefficients was prepared by Excel software (2007).

RESULTS

The result revealed that the concentration of 10^7cfu.ml^{-1} inhibited the growth of saprolegnia. Increase in the growth and diameter of colonies started in plates containing 10^6 cfu.ml^{-1} of bacterial solution

and continued to that in 10^3 cfu.ml^{-1}. On the fifth day, the colonies completely filled the control petri dishes. There was no significant difference between colony diameter on the second and fifth day in plates containing 10^3 cfu.ml^{-1} bacterial concentration (P>0.05) while significant differences were detected between colony diameter in dishes with 10^3 cfu.ml^{-1} concentration and control plates on the second and fifth days (P<0.05). Although the observations revealed increased diameter of fungi colony from the second day to the fifth day by a reduction in concentration of bacteria from 10^6 to 10^4 cfu.ml^{-1}, no significant differences were detected (P>0.05). The fungal growth increased significantly in control treatments (P<0.05). Bacteria had an inhibitory effect on growth rate of saprolegnia in concentrations applied in the present trial and this inhibitory effect was increased significantly by increasing the bacterial concentration from 10^4 to 10^7 (P<0.05) (Table 1). Noteworthy, the bacteria were grown in all bacterial control treatments.

Table1. Fungi colony diameter in bacterial and control petri dishes after 2 and 5 days.(Mean±SD).

Concentration of bacteria Cfu.ml^{-1}	Colony diameter after 2 days(cm)	Colony diameter after 5 days(cm)	P value
10^7	$0.00\pm0.00^{a}{}_{A}$	$0.00\pm0.00^{a}{}_{A}$	1
10^6	$2.03\pm0.149^{a}{}_{B}$	$2.41\pm0.243^{a}{}_{B}$	0.08
10^5	$2.94\pm0.248^{a}{}_{C}$	$3.52\pm0.379^{a}{}_{C}$	0.09
10^4	$3.44\pm0.232^{a}{}_{D}$	$4.36\pm0.431^{a}{}_{D}$	0.2
10^3	$4.73\pm0.058^{a}{}_{E}$	$7.07\pm0.58^{a}{}_{E}$	0.0
Control of fungi	$4.76\pm0.098^{a}{}_{E}$	$7.2\pm0.000^{b}{}_{E}$	0.0

Different letters (A-D) indicate significant difference in each column (P<0.05).
Different letters (a-b) indicate significant difference in each raw (P<0.05).

The relationship between the concentration of bacterial and fungal growth in plates showed that by increasing the bacterial concentration, fungal growth decreased in diameter after two (R=0.7069) and five days (R=0.8258) incubation (Fig. 2).

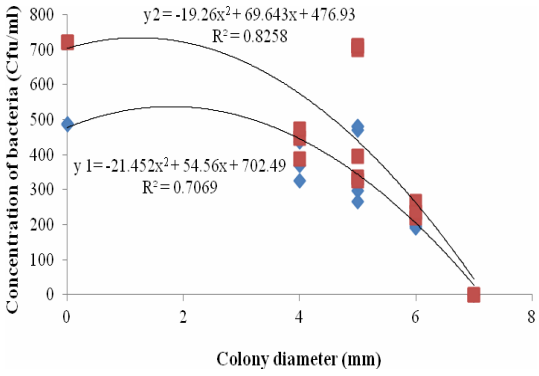

Fig1. Relationship between concentrations of bacteria and colony diameter after two (y1) and five days (y2) incubation.

DISCUSSION

In the present study, attempts were made to identify the inhibitory effect of *P. aeroginosa* on growth of *Saprolegnia* and to determine the minimum inhibitory concentration (MIC) of bacteria on pathogenic fungi of the sturgeon eggs. The presence of bacteria can reduce the growth rate of *Saprolegnia* and diameter of hyphal growth in each plate at the same time. It was revealed that 10^7cfu.ml^{-1} and 10^4 cfu.ml^{-1} concentrations had the maximum and the minimum inhibitory effects on fungal growth rate, respectively. By increasing the concentration of bacteria, the observations showed the reduction of hyphal growth diameter. In the 10^3cfu.ml^{-1} concentration, the bacteria could not affect on growth of *Saprolegnia*.

Antagonistic activity of some bacteria (in vitro) has been previously shown by many authors. Osman *et al*. (2008) controlled the saprolegniosis with non pathogenic *Aeromonas* strain (NPAS) taken from intestinal swabs of *Oreochromis niloticus* as a bath of *Aeromonas* suspension two times for three days. In this experiment, for testing the bacteria in vitro, hyphal tips obtained from a culture of *Saprolegnia*, which was grown on Sabourad's dextrose agar (SDA) at 25^{o}C, were inoculated onto the prepared (NPAS) plates. In the first part of the plate hyphal tips were inoculated onto the area containing (NPAS) while inoculation in the second half of the plate served as a control to observe the Saprolegnian hyphae growth. The top of the plate containing NPAS had no growth of the hyphae of *Saprolegnia* indicating the potential of NPAS as a biological control agent. Husein *et al*. (2011) repeated this research with *Saprolegnia* isolated from *Mugil cephalus* and reported the same results. However, no comparison was conducted on the bacterial inhibitory effects at different concentrations in these studies.

To confirm the results of antagonistic activity of bacteria in vivo, Osman *et al*. (2008) diluted the bacteria grown in Trypticase Soy Broth (TSB) in concentrations of approximately 10^6-10^8 cells/ml in ten liters of water in tanks containing natural infected *O. niloticus* with saprolegniosis. Hyphal masses were observed floating on the water column after overnight exposure to NPAS and the fish appeared to have recovered as judged by the absence of *Saprolegnia* growth although the wound remain unhealed.

Lategan *et al*. (2004) showed the inhibitory effect of *Aeromonas media* A199 (10^5 cfu.ml^{-1}) for controlling saprolegniosis in *Anguilla australis*. Eels were challenged in the presence of physiological and physical stress the same as preceding the winter outbreaks of saprolegniosis in farms. The results showed morbidity was low, 27% in A199-treated tanks, in comparison to 44% recorded for the non-treated control tanks.

Lategan *et al*. (2004) tried the *Aeromonas media* A199 on silver perch, *Bidyanus bidyanus,* for controlling *Saprolegnia* growth, and found that the daily addition of A199 to tanks during the winter outbreak of saprolegniosis significantly increased survival rate ($P<0.05$).

Hussien *et al*. (2010) tried the biocontrolling effect of *Aeromonas* sp. taken from intestinal swabs of *Mugil cephalus* in 10^6-10^8 concentrations and showed that *Aeromonas* could play a significant role in the control of *Saprolegnia*.

The general mechanism of biological control can be divided into direct and indirect effects of the biocontrol agent. Direct effects include competition for nutrients or space; it is a common mechanism for the control of fungi where the antagonist and the pathogen are closely related. Since they are closely related, both will compete for the same nutrient and site of infection (Verschere *et al*., 2000).

Production of antibiotic and lytic enzymes is one of the important mechanisms to control fungal infection. A number of highly effective disease-suppressive agents are found among the fluorescent Pseudomonads, making this group of bacteria. Indirect effects include all those aspects that produce morphological and biochemical changes in hosts (Gohel *et al*., 2006).

Minaxi and Saxena (2010) revealed that *P. aeroginosa* RM-3 produce extracellular chitinase enzymes and an important antibiotic, Phenazine and had biocontrol

potential of different phytopathogenic fungi in dual plate and liquid assays. *P. aeroginosa* produced extra cellular chitinase enzyme and an important antibiotic, phenazine that caused morphological abnormalities, perforation, fragmentation, swelling, shriveling and lysis of hyphae of pathogenic fungi (Minaxi & Saxena, 2010).

Osman *et al*. (2008) and Husein *et al*. (2010) suggested that the ability of NPAS to control saprolegniosis was related to its ability to liquefy gelatin of fungi, the direct effect of gelatin hydrolase on saprolegnia growth. NPAS is considered as gelatin positive (Holt et al., 1993).

Parenthetically the other candidate for the inhibitory activity for saprolegnia is cellulase, an enzyme produced by NPAS (Hussein and Hatai, 2001).The saprolegniacae have cellulose rather than chitin in their cell wall (Dick, 1990). However, there are some reports that discussed in vitro inhibition of saprolegnia sp. by a germ negative rod, *P. fluorescens* by Bly (1996) and Hatai (1988). They reported that inhibition of saprolegnia by bacteria was not related to the secretary substance but rather to the result of competition.

In conclusion, results of this investigation showed the potential of *P. aeroginosa* as a biological agent to control saprolegniosis. To investigate the strategy of the bacteria in order to control fungal growth, more studies are needed.

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ممانعت از رشد ساپرولگنیای جداشده از تخم تاس ماهی ایرانی (*Acipenser persicus*) بوسیله سودوموناس آئروژینوزا(*Pseudomonas aeroginosa*,PTCC:1430) در آزمایشگاه

ع.آقایی مقدم[1]، ع. حاجی مرادلو[2]، م. قیاسی[3]، ر. قربانی[2]

1-مرکز تحقیقات آبزیان آبهای داخلی گرگان، استان گلستان، ایران
2- دانشگاه علوم کشاورزی و منابع طبیعی گرگان، استان گلستان، ایران
3- پژوهشکده اکولوژی دریای خزر، ساری، استان مازندران، ایران

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چکیده

ساپرولگنیازیس یکی از عوامل مهم کاهش بقای تخم در هچری‌های ماهیان خاویاری است. مواد شیمیایی زیادی جهت کنترل قارچ زدگی تخم‌ها مورد استفاده قرارمی گیرد. در این مطالعه سعی در معرفی باکتری گرم منفی، سودوموناس آئروژینوزا بعنوان عامل کنترل زیستی ساپرولگنیا در آزمایشگاه شده است. ساپرولگنیای مورد آزمایش از تخم‌های تاس ماهی ایرانی (*Acipenser persicus*) قارچ زده در هچری جدا سازی و در آزمایشگاه خالص گردید. باکتری در محیط (PDB) پوتیتو-دکستروز-براث،رشد داده شد و سپس جهت مواجهه با قارچ در 5 غلظت (10^3-10^4-10^5-10^6-10^7cfu.ml^{-1}) آماده گردید. قارچ و باکتری در پتری دیش‌های واجد محیط کشت (SDA) درآزمایشگاه مواجهه داده شدند. نتایج نشان داد که بین غلظت های$^{-1}$cfu.ml 10^7-10^6-10^5-10^4و شاهد در رشد پرگنه قارچ تفاوت معنی داری وجود دارد($p<0.05$) وباافزایش غلظت باکتری، رشد ریسه‌های قارچ کاهش می‌یابد. بیشترین غلظت (10^7) رشد قارچ را کاملا متوقف نموده و کمترین غلظت موثر$^{-1}$cfu.ml 10^4 بود.بین قطر پرگنه قارچ در پتری دیش های شاهد و غلظت$^{-1}$cfu.ml 10^3 باکتری تفاوت معنی داری مشاهده نشد($p>0.05$). نتایج این تحقیق نشان داد که سودوموناس آئروژینوزا (PTCC:1430) می‌تواند بعنوان عامل کنترل کننده زیستی ساپرولگنیازیس مطرح شود.

* مولف مسئول