

ANTIBACTERIAL ACTIVITY OF THREE WILD WOOD-DECAYING FUNGI IN SOUTHERN VIETNAM TOWARD *VIBRIO PARAHAEMOLYTICUS* BACTERIUM IN AQUACULTURE WASTEWATER

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SUMMARY

This research demonstrated antibacterial activity of wood-decaying fungi to treat the aquaculture pathogenic bacterium, *Vibrio parahaemolyticus* (*V. parahaemolyticus*). Three wild wood-decaying fungi (WDF) collected in Southern Vietnam including *Flavodon flavus* (*F. flavus*), *Physisporinus vitreis* (*P. vitreis*), and *Schizophyllum commune* (*S. commune*) were tested to treat *V. parahaemolyticus* presented in aquaculture wastewater. Fungal adaptation to different salt concentrations at 0, 10, 15, 20, and 30‰ was tested for the assessment of application potential of these fungi in the aquaculture farming. Fungal mycelium could adapt differently to saline conditions. All three strains grew well at low salt concentration (0-10‰), but only *F. flavus* survived up to 20‰ NaCl. For the antibacterial ability toward *V. parahaemolyticus*, two experiments using mycelium culture broth and mycofiltration were performed. Results revealed that all the three species of WDF effectively inhibited *V. parahaemolyticus*. Thus, mycelium culture broth of *P. vitreis*, *S. commune*, and *F. flavus* could inhibit 98.7, 96.5 and 97.8% *V. parahaemolyticus*, respectively after 8 hours. Even higher inhibitory effects were observed in the mycofiltration experiment, the efficiency reached 100% for *P. vitreis*, and 98% for *F. flavus* and *S. commune* after 8 hours. The obtained results showed very good application potential of myco-remediation technique using WDF in the treatment of *V. parahaemolyticus* in aquaculture farming. Mycofilter can be used as a new approach for controlling aquaculture disease, specifically *Vibrio* sp. treatment based on the antibiotic capability of these WDF. The reduction in pH of the mycelium broth can illustrate for the acidification during the mycelium development and could link with the bactericide capacity of these WDF.

Keywords: *Flavodon flavus*, mycelium culture broth, mycofiltration, *Physisporinus vitreis*, *Schizophyllum commune*, *Vibrio parahaemolyticus*, wood-decaying fungi

INTRODUCTION

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a gram negative, curved rod shaped bacterium inhabiting in marine environment (Kim *et al.*, 1999). This bacterium can cause infection diseases in marine animals, and consequently in human consuming contaminated seafood. The infection dose in human is upon 10^7 - 10^8 organism ingestion (Yeung, Boor, 2004). During 2013, some strains of *V. parahaemolyticus* were reported as the etiological agent of the acute hepatopancreatic necrosis syndrome (AHPNS/ESM) that caused the collapse of the shrimp aquaculture in Asia and Mexico (Tran *et al.*, 2013). Bacteria form biofilms

protecting themselves from unfavorable factors in the surrounding environments, including antibiotics and other treatments in aquaculture farming. *V. parahaemolyticus* has high tolerance to salinity, pH, temperature and easily grip on the aquatic planktons. Only a small number of *Vibrio* strains are pathogenic to shrimps. Many of them are the opportunistic pathogens. They grow and cause disease only when the hosts are in stress and unable to fight them. *V. parahaemolyticus* are common in the estuary and freshwater environments. The pathogens causing AHPND present in many shrimp species and at various development stages of the animals. They can easily break out, spread rapidly and cause the disease which is hard to be

controlled. Due to the complex nature of the *Vibrio* varieties, controlling the pathogenic bacteria causing EMS/AHPND becomes very challenging. Commonly, antibiotics are used for the treatment of shrimp diseases, but for the case of EMS/AHPND this approach is so far ineffective.

In recent years, several techniques were used to control *V. parahaemolyticus* during shrimp farming. *Vibriosis* was controlled by rigorous water management and sanitation to prevent the entry of *vibrios* in the culturing water (Baticados *et al.*, 1990) and to reduce stress on the shrimps (Lightner *et al.*, 1993). Good site selection, pond design and pond preparation were also important (Nash *et al.*, 1992). An increase in daily water exchange and a reduction in pond biomass by partial harvesting were recommended to reduce mortalities caused by vibriosis. Draining, drying and administration of lime/dolomite to ponds following harvest was also recommended (Anderson *et al.*, 1988). Sand filtration could remove bacterial contamination, but the limitations of the method were low hydraulic loading rate and high maintenance requirements (Bright, Bulgheresi, 2010; Davies, Bavor, 2000). According to some recent studies, the inactivation effect of X-ray treatments on *V. parahaemolyticus* (Mahmoud, 2009) and the application of chitosan, chlorine for reducing pathogens in shrimps (Chaiyakosa *et al.*, 2007) were recognized for *V. parahaemolyticus* treatment. Other methods such as ozonation, anaerobic digestion, antibiotics and biofilters have been applied to disinfect aquaculture wastewater before reusing in ponds or discharging to the environment. These techniques show efficiency in reducing the COD, total nitrogen, phosphorus, suspended solid, etc. and pathogens as well (Summerfelt, 2003; Mirzoyan, *et al.*, 2010; Robertson, Philips, 1995). However, the physical-chemical approaches usually show drawbacks in terms of investment cost, operating complexity and the most important, the increasing resistance of aquaculture pathogens toward various antibiotics (Summerfelt, 2003).

In this study, a new approach of using wild WDF collected in Vietnam to control the pathogenic bacterium *V. parahaemolyticus* in aquaculture wastewater was demonstrated. The WDF are known for various extracellular enzymes, in particular, the highly effective ligninolytic enzymes (Pointing, 2001; Hatakka, 1994). These enzymes play an important role in degrading persistent organic compounds and therefore would be an effective

approach to treat organo-chemical wastes (Pointing, 2001; Bumpus, Aust, 1987). Besides, the lignin degrading enzymes and mycelium of these fungi have been reported as potential antimicrobial agents (Folman *et al.*, 2008). Thus, some WDF are capable of trapping bacteria in their mycelia (Taylor *et al.*, 2015) or inducing acidification in their environment and by the way inhibit other bacteria (de Boer *et al.*, 2010). Though the mechanistic nature of these effects are not fully understood, the antibacterial capability of WDF makes a great interest in the application of them for controlling bacterial pathogens in aquaculture, in particular the most common *V. parahaemolyticus*. Three strains of wild WDF collected in Southern Vietnam were investigated for their antibacterial activity against *V. parahaemolyticus*. Mycelium culture broth, and mycolfilter of these species were evaluated for their *V. parahaemolyticus* inhibition. These fungi were also tested for their adaptation in saline water in order to apply for further field treatment.

MATERIALS AND METHOD

Wood-decaying fungi (WDF)

Three species of wild wood-decaying fungi including *Flavodon flavus* (*F. flavus*), *Physisporinus vitreus* (*P. vitreus*), and *Schizophyllum commune* (*S. commune*) were collected and isolated from the fruiting body grew on rotting trees located in Linh Trung, Hochiminh city, Southern Vietnam (Fig. 1) and preserved at the Biotechnology Laboratory, International University – Hochiminh city, Vietnam. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were used for isolation and enrichment for WDF (Atlas, 2010). All three strains were identified by 28S rDNA amplification and sequencing. These strains were preserved on PDA plates for further studies.

Mycelium culture broth and bacteria culture

Mycelium culture broth was prepared by culturing each strain of WDF in a 100 ml PDB medium pH 7, 120 rpm shaking at room temperature (24-25°C) for 7 days. Bacterium *V. parahaemolyticus* 902 was provided by Biotechnology Laboratory, International University – Hochiminh city. The bacterium was cultivated in Tryptone Soya Broth medium at 30°C for 24 hours before experiments. The cell density after 24 hours reached 10⁹ CFU/ml.

Aquaculture wastewater

Aquaculture wastewater was collected from shrimp ponds with salinity of 13‰, pH of 7.2 as

measured by pH meter and refractometer (Research Institute for Aquaculture No. 2 (RIA2), Ho Chi Minh City).

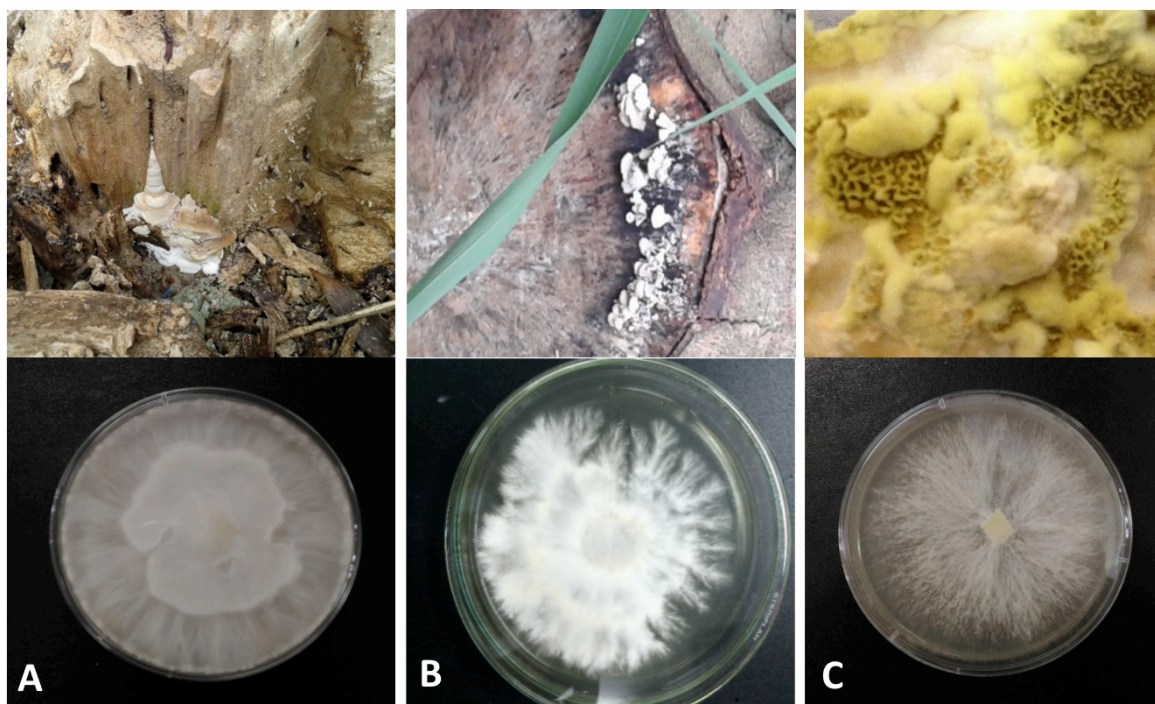


Figure 1: Pictures of fruiting body (upper images) and mycelium growth in petri disks (lower images) of three wild wood-decay fungi. A: *P. vitreus*; B: *S. commune*; C: *F. flavus*.

Salinity adaptation of WDF

Five day old WDF cultures grown in PDB medium were transferred to Erlenmeyer bottles containing fresh PDB medium with different salinities at 10, 15, 20 and 30‰ NaCl and incubated for five days. For each strain, the experiment was conducted in nine replicates. The salinity tolerance of fungal strains was evaluated based on their growth rate and the morphology of mycelia under the tested conditions.

Antibacterial activity of WDF

The ability of the WDF strains to inhibit *V. parahaemolyticus* was evaluated in two experiments as following:

Mycelium culture broth and aquaculture mixture

For each fungous strain, a 100 mL of five-day culture broth was mixed with 100 mL aquaculture

wastewater. This mixture was then inoculated with 100 μ L of 24-hour liquid culture of *V. parahaemolyticus*. Control bottle contained only 200 ml aquaculture wastewater and 100 μ L of 24-hour liquid culture of *V. parahaemolyticus*. The experiments were conducted in triplicate. Number of *V. parahaemolyticus* cells survived after the treatments was determined via colony counting on TCBS plates after 4 and 8 hours of incubation.

Mycofiltration

Bagasse was disinfected by immersing in 5% Ca(OH)₂ solution for 24 hours, washed with water and dried. Substrate bags of 200 mL volumes were prepared by mixing the dried bagasse with rice bran sterilizing at 121°C for 15 minutes. Fungal strains were cultured in the substrate bags which were kept in dark and cool place for the mycelium development. After 3-4 weeks, the fungus mycelia

fully covered the bagasse substrate and such kind of mycofilter was used in the experiments of inhibiting *V. parahaemolyticus*. To each mycofilter of 200 ml volume, 100 mL of aquaculture wastewater with 13‰ of salinity containing $\sim 3 \times 10^4$ CFU/mL of *V. parahaemolyticus* was added. The experiments were conducted in triplicate. The control experiment was prepared in a similar way, however fungous seeding was omitted.

Statistical analysis

The data was analyzed by SPSS software version 18 using one-way ANOVA and Turkey Post Test at 0.05 level significance. All results were shown as mean standard deviation.

RESULTS AND DISCUSSION

Salinity tolerance of the WDF

All three strains could grow well at low salt concentrations, ranging from 0-10‰. Above 10‰, their growth became slower and the hyphae's color turned brown instead of white as for the growth at low salt concentrations. The exception case was *F. flavus* that has survived at 20‰. No growth was observed at the salinity of 30‰ (Table 1). Thus, at the salinity of fresh and brackish water below 15‰, these WDF strains would have possibility to adapt and compete for their growth in the aquaculture environments.

Table 1. Survival ratio of WDF (out of total 9 replicates of the experiments) at different salt concentrations.

Fungal strain	Salt concentrations				
	0‰	10‰	15‰	20‰	30‰
<i>P. vitreus</i>	8/9	7/9	0	0	0
<i>S. commune</i>	8/9	9/9	7/9	0	0
<i>F. flavus</i>	9/9	9/9	8/9	6/9	0

Tolerance of fungi toward high salt concentrations has been also reported elsewhere. Arfi *et al.* (2013) showed that mangrove fungus *Pestalotiopsis* sp. grew at salt of 3‰, however its lignocellulolytic enzyme composition under high salt concentration had changed. In the same report, ten basidiomycetes strains were isolated from decayed wood, three of them produced biomass under high salt condition, while seven did not grow. The authors speculated that basidiomycetes fungi responded in different ways to saline conditions, varying from highly sensitive to moderate halophilic (Arakaki *et al.*, 2013). The most halophilic isolate was *Dacryopinax elegans* (*D. elegans*) which had an improvement of its growth in the presence of NaCl. It has also revealed that glycerol and salt led to increase the manganese peroxidase production by *D. elegans*, whereas did not influence the lignin peroxidase and laccase. In this study, *F. flavus* was found most adapted to high saline environment, it could grow at salt concentration up to 20‰, but mycelium appearance and color had changed.

Antibacterial activity of the mycelium culture broth

The result of the mycelium culture broth and aquaculture mixture experiments showed high inhibitory effects against *V. parahaemolyticus* (Fig. 2). From the initial cell density of *V. parahaemolyticus* at 2.10^4 - 3.10^4 CFU/mL, after 8 hours of incubation 85% to 96% of the bacterium was killed. Among the three strains, *P. vitreus* was the most effective, reaching an inhibitory ratio at 92.3% after first 4 hours, and up to 98.7% after the next 4 hours. The other two fungus strains showed slightly lower inhibitory effects against *V. parahaemolyticus*. After 4 hours of incubation, the *F. flavus* and *S. commune* inhibited 60.4% and 82 % of the bacterium, respectively. After 8 hours, totally 96.5% and 97.8% of *V. parahaemolyticus* was removed by *S. commune* and *F. flavus*, respectively. Besides, a light acidification in the culture medium of the three WDF strains was observed, pH value decreased from 7 reduced to 5 - 6 after 7 days growth (Table 2).

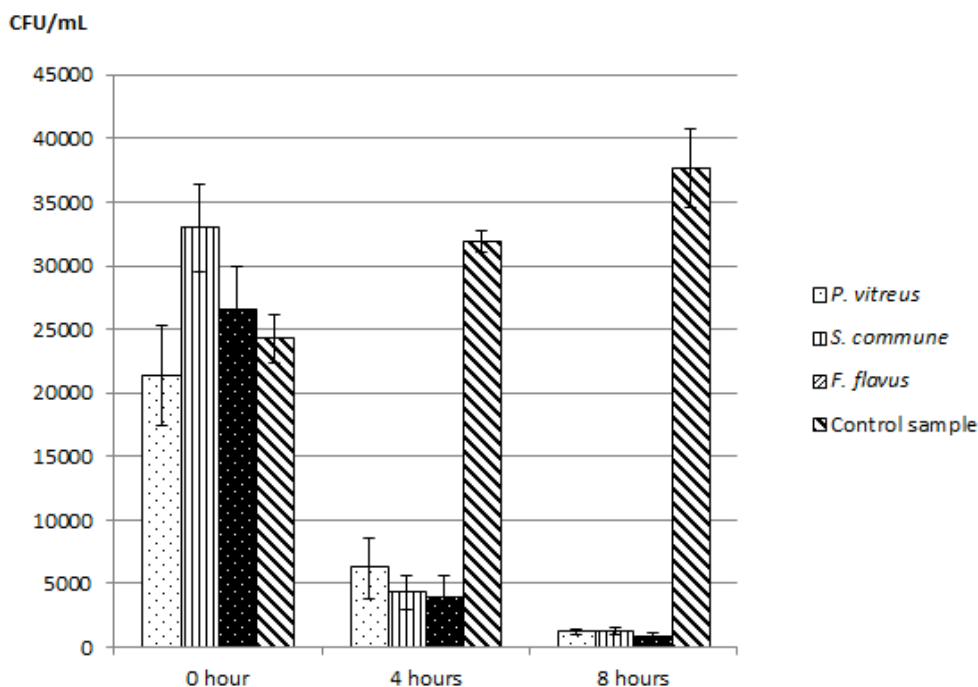


Figure 2. Removal of *V. parahaemolyticus* by mycelium culture broth. Columns represent mean \pm SD values of 3 replicates per treatment.

These results proved that mycelium culture broth of these fungi had the ability to inhibit *V. parahaemolyticus* in a certain period of time. It was proposed that the antibacterial activity against this bacterium of the fungal strains was due to metabolites produced by the fungi during their growth. Secondary metabolites produced by other fungal strains have been reported with the antimicrobial activities in several publications. Svahn *et al.*, (2012) reported gliotoxin from *Aspergillus fumigatus*, Vaz *et al.* (2009) found antimicrobial effects of metabolites from 22 endophytic fungi isolated from leaves and root of terrestrial orchids in semideciduous forest. Aqueous extracts of edible mushrooms had also showed antimicrobial activities, apparently by the polysaccharide contents. The extraction from *Cordyceps sinensis* inhibited growth of *Bacillus subtilis* and *Staphylococcus epidermidis*, whereas extraction from *Pleurotus australis* restricted the growth of *Staphylococcus epidermidis* (Ren *et al.*, 2014).

Although the mechanism of the antibacterial activity remains unknown, it can be assumed that extracellular substances and enzymes may take

responsibility in this case. Some of these substances might be induced by the presence of *V. parahaemolyticus* and turn into toxic to these bacteria as a method of competition and self-protection (Tornberg, Olsson, 2002). Similar to the pH reducing phenomenon in fungal cultures noticed in this study, the research on white rot fungus *Hypholoma fasciculare* (*H. fasciculare*) by De Boer *et al.* (2010) proved that antibacterial effect of the fungal cultures might link to the rapid acidification during the fungal growth.

Table 2. pH value of 7-day mycelium culture broth of WDF with initial pH 7.

White rot fungi	pH value
<i>P. vitreus</i>	6.12 \pm 0.01
<i>S. commune</i>	5.39 \pm 0.17
<i>F. flavus</i>	4.97 \pm 0.38

Antibacterial activity of the mycofilter

The mycofilters made from the fungal mycelia grew on solid substrates such as bagasse in this study

had dense but permeable networks of mycelia allowing wastewater flow through and good contact between the mycelia and *V. parahaemolyticus* cells. Of the three fungi, *P. vitreus* mycofilter demonstrated the most effective, reaching 98% bacterial removal after 4 hours of treatment (Fig. 3). After 8 hour treatment, the removal efficiency of mycofilters of all three fungal strains was as high as 98 - 99%.

The capability of the mycofilters to remove bacterial cells might due to the network of mycelia functioning as traps for the bacterial cells when the

wastewater flow through. Taylor *et al.* (2015) showed that mycofilters containing mycelia from *Stropharia rugosoannulata* could remove > 90% of *Escherichia coli* cells in the wastewater effluent. Another mechanism of the bacterial removal by the mycofilters might due to the antimicrobial activities of metabolites released by fungal mycelia. In practice, both of these mechanisms could happen at the same time. This hypothesis explained why the efficiency of the treatment using mycofilters was often higher than that of mycelium cultures as shown above.

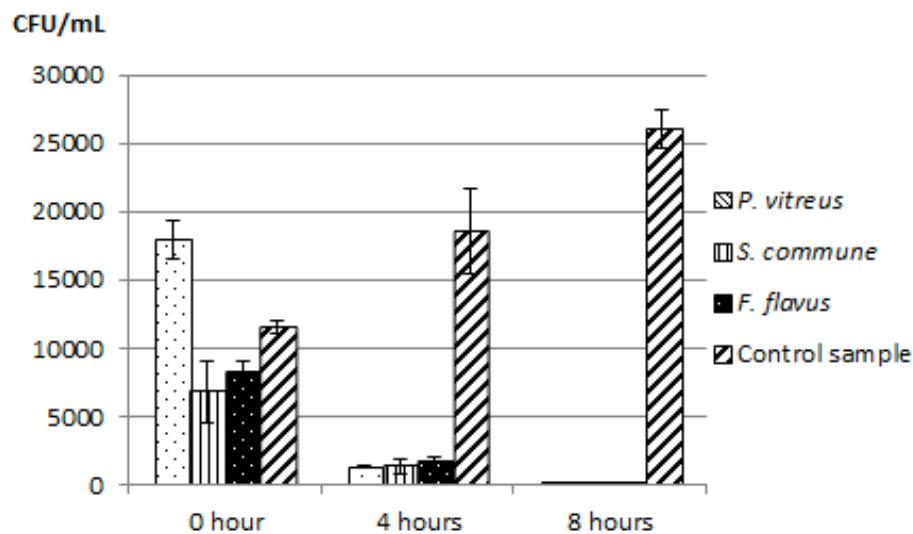


Figure 3. Removal of *V. parahaemolyticus* by mycofilters. Columns represent mean \pm SD values of 3 replicates per treatment.

CONCLUSION

Two experiments of *V. parahaemolyticus* treatment using three different fungus strains showed extremely high efficiency, up to 98.7% of bacteria removed for mycelium culture broth and 99.8% for mycelium mycofilter treatment. It is shown that mycofilter can be used as a new approach for controlling aquaculture disease, specifically *Vibrio* sp. treatment based on the antibiotic capability of these WDF. The reduction in pH of the mycelium broth can be illustrated for the acidification during the mycelium development and could link with the bactericide capacity of these WDF. However, the mechanism still needs further to be explored.

Conflicts of interest: The authors declare no conflict of interest.

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HOẠT TÍNH KHÁNG KHUẨN CỦA BA CHỦNG NẤM MỤC GỖ PHÂN LẬP TẠI MIỀN NAM VIỆT NAM ĐỐI VỚI VI KHUẨN *VIBRIO PARAHAEMOLYTICUS* TRONG NƯỚC THẢI THỦY SẢN

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TÓM TẮT

Nghiên cứu hướng đến mục tiêu ứng dụng khả năng kháng khuẩn của các chủng nấm mục gỗ để xử lý vi khuẩn gây bệnh trong thủy sản *Vibrio parahaemolyticus* (*V. parahaemolyticus*). Ba chủng nấm mục gỗ (WDF) được thu thập ở phía nam Việt Nam, bao gồm *Flavodon flavus* (*F. flavus*), *Physisporinus vitreus* (*P. vitreus*) và *Schizophyllum commune* (*S. commune*) được sử dụng để xử lý *V. parahaemolyticus* trong nước thải thủy sản. Tính chịu mặn của các chủng nấm được đánh giá ở các độ mặn khác nhau là 0, 10, 15, 20, and 30‰, qua đó phản ánh khả năng ứng dụng các chủng này trong xử lý môi trường của ngành nuôi trồng thủy hải sản. Kết quả nghiên cứu cho thấy các chủng nấm có khả năng chịu mặn ở nồng độ muối thấp (0-10‰), trong đó, *F. flavus* có thể chịu được độ mặn lên đến 20‰ NaCl. Nghiên cứu này đồng thời đánh giá khả năng kháng khuẩn *V. parahaemolyticus* của các chủng nấm thông qua môi trường nuôi nấm ở dạng lỏng và dạng hệ lọc bằng hệ sợi tơ nấm trên cơ chất bã mía. Kết quả cho thấy cả ba chủng nấm này đều ức chế được *V. parahaemolyticus*. Cụ thể, môi trường nuôi dạng lỏng của *P. vitreus*, *S. commune* và *F. flavus* có thể khử 98,7, 96,5 và 97,8% *V. parahaemolyticus*, tuần tự, sau 8 giờ xử lý. Đối với hệ lọc trên cơ chất bã mía, hiệu quả xử lý khuẩn của các chủng nấm này cao hơn nhiều, đạt 100% đối với *P. vitreus* và 98% với *F. flavus* và *S. commune* sau 8 giờ xử lý. Các kết quả này cho thấy tiềm năng ứng dụng rất tốt của kỹ thuật xử lý môi trường bằng hệ lọc từ hệ sợi tơ nấm trên cơ chất rắn của các chủng WDF trong việc xử lý *V. parahaemolyticus* trong ngành thủy sản.

Từ khóa: *Flavodon flavus*, môi trường nuôi lỏng, hệ lọc trên cơ chất, *Physisporinus vitreus*, *Schizophyllum commune*, *Vibrio parahaemolyticus*, nấm mục gỗ