Fishery Technology 62 (2025) : 153 - 156

Research Note

High-Throughput Approach for Evaluating Antibacterial Activity of Fish Epidermal Mucus

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Abstract

Fish epidermal mucus (FEM) is a source of antimicrobial peptides (AMP) that play an important role in the innate immunity of fish. Currently, there are no simple and quick protocols for screening FEM for antimicrobial activity. The present work describes the optimization of a modified optical density reduction (MODR) method to screen the FEM for antibacterial activity against various bacteria within 8 h. Reduction in optical density values in the bacteria compared with positive control suggest FEM sample exhibits antibacterial activity. The MODR approach is a low-cost, rapid and solvent-free method, and can be used to screen the FEM for potential antibacterial activity.

Keywords: Fish mucus, fish epidermal mucus, antimicrobial activity, antimicrobial peptide

Introduction

Fish epidermal mucus (FEM) is a slimy discharge composed of mucins, inorganic salts, immunoglobulin, proteins, and lipids; which act as a physical barrier and protects the fish from abrasion, toxicity, harmful chemicals, and toxins. The continual secretion and replacement of fish skin mucus helps to avoid the colonisation of potentially pathogenic bacteria. It also protects the fish from predators and parasites (Reverter, Tapissier-Bontemps, Lecchini, Banaigs, & Sasal, 2018). FEM acts as an interface between fish and its surroundings and performs essential physical, ecological and communication roles that are crucial for fish shoaling, synchronised spawning, probing warning signals and habitat marking (Davies, Duffy, Bennie, & Gaston, 2014). FEM contains immune-related proteins such as lysozymes, phosphatases, esterases, proteolytic enzymes, complement factors, lectins, immunoglobulins, and C-reactive proteins that eradicate pathogens and initiate the immunological cascade during an infection and continually encounter, monitor, and manage the pathogens in the aquatic environment infection (Reverter et al., 2018). FEM of most fishes consists of bioactive low-molecular-weight compounds called antimicrobial peptides (AMP) (Silva, Silva, & Ribeiro, 2018). Recent research on the antimicrobial capabilities of fish skin mucus against human and fish infections indicates potential future therapeutic use in both human and animal illnesses (Rakers et al., 2013). AMPs are also called as host defence peptides and are responsible for the innate defence mechanism against invading bacteria/viruses (Radek & Gallo, 2007). AMPs comprise of short chain of amino acids ranging between 12 and 15 amino acids (Chen et al., 2013). Being short-chain, AMPs are generally thermostable. Pleurocidin from winter flounder, cathelicidins from rainbow trout, defensins from zebrafish, piscidins from hybrid striped bass, dicentracin from sea bass, hepcidin from channel catfish and epinician from the groupers are some of the extensively studied AMPs (Dash, Das, Samal, & Thatoi, 2018). AMP extraction from natural sources is often associated with the process of drug development (Saucedo-Vázquez et al., 2022). The screening of AMPs from natural sources is important for the new drug discovery

Received 14 June 2024; Revised 22 November 2024; Accepted 11 December 2024

Handling Editor: Dr. B. Madhusudana Rao

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process. However, isolation of AMPs from the FEM is a complex and time-consuming procedure and requires more volume of mucus for the screening. To circumvent this problem, a new approach was developed that is rapid and requires less volume to analyze large number of samples. In the present experiment, the MODR method has been employed to assess the antibacterial activity of FEM against various pathogens in a single test. It is a modified version of the growth curve analysis method.

Materials and Methods

Live fish species i.e., *Pangasianodon hypophthalmus* (Sauvage, 1878) were transported to the laboratory and euthanized by the application of ice. After 10 min, the fish mucus was carefully removed from the fish specimen using a Bard Parker blade and then transferred to a 15 mL glass test tube. The accumulated FEM was quantified by measuring its weight. Subsequently, the FEM was diluted with normal saline in a ratio of 1:3. The contents were thoroughly mixed for 2 min using a vortex. Subsequently, the supernatant was filter sterilized and stored in the pre-sterilized cryovials and preserved at a temperature of -80^{o}C until the commencement of analysis. The study utilised five bacterial species, namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae* and *Salmonella.*

A sterile 96 well-micro titre plate, generally used for tissue culture purposes (Visnuvinayagam, Thangavel, Lalitha, Malmarugan, & Sukumar, 2015), is used for the antibacterial assay method *i.e.* MODR. Initially, 100 µL of the filtered FEM was added in the first three wells of the 96 well micro titre plate (3-wells, i.e., in triplicates). For positive and negative control, 100 µL of normal saline was added instead of mucus. Then 100 µL of 2X concentration of cation adjusted Mueller Hinton Broth (MHB) was added to all the wells. Then 20 µL of bacterial culture consisting of 5×10^5 cfu/mL was added to all the wells except negative control. In the negative control, 20 µL of normal saline was added separately. Then, the OD value was observed immediately after adding the culture at 600 nm using a multimode microplate reader (Synergy HI, BioTek, Agilent, USA). The OD_{600} values were recorded at every 2h intervals up to 12h and analysed for interpretation.

Results and Discussion

In the recent decade, research on fish epidermal mucus (FEM) has been growing owing to the identification of bioactive compounds and their potential therapeutic use in human medicine and aquaculture. In addition, FEM can be utilised to identify diseases and monitor environmental contaminants without harming the fish. FEM content varies with the fish species, sex, developmental stage, diseases, distress, water temperature and pH. Significantly, the infection and stress condition of the fish, changes the mucous composition and production. So, isolating mucus with potential antimicrobial activity is highly important for any practical application. In this connection, as per the conventional procedure, separating bioactive molecules and checking antimicrobial activity is costly and time-consuming, which may not be feasible for practical application. A high throughput method is needed to quickly evaluate the FEM for antimicrobial activity. Accordingly, a modified optical density reduction method (MODR) has been optimised to screen the FEM for antibacterial activity against various pathogens.

In the present study, the antibacterial activity was measured by the OD method of analysis. If any test material possesses antibacterial activity, it will not allow the bacteria to grow; hence, the OD value of the bacteria will be in the static condition. Based on this thumb rule, the duration of the OD value retained at the normal level is considered as an antibacterial effective period. In this concept, the FEM was added in the 96 well micro titre plates in triplicate and the OD_{600} values were recorded at

Table 1. Reduced OD_{600} of fish epidermal mucus against *S. aureus*

	Positive Control	Negative Control	FEM
0 h	0.12±0.01^{A}	0.10±0.002^{A}	0.13±0.0035^{A}
2 h	0.19±0.003^{A}	0.12±0.012^{B}	0.15±0.0056^{B}
4 h	0.29±0.007^{A}	0.15±0.007^{B}	0.15±0.0063^{B}
6 h	0.32±0.0035^{A}	0.14±0.0035^{B}	0.15±0.0205^{B}
8 h	0.33±0.0028^{A}	0.14±0.0190^{B}	0.19±0.0077^{B}
10 h	0.35±0.0007^{A}	0.15±0.0431^{B}	0.38±0.1322^{A}

**Means with different superscripts indicate significant difference at 5% level (P<0.05)*

every 2h interval. In the analysis, it has been observed that only in *S. aureus* inoculated wells there was a reduction in the OD_{600} value of the mucus sample up to 8h (Table 1). From the 10^{th} h onwards, the OD_{600} values started increasing. At the same time, positive control wells showed a continuous increase in the OD_{600} value at each 2h observation. In the case of negative control wells, there was no increase in the OD_{600} value and the values were stable value up to 12h. The OD_{600} value of the other pathogens showed continuous increase and the OD_{600} value (Fig. 1) in these wells were higher than the positive control. It may be because the mucus material may be utilised for bacterial growth.

Antimicrobial function of mucus is well-known, but the separation of antimicrobial compounds from mucus is relatively new and the separation of the bioactive molecules from mucus is much more complicated (Ebran, Julien, Orange, Auperin, & Molle, 2000). So, isolating mucus with potential antimicrobial activity is highly important in any practical application. In this connection, as per the conventional procedure, separating bioactive molecules and checking its antimicrobial activity is costly and time-consuming, which may not be feasible for practical application. A high throughput method is needed to evaluate the antimicrobial activity of the FEM to screen the various pathogens quickly. A 96-micro well micro titre method was recently exploited to screen greater number of samples in a single plate (Visnuvinayagam et al., 2020).

The antibacterial activity was analysed using the optimized optical density reduction method. Here, the control (untreated) growth curve was compared with the treatment to assess the antimicrobial activity. Owing to the antibacterial activity of the treatment, the inhibition of the growth of the bacteria can be compared with the control (without treatment material and with bacterial culture). Here the main advantage is that a large number of samples with various bacteria can be analysed quickly.

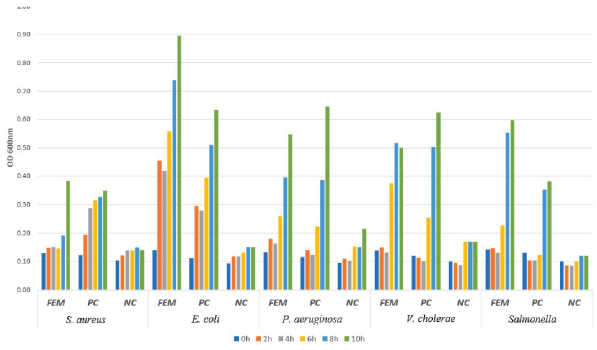

Fig. 1. The OD_{600} value of the fish epidermal mucus against various pathogens

OD_{600} value for the mucus sample with *S. aureus* is lower than its control. In the case of other pathogens, the mucus sample had a higher value than the positive control.

Based on the MODR approach 15 samples can be tested with a single bacterium i.e., each sample needs three wells (triplicate) for a sample; similarly, three wells for the positive control. Overall, three wells can be used for the negative control.

Fish have co-evolved under selective pressure by developing a complex network of defence mechanisms, such as the adaptive immune system, especially through mucosal barriers responsible for fending off pathogens on first contact by production of AMPs. That is because there is a greater degree of organization in the distribution of leukocytes in fish, for instance, in the mucosal tissues of the stomach, gills, and skin, as opposed to the liver or gonads (Patrulea, Borchard, & Jordan, 2020). The relevance of the mucosal layer and epithelial scaffold in immunology is shown by the fact that disruption enhances infection incidence and severity (Liu et al., 2015). Hence, while screening the fish from different ponds, if any FEM has higher AMPs, then there would be a possibility of infection.

The modified optical density reduction method (MODR) is a high throughput method that can be used to screen the fish mucus sample against various pathogens and may also find application for predicting the infection in fish. Since the antimicrobial activity of the mucus varies based on the stress and infection, a suitable fish or pond may be checked for bioactive compounds before proceeding to the bulk collection of mucus, which will be a more practical approach for the high-yield component. However, the low-cost, hassle-free method needs a multi-well micro titre plate reader.

Acknowledgments

The authors wish to thank The Director, ICAR-CIFT for the support and facility to carry out the present study. Technical Assistance provided by Smt. M. Rekha, Asst. Chief Technical Officer and Smt. Reshmi K. Technical

Assistant, MFB Division of ICAR-CIFT are also greatly acknowledged.

References

Chen, Y., Zhao, H., Zhang, X., Luo, H., Xue, X., Li, Z., & Yao, B. (2013). Identification, expression and bioactivity of *Paramisgurnus dabryanus* â-defensin that might be involved in immune defense against bacterial infection. *Fish & Shellfish Immunology*, *35*(2), 399-406. https://doi.org/10.1016/j.fsi.2013.04.049.

Dash, S., Das, S. K., Samal, J., & Thatoi, H. N. (2018). Epidermal mucus, a major determinant in fish health: a review. *Iranian Journal of Veterinary Research*, *19*(2), 72-81.

Davies, T. W., Duffy, J. P., Bennie, J., & Gaston, K. J. (2014). The nature, extent, and ecological implications of marine light pollution. *Frontiers in Ecology and the Environment*, *12*(6), 347-355. https://doi.org/10.1890/130281.

Ebran, N., Julien, S., Orange, N., Auperin, B., & Molle, G. (2000). Isolation and characterization of novel glycoproteins from fish epidermal mucus: correlation between their pore-forming properties and their antibacterial activities. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1467*(2), 271-280. https://doi.org/10.1016/S0005-2736(00)00225-X.

Liu, X., Wu, H., Liu, Q., Wang, Q., Xiao, J., & Zhang, Y. (2015). Skin injured Zebrafish, Danio rerio, are more Susceptible to Vibrio anguillarum Infection. *Journal of the World Aquaculture Society*, *46*(3), 301-310. https://doi.org/10.1111/jwas.12188.

Saucedo-Vázquez, J. P., Gushque, F., Vispo, N. S., Rodriguez, J., Gudiño-Gomezjurado, M. E., Albericio, F., Tellkamp, M. P., & Alexis, F. (2022). Marine arthropods as a source of antimicrobial peptides. *Marine Drugs*, *20*(8), 501. https://doi.org/10.3390/md20080501.

Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. *Frontiers in Microbiology*, *9*, 594. https://doi.org/10.3389/fmicb.2018.00594.

Patrulea, V., Borchard, G., & Jordan, O. (2020). An update on antimicrobial peptides (AMPs) and their delivery strategies for wound infections. *Pharmaceutics*, *12*(9), Article 840. https://doi.org/10.3390/pharmaceutics12090840.

Radek, K., & Gallo, R. (2007). Antimicrobial peptides: natural effectors of the innate immune system. *Seminars in Immunopathology*, *29*, 27-43. https://doi.org/10.1007/s00281-007-0064-5.

Rakers, S., Niklasson, L., Steinhagen, D., Kruse, C., Schauber, J., Sundell, K., & Paus, R. (2013). Antimicrobial peptides (AMPs) from fish epidermis: perspectives for investigative dermatology. *Journal of Investigative Dermatology*, *133*(5), 1140-1149. https://doi.org/10.1038/jid.2012.503.

Reverter, M., Tapissier-Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2018). Biological and ecological roles of external fish mucus: a review. *Fishes*, *3*(4), Article 41. https://doi.org/10.3390/fishes3040041.

Visnuvinayagam, S., Thangavel, K., Lalitha, N., Malmarugan, S., & Sukumar, K. (2015). Assessment of the pathogenicity of cell-culture-adapted Newcastle disease virus strain Komarov. *Brazilian Journal of Microbiology*, *46*(3), 861-865. https://doi.org/10.1590/S1517-838246320140051.

Visnuvinayagam, S., Murthy, L. N., Parvathy, U., Jeyakumari, A., Rawat, K. P., Khadar, S. A., & Sarma, K. S. S. (2020). Destructive dose determination of electron beam irradiation for pathogenic bacteria in water medium by 96 well plate assay. *Journal of Environmental Biology, 41*, 1013-1017. http://doi.org/10.22438/jeb/41/5/MRN-1259.