International Journal of Biotechnology and Biomedical Sciences
p-ISSN 2454-4582, e-ISSN 2454-7808, Volume 2, Number 1; January-June, 2016 pp. 78-82

http://www.krishisanskriti.org/Publication.html

Saprolegniasis: Ubiquitous Fungal Disease in Freshwater Fishes and Biotechnological Remedies

S. Krishna Sundari[1*], Devesh Raizada[2], Norbu Jamtsho[3], Juhi Singh[4] and Manu Goel[5]

[1,2,3,4,5]Jaypee Institute of Information Technology, A-10, Sector-62, NOIDA, Uttar Pradesh, India.
E-mail: [1]krishna.sundari@jiit.ac.in, [2]deveshraizada2495@gmail.com, [3]nortsho7@gmail.com, [4]juhisingh1.618@gmail.com, [5]manugoel0811@gmail.com

Abstract—*Saprolegniasis is the most commonly occurring fungal disease in freshwater fishes and has a huge impact on the aquaculture sector. The susceptibility to this disease is largely affected by lethal concentrations of chemicals like copper, cyanide, ammonia and nitrate, besides these chemical factors is skin damage and presence of other pathogenic agents also account for increased susceptibility to infection. The present review discusses existing methods to control Saprolegniasis, including the use of malachite green that has high anti-fungal properties, however it is also one of the most controversial chemicals in use because of its toxicity and carcinogenic effects. The study suggests need for incorporating biotechnological remedies which will not only be efficient but are also environmentally friendly. In our paper, we talk about various emerging biotechnological strategies that involve targeting important fungal enzymes like chitin synthase and cyclooxygenase for inhibiting essential biological pathways, use of pathogens like bacteria that have biocidal effects and even vaccination of fishes. Even though we still seek methodologies that can give us desirable in vivo results, but the current developments have certainly opened new gates for further investigation and establishment of sustainable methods for treatment of this disease.*

Keywords*: Saprolegniasis, Malachite green, Chitin synthase, Aquaculture, Antifungal agent.*

1. INTRODUCTION

Fisheries is an important sector in India, because of it's large contribution to the country's economy, offering and employment security. India constitutes to ~6.3% of the total global fish production. It is also the second largest country in terms of aquaculture productions. 95% of all aquaculture productions are freshwater aquaculture. It is majorly the carps, namely Rohu, Catla and Mrigal [19] that account for the country's whooping contribution, i.e. 70 to 75 percent of the total freshwater production. So with this heavy reliance on freshwater forms it is necessary to maintain healthy production chain. The biggest threat to the aquaculture production comes from fungal infections.

Most prominent fungal diseases affecting freshwater fishes are Dermocystidiosis, Ichthyophonosis, Branchiomycosis, and Oomycetosis[1]. Saprolegniasis, a type of Oomycetosis is found to be the most common fungal infection in fishes. This disease infects almost all the economically important fishes and other water forms. It is widespread in all freshwater ecosystems around the world.
Most frequently used chemicals for the treatment of Saprolegniasis in aquaculture are Malachite Green, Formalin and Hydrogen peroxide. These chemical compounds have shown to be adversely affecting the aquatic life forms as well as human health. Also chemical methods pose various problems resulting in immunosuppressive, teratogenic, carcinogenic and mutagenic effects on repeatedly treated fish. The major reason behind the restriction imposed on the use of such chemicals is their possible carcinogenic and mutagenic effects due to bioaccumulation, biomagnifications inhuman populations[20]. Hence, there is an urgent need for the development of safer alternate treatments employing new biotechnological approaches such as targeting essential enzymes for drug designing, use of other pathogens (strains of *Pseudomonas*) with biocidal effects and the development of vaccines (as shown in fig 1.). Our review provides a critic on existing methodologies and offers a detail account of latest developments in biotechnological research targeted to treatment of Saprolegniasis.

2. SAPROLEGNIASIS

Saprolegniasis is a fungal disease of freshwater fishes caused by species of *Saprolegnia.* The disease affects the epidermal tissue and forms characteristically white or grayish cotton like masses on the skin or gills of fishes. Hence it is commonly known as "cotton wool disease"[1]. The infection in its initial stage might be small but can rapidly cover the entire body surface of the fish. *Saprolegnia* being an opportunistic parasite invades the fish when it is mechanically damaged, or is already suffering from a parasitic or bacterial infection. The fungus also infects fish eggs by adhering to and then penetrating the cell membrane. The infection spreads from diseased to healthy eggs through positive chemotaxis. *Saprolegnia* leads to necrosis and epidermal damages due penetration of hyphae. In later stages the fish suffers from osmoregulatory dysfunctions, protein deficiency, atrophy of

skeletal muscles and lack of collagen synthesis. The life cycle of *Saprolegnia* includes both sexual as well as asexual reproduction. The motile primary zoospore produced is very short lived and germinates soon after its release. The secondary Zoospores are considered as the main infection causing phase of the fungus as they repeatedly reencyst until a suitable host is found [2].

3. CONDITIONS ACCELERATING THE OCCURRENCE OF SAPROLEGNIASIS.

Stress can be considered an important factor that challenges homeostasis i.e. tries to act against and alter all parameters that lie in a normalized range for the fish existing in a sustainable biological condition. Under stress, a primary stress hormone cortisol is secreted that triggers anti-stress and anti-inflammation pathways. However, elevated cortisol levels lead to the suppression of the immune system. A higher concentration of this hormone blocks the interaction of T-cell lymphocytes with interleukins which in turn inhibits T-cell proliferation.

During chemical treatment heavy dosages of some chemicals were used for a small duration to see the effect of acute stress on the susceptibility of the host to the disease. 0.25 mg/L of Copper, 0.07 mg/L, 0.5 mg/L and 0.24 mg/L of cyanide, ammonia and nitrite were used respectively for duration of 10 minutes. This short exposure shot up cortisol levels in the fishes and when fish subjected to chemical stress were later exposed to *Saprolegnia parasitica,* increased levels of cortisol hormone were observed reflecting its commanding effect on the disease susceptibility. All fishes that had cortisol levels greater than 370ng/ml were infected [3].

This certainly tells a lot about the pathogen that as previously discussed is an opportunistic one. Therefore, when the cortisol levels in the host increased there was a suppressive effect on the host defense mechanism facilitating largely infecting the fish.

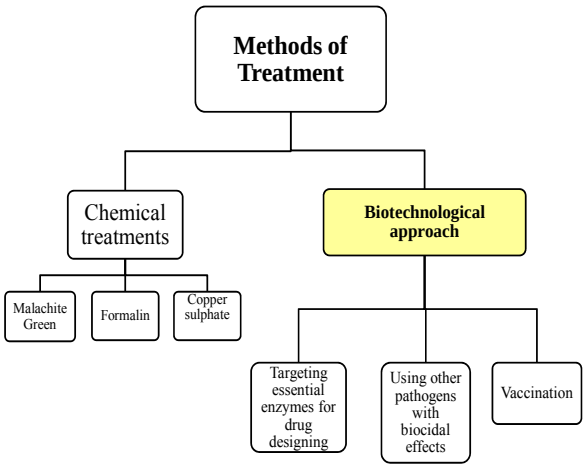

Fig. 1: Different methods of treatment against Saprolegniasis

4. CHEMICAL TREATMENT

The most widely used chemical treatment is that of Malachite Green (MG) or the triarylmethane dye $[C_6H_5C\ (C_6H_4N\ (CH_3)_2)_2]$ Cl. It shows a high antifungal property for treating fungi infected fishes and even to prevent fish eggs from fungal infection. Chemical treatment can be basically of two types- Tropical bath and Multicomponent. The Tropical bath uses a mixture of MG and water and can be further classified into short term bath and long term baths for 1 to 2 hours at dosage of 6.67 mg/L and for 6 days @ 0.2-0.5mg/L respectively. The multicomponent treatment uses a mixture of Malachite green in aqueous Formalin [5].

It is not actually MG but its alcohol form that passes through the cellular membrane.

$[C_6H_5C\ (C_6H_4N\ (CH_3)_2)_2]Cl + H_2O \rightarrow C_6H_5C\ (OH)\ (C_6H_4N\ (CH_3)_2)_2 + HCL$

The cationic part of MG takes an alcohol form after the addition of a hydroxyl ion from water which has higher lipid solubility than MG. It is then metabolized and reduced to Leuco Malachite Green (LMG) which gets accumulated inside the fish and persists for long durations. The exact mechanism describing the chemical's antifungal efficacy is yet to be researched. Although the chemical MG is quite effective, its toxicity cannot be ruled out. Several parameters affect the toxicity of MG such as time of exposure, pH value, concentration used and the temperature [4]. The presence of humic substances in water in which MG is mixed reduces its toxicity but when Ca^{2+} concentrations in the water are high, they bind to the binding-sites of these humic substances disallowing them to interact with Malachite Green, hence making it essential to choose the right quality of water during aquaculture. [5]. Some of the characteristic observations in fish upon MG toxicity are increased skin slime, green coloration of muscle tissue and even disoriented movement, not only this but it also caused changes in the RBC and WBC count of some carp fishes. In humans it is reasoned that Malachite Green has cytotoxic and carcinogenic effects. The use of MG was banned by EU (European Union) in 2002 [5].

5. BIOTECHNOLOGICAL REMEDIES

5.1 Chitin synthase in Saprolegnia – A novel target for producing anti-Oomycete drugs

Oomycetes have a very low concentration of chitin when compared to other true fungi species [6], but it has been experimentally observed that chitin in *Saprolegnia monica* has a very essential role to play irrespective of its scanty presence. The vitality of chitin in this organism resides in its ability to maintain cell wall integrity at the hyphal tip. Chitin synthase is an enzyme essential for the synthesis of chitin. The studies done on this enzyme involved *in vitro* observations and a bioinformatics analysis [7].

Nikkomycin Z, an inhibitor of chitin synthase was found to be better than another commonly used inhibitor- Polyoxin D [18] and was chosen for *in vitro* studies. It was found that the inhibitor was able to curb mycelial growth at the tip, made cells to swell and finally burst the cells due to cell lysis. The few cells that survived were reported to have a lot of morphological defects and abnormalities though the exact mechanism of the inhibitor is yet to be discovered. However, the discovery of MIT (microtubule interacting and trafficking) domains in a bioinformatics analysis can be considered responsible for membrane targeting and intracellular trafficking in chitin synthases of *Saprolegnia monica* that have never been reported in any other chitin synthase complements in earlier *in vitro* observations. It was reasoned that these domains deliver the enzymes to the apex where they catalyze the formation of chitin and when Nikkomycin Z is used it was able to control mycelial growth at the tip. Despite it's role, use of Nikkomycin Z as an efficient drug to treat Saprolegniasis fails owing to the requirement of higher concentrations (200microM) as per the reported data; even though the high concentrations make it almost impractical for use. There is no doubt that it opens areas for research and investigation targeting chitin synthases and producing drugs that will fight the infection efficiently at lower concentrations [7].

5.2 Prostaglandin E2 in Oomycetes

The presence of prostaglandin E2 an active lipid compound has only recently been discovered in *Saprolegnia parasitica* and is the only example of such compounds in Oomycetes. Prostaglandin E2 in humans have diverse-effects for example it plays an important role in labor where it helps in uterine contraction and also in inducing fever but its exact role in Oomycetes still requires reinforcing evidence and further research though what has been reported suggests that it has two very important roles to play, firstly, in suppressing the host's immune system and secondly in mycelial growth [13]. Here we shed some light on curbing the growing mycelium using inhibitors that will interfere with the biosynthesis of Prostaglandins.

Cyclooxygenase(COX) is the enzyme that catalyzes the production of Prostaglandin H2 from Arachidonic acid. (See fig. 2) Cyclooxygenase inhibitors like aspirin acetylate serine in the active site of COX hence barring its attachment to Arachidonic acid.

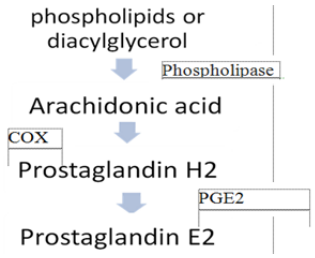

Fig. 2: Biosynthesis of Prostaglandin E2.

The studies on *Saprolegnia parasitica* report a putative phospholipase and a putative prostaglandin E2 synthase activities. Even though no enzyme homologous to COX has been discovered, however use of COX inhibitors like Aspirin was able to control the mycelial growth. This kind of paradox has previously been discovered in *Candida spp.* of true fungi [8, 9, 10, 11, 12] where no clear homologue to COX was discovered but still COX inhibitors were found to be effective. This indicates that there might be some other enzyme having similar functions showing similar response to these inhibitors. Though the actual mechanism is yet to be discovered, their effects under *in vitro* conditions cannot be underestimated [13]

5.3 Antifungal activity of bacterial strains

The bacterial strains of the genera *Alteromonas, Pseudomonas*, and *Aeromonas* are natural competitors of *Saprolegnia* [14]. They are found in the lesions of the fish and produce certain antifungal substances. These microbes can be effectively used as a method of biocontrol for the disease. Particularly species of *Pseudomonas* were shown to have major antagonist effects on the growing fungi. *Pseudomonas H6* produces viscosin-like lipopeptide surfactants which lead to a significant reduction in attachment of *S. dicina* hyphae to salmon eggs. The fungal growth was effectively curbed by using biosurfactants at concentrations in the range of 15-100 µg/ml [15]. The surfactants were also reported to inhibit the germination of cyst showing strong zoosporicidal activity and were also responsible for swelling and branching of hyphae. The bacteria were also observed to form a protective biofilm that prevents the fungal infection. The biofilm provides an ecological niche for the growth and proliferation of the bacteria eliminating the growth of the fungi. However the most important drawback in using live bacteria for the treatment is that of causing possible secondary infections. Further studies regarding the use of bacteria for biocontrol of saprolegniasis should be performed [15].

5.4 Vaccination against Saprolegniasis

Vaccinating the fish against the pathogen is another reported alternative for treating Saprolegniasis. Saprolegniasis resistant fishes were used to identify proteins in the *S.parasitica* genome that were recognized by the host antibodies and a serine protease (SpSsp1) with an indentified subtilase domain in the genome of *S.parasitica* [16].

Studies on SpSsp1 show that this protein in rich quantities has the ability to degrade immunoglobulinM (IgM) in trout [17] and hence poses a major threat to the fish's immune system. But Saprolegniasis resistant fishes that were able to immunologically recognize the protein tend to show a direct relation between the recognition of SpSsp1 and development of a strong immune response against the pathogen. Hence the use of SpSsp1 as a vaccine can be proposed. This was further tested in certain laboratories via ELISA where immunized fishes were able to mount an increased production of antibodies against the protein [17].

An important point of observation should be that SpSsp1 is very similar to serine proteases from some bacteria especially *Streptomyces griseus* with which it shares a significant similarity in the subtilisin domain, bringing in the possibility that SpSsp1 might have cross reacted i.e. the antibodies acting against the speculative protein might have acted in response to a bacterial infection [17]. The studies certainly reveal that some proteins in the *S. parasitica* genome can be used as a vaccine and further researches focusing on SpSsp1 hopes to tie all loose ends and explore the potential that the protein might have to act as an effective vaccine against the disease.

6. CONCLUSION

Biotechnological remedies mark some novel and milestone discoveries, chitin synthase inhibition and bacterial strains fighting the infection all pose as major methods of treatment of Saprolegniasis but Inhibitors like Nikkomycin Z are required in high concentrations while the use of bacteria with biocidal effects often leads to secondary infections. The hunt for a more effective and efficient mode that can successfully replace the use of toxic chemicals is still on.

7. ACKNOWLEDGEMENT

We acknowledge the support, guidance and opportunity provided by the Department of Biotechnology, Jaypee Institute of Information Technology, Noida.

REFERENCES

[1] http://www.fao.org/fishery/countrysector/naso_india/en

[2] N.Ramaiah. "A review on fungal diseases of algae, marine fishes, shrimps and corals", Indian Journal of Marine Science, 2006, 35(4):380-387.

[3] Roy P.E.Yanong, VMD, "Fungal diseases of fish". The Veterinary Clinics Exotic Animal Practice, 2003, 6:377–400.

[4] Prost.M. and Sopinsika A. Reaction of mucous cell of the carp (*Cypriuns caprio* L) to an insecticide as a biological indicator of water pollution, 1989 Med Water.

[5] Fitzpatrick, M.S., Schreck, C.B., and Chitwood, R.L. Evaluation of three candidate fungicides for treatment of adult spring chinook salmon. Prog. Fish-Cul, 1995, 57: 153-155.

[6] M. Carballo,M. J. Munoz, M. Cuellar, and J. V., "Effects of Waterborne Copper, Cyanide, Ammonia, and Nitrite on Stress Parameters and Changes in Susceptibility to Saprolegniosis in Rainbow Trout (*Oncorhynchus mykiss*)".Applied And Environmental Microbiology, 1995,2108–2112.

[7] E. Sudova, J. Machova, Z. Svobodova,T. Vesely3, 'Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish". Veterinarni Medicina, 2007, 52(12): 527–539.

[8] Shivaji Srivastava, Ranjana Sinha, D. Roya. "Toxicological effects of malachite green". Aquatic Toxicology, 2004, 66: 319–329.

[9] Melida H, Sandoval-Sierra JV, Dieguez-Uribeondo J, Bulone V. "Analyses of extracellular carbohydrates in oomycetes unveil the existence of three different cell wall types". Eukaryot. Cell, 2013, 12:194–203.

[10] Gea Guerriero, Mariano Avino, Qi Zhou, Johanna Fugelstad, Pierre-Henri Clergeot, Vincent Bulone, 'Chitin Synthases from Saprolegnia Are Involved in Tip Growth and Represent a Potential Target for Anti-Oomycete Drugs". Plos Pathogen, 2010, 6(8).

[11] Gaughran JP, Lai MH, Kirsch DR, Silverman SJ, "Nikkomycin Z is a specific inhibitor of Saccharomyces cerevisiae chitin synthase isozyme Chs3 in vitro and in vivo". Journal of Bacteriology, 1994, 176: 5857–5860.

[12] Rodrigo Belmonte, Tiehui Wang, Gary J. Duncan, Ida Skaar,Hugo Melida, Vincent Bulone, Pieter van West, Christopher J. Secombesa, "Role of Pathogen-Derived Cell Wall Carbohydrates and Prostaglandin E2 in Immune Response and Suppression of Fish Immunity by the Oomycete Saprolegnia parasitica". Infection and Immunity, 2014, 82(11): 4518–4529.

[13] Erb-Downward JR, Noverr MC. "Characterization of prostaglandin E2 production" by Candida albicans. Infect. Immun, 2007, 75:3498–3505.

[14] Stepanovic S, Vukovic D, Jesic M, Ranin L. "Influence of acetylsalicylic acid (aspirin) on biofilm production by Candida species". J. Chemother. 2004.16:134–138.

[15] Alem MA, Douglas LJ. 2004. "Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of Candida albicans. Antimicrob. Agents Chemother. 48:41–47.

[16] Ells R, Kock JL, Albertyn J, Kemp G, Pohl CH."Effect of inhibitors of arachidonic acid metabolism on prostaglandin E(2) production by CandidaalbicansandCandidadubliniensisbiofilms". Med.Microbiol.Immunol. 2011, 200:23–28.

[17] Abdelmegeed E, Shaaban MI. "Cyclooxygenase inhibitors reduce biofilm formation and yeast-hypha conversion of fluconazole resistant Candida albicans". J. Microbiol. 2013, 51:598–604.

[18] Mortada M.A.Hussein and K.Hatai, "In Vitro Inhibition of Saprolegnia by Bacteria Isolated from Lesions of Salmonids with Saprolegniasis". Fish Pathology, 2001, 36(2):73-78.

[19] Yiying Liu, Elzbieta Rzeszutek, Menno van der Voort, Cheng-Hsuan Wu, Even Thoen, Ida Skaar, Vincent Bulone, Pieter C. Dorrestein, Jos M. Raaijmakers, Irene de Bruijn, "Diversity of Aquatic Pseudomonas Species and Their Activity against the Fish Pathogenic Oomycete Saprolegnia". Plos One, 2015, 10:1371.

[20] Kirsty L. Minor, Victoria L. Anderson, Katie S. Davis, Albert H. Van Den Berg, James S. Christie, Lars Lobach, Ali Reza Faruk, Stephan Wawra, Chris J. Secombes, Pieter Van West, "A putative serine protease, SpSsp1, from saprolegnia parasitica is recognized by sera of rainbow trout, *Oncorhynchus mykiss"*. Fungal Biology, 2014, 118: 630-639.

[21] Rays H. Y. Jiang, Irene de Bruijn, Brian J. Haas, Rodrigo Belmonte, Lars Lobach , James Christie, Guido van den Ackerveken, Arnaud Bottin, Vincent Bulone, Sara M. Diaz-Moreno, Bernard Dumas, Lin Fan, Elodie Gaulin, Francine Govers, Laura J. Grenville-Briggs, Neil R. Horner, Joshua Z. Levin, Marco Mammella9 , Harold J. G. Meijer7 , Paul Morris, Chad Nusbaum, Stan Oome, Andrew J. Phillips, David van Rooyen, Elzbieta Rzeszutek, Marcia Saraiva, Chris J. Secombes, Michael F. Seidl8, Berend Sne, Joost H. M. Stassen, Sean Sykes,

Sucheta Tripathy, Herbert van den Berg, Julio C. Vega-Arreguin, Stephan Wawra, Sarah K. Young, Qiandong Zeng, Javier DieguezUribeondo, Carsten Russ, Brett M. Tyler, Pieter van West. "Distinctive Expansion of Potential Virulence Genes in the Genome of the Oomycete Fish Pathogen Saprolegnia parasitica". Plos Genetics, June 2013, 9:6.