J. Soils and Crops 32 (2) 398-403, December 2022

ISSN 0971-2836 (Print)
ISSN 2582-2756 (Online)

IMPORTANT OF *Bacillus thuriengensis* IN BACTERIAL DISEASES OF FISH AND THEIR CONTROL IN AQUACULTURE

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ABSTRACT

In the present research work applications of *Bacillus thuringiensis* against important bacterial diseases of fishes has been studied. *Bacillus thuringiensis* is a biological potent agent for control of microbial fish diseases. It is well-known that *Bacillus thuringiensis* is biological control agent against the various insects and pests. Demanding aquaculture in the rural water bodies of Chandrapur and Gadchiroli with very little infrastructure development may bring-about socioeconomic development in many parts these tribal districts. Due to the need to effectively prevent and control disease outbreaks, biological control agent has become an important in intensive in aquaculture. From this study following results were observed; for all the fish samples ranged between 1 x 10^6 and 20 x 10^6 cfu ml^{-1}. The skin had the highest number of bacteria with 19.88 x 10^6 cfu ml^{-1} in *Channa marulias*. Maximum inhibition of *Pseudomonas sp.* was observed with *Bacillus thuringiensis* Strain - I and *Klebsiella sp.* with *Bacillus thuringiensis* Strain - II. Farmers can adopt this aquaculture technique to get additional benefit.

(Key words: *Bacillus thuringiensis*, bacterial diseases, aquaculture, biological control)

INTRODUCTION

Biological control of insects using *Bacillus thuringiensis*

Bacillus thuringiensis is a facultative an aerobic, gram positive bacterium that forms characteristic protein inclusions adjacent to the endospore. *Bacillus thuringiensis* subspecies can synthesize more than one parasporal inclusion. *Bacillus thuringiensis* produces parasporal crystalline inclusions, which are toxic for certain invertebrates, especially species of insect larvae belonging to the insect orders Coleoptera, Diptera and Lepidoptera. The parasporal inclusions are formed by different insecticidal crystal proteins (ICP). The crystals have various shapes (bipyramidal, cuboidal, flat rhomboid, spherical or composite with two crystal types), depending on their ICP composition. A partial correlation between crystal morphology, ICP composition, and bioactivity against target insects has been established.

Different domains of the ICP are responsible for host susceptibility (receptor recognition) and toxicity (pore formation). The *Bacillus thuringiensis* subspecies represents a group of organisms that occur naturally and can be added to an ecosystem to achieve insect control (Andrews *et al*., 1987; Stahly *et al*., 1991). In this monograph, a natural habitat is considered to be one where *Bacillus thuringiensis* can be isolated when there has been no previous history of application of the organism to that ecosystem, whereas a treated habitat is one where *Bacillus thuringiensis* can be isolated after a previous history of application of the organism for insect control. Insecticides formulated with *Bacillus thuringiensis* are being manufactured and used worldwide. These commercial *Bacillus thuringiensis* products may be applied as an insecticide to foliage, soil, water environments and food storage facilities.

Effects of *Bacillus thuringiensis* on non-target organisms

Studies on mammals, particularly those on laboratory animals, have evaluated possible infectivity and toxicity of various *Bacillus thuringiensis* preparations, which include the ICPs, vegetative cells and spores. The ICPs, spores and vegetative cells of the *Bacillus thuringiensis* subspecies, which were administered by different routes, were mostly non-pathogenic and non-toxic to the various animal species tested. The vegetative cells and/or spores of *Bacillus thuringiensis* were demonstrated to persist for weeks without causing adverse effects. *Bacillus thuringiensis* has not been observed to adversely affect birds, fish or many other non-target aquatic vertebrates tested in a large number of laboratory and field studies. Relatively few species of aquatic invertebrates are susceptible to *Bacillus thuringiensis* under either laboratory or field conditions. *Bacillus thuringiensis* does not adversely affect earthworms.

Major bacterial diseases affecting aquaculture fish

Many bacterial disease agents have been detected in aquaculture. Since common carp and rainbow trout are

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the predominant fish cultured in Serbia, with 149 registered fishponds, of which 77 are for carp farming and 68 are for trout farming (Relic and Markovic, 2021), most of the disease agents have been isolated from these species. The most noteworthy bacterial diseases in aquaculture are motile aeromonad septicemia (MAS), Pseudomonas septicemia, furunculosis, yersiniosis, lactococcosis, flavobacteriosis (including columnaris disease), and bacterial kidney disease (BKD).

Motile aeromonad septicemia (MAS)

MAS caused by *Aeromonas hydrophila* and other aeromonads, such as *A. sobria, A. veronii*, and *A. caviae*, is one of the most important opportunistic infections in freshwater fish aquaculture (Pekala-Safinska, 2018; Shulz *et al*., 2020). Motile aeromonads like *A. hydrophila* can put an economic burden on aquaculture production, with outbreaks in fish farms causing high mortality rates (Zhang *et al*., 2020; Pridgeon *et al*., 2011).

Pseudomonas septicemia

Pseudomonads are opportunistic fish pathogens, mostly causing secondary infection, but also acting as primary pathogens. In many fish species, pseudomonads cause a generalized infection with ulcerative syndrome, hemorrhagic septicemia and high mortality rate (Algammal *et al*., 2020). Various *Pseudomonas sp.* are known as fish pathogens, with *P. fluorescens* and *P. putida* causing severe losses in rainbow trout aquaculture (Oh *et al*., 2019). Pseudomonads are common disease agents in different aquatic animals, including rainbow trout in Serbia (Jeremic *et al.,* 2005, Jeremic *et al*., 2011, Radosavljevic *et al*., 2013). Due to the facts that most pseudomonads are naturally resistant to several antibiotics (De Kievit *et al*., 2001) and common therapy for the pseudomonas infection in aquaculture is antibiotic treatment, problems with antimicrobial resistance are often present. Various experimental vaccines have been used for prevention of the diseases caused by pseudomonads (Romalde *et al*., 2005). Formalin inactivated whole cell vaccines have been developed for *P. anguilliseptica* and *P. plecoglossicida* (Austin and Austin, 2016), but currently, no commercial vaccine is available for aquaculture (Duman *et al*., 2021).

Furunculosis

Furunculosis, an important disease of cultured salmonids caused by *Aeromonas salmonicida* subsp. salmonicida (sometimes called typical *A. salmonicida*) is one of the oldest known fish diseases (Austin and Austin, 2016). The disease caused by different strains of *A. salmonicida* has been reported in more than 50 different fish species worldwide (Austin and Austin, 2016). *A. salmonicida* subsp. salmonicida is often detected in rainbow trout farms in Serbia, and the outbreaks result in high mortalities of infected trout (Jeremic *et al*., 2005, Jeremic *et al*., 2011, Radosavljevic *et al*., 2013). However, the incidence of furunculosis has been reduced in food fish by using vaccines (Braden *et al*., 2019).

Yersiniosis

Yersiniosis, also known in fish as enteric red mouth disease, is caused by *Yersinia ruckeri*, and is one of the most important diseases in rainbow trout, causing significant economic pressure in aquaculture (Austin and Austin, 2016). It was detected in the USA in the 1950s, and it is now present in aquaculture all over the world (Plumb and Hanson, 2011). In Serbia, the disease was first diagnosed in 1987 (Ocvirk *et al*., 1987), but is now widespread and has become one of the most prevalent trout pathogens in Serbia (Jeremic *et al*., 2005; Radosavljevic *et al*., 2013). In the study by Jeremic *et al.* (2011), Yersiniosis was present on the majority of examined trout farms in one-year-old rainbow trout. Vaccination has been shown to be the most successful strategy, providing protection against this disease in fish (Skov *et al*., 2018).

Lactococcosis

Lactococcosis is systemic disease of rainbow trout, caused by *Lactococcus garvieae*, and is one of the major threats during the warm period (Austin and Austin, 2016). Surviving fish can suffer from chronic or persistent infections. It has been a cause of economic losses in rainbow trout aquaculture, especially in many South European countries (Radosavljevic *et al*., 2020; Vendrell *et al*., 2006). The disease is present in rainbow trout farms in Serbia and results in high mortalities of infected fish (Radosavljevic *et al*., 2020). Vaccination seems the best approach to control this disease (Tanrikul, 2012).

Flavobacteriosis

Three important diseases of rainbow trout are caused by Flavobacterium. *Columnar* is disease (caused by *F. columnare*), bacterial cold-water disease (caused by *F. psychrophilum*) and bacterial gill disease (caused by *F. branchiophilum*) (Starliper, 2011). Other Flavobacterium species (*F. scophtalmum, F. balustinum, F. hydatis, F. johnsoniae* and *F. oncorhynchi*) can also cause disease in fish (Austin and Austin, 2016). In Serbia, *F. columnare, F. psychrophilum and F. branchiophilum* were identified in diseased rainbow trout (Jeremic *et al*., 2005, Jeremic *et al.,* 2011, Radosav ljevic *et al*., 2013). In order to prevent and control the diseases caused by Flavobacterium in aquaculture, various preventive and curative measures have been implemented, including vaccination (Declercq *et al.,* 2013). Many attempts have been made to produce commercial vaccines for preventing diseases caused by flavobacteria (mostly *F. psychrophilum*) (Hoare *et al*., 2017; Madetoja *et al.,* 2006). Despite the important advances that have been made, new strategies and initiatives for development of commercial vaccine are still needed (Gomez *et al*., 2014).

Bacterial kidney disease (BKD)

This disease is caused by *Renibacterium salmoninarum,* a Gram-negative bacterium that targets the kidney of infected fish, causing creamy white granulomatous lesions (Austin and Austin, 2016). It is a chronic disease with high mortality rates, particularly in salmonids

(Delghandi *et al*., 2020; Zrncic and Radosavljevic, 2017). Since there are no available therapeutic procedures or any commercial vaccine for complete eradication of the causative agents, BKD is hard to control (Delghandi *et al*., 2020). Rainbow trout are affected at all stages of life, and the disease has a serious impact on the aquaculture (Johansen *et al*., 2011). Various factors in the production system (food, good practice, size and age of fish, etc.) can contribute to the development of BKD (Jónsdóttir *et al*., 1998). The disease is present in rainbow trout farms in Serbia, resulting in a high mortality rate in infected fish (Radosavljevic *et al*., 2012, Radosavljevic *et al*., 2015).

MATERIALS AND METHODS

The study organism

Channa marulius

Channa marulius is native to South Asia. In South India it is commonly found in reservoirs of eastern Vidarbha region. It is a faster growing fish than most of the other species of the genus. It is a carnivorous species. It is marketed live and fetches high prices in the market.

The following methodology could be applicable for study of microbial fish diseases;

Sampling for disease diagnosis

The ideal specimens for disease investigation or health monitoring are live fish. Samples can be taken from these either on site or transported live to the appropriate laboratory. Transport of live fish, place the fish, representative of the problem, in a plastic bag filled to approximately a third with water and two thirds oxygen. Seal the bag with cable ties or equivalent and place in another bag and seal again. Then place the bag on ice or cool-packs in an insulated box e.g. polystyrene, place more ice on top and seal the container. The maximum transport time depends on water temperature, and the ratio between biomass, water volume and oxygen. As a rough guide the transport time should not exceed 12 hours and the biomass should not exceed one third of the water volume. Transport time is significantly reduced if oxygen is not used.

All samples must be chilled to as close to 0°C without freezing. Pack samples in ice and in an insulated container and dispatch. The maximum transport time is 24 hours.

Parasitological sampling of fish

- **Examination of skin:** first stun the fish with a sharp blow to the head. Then take scrapings for microscopic examination using a scalpel and scrape from front to back of the fish or around the fins. Place scraping on a clean glass slide with a drop of water from the holding facility and cover with a coverslip.
- **Examination of gills:** following gross examination of the gills, clip a small portion of gill lamellae with sharp scissors and place on glass slide (alternatively scrap the gill lamellae with a scalpel), add a drop of the holding tank water and cover with a coverslip. Examine under low power with high contrast or phase.
- **Examination of other organs:** any other organs suspected of having parasitic infection can have squash preparations made from small sub-samples of tissue and examined similarly using light microscopy.

Bacteriological sampling of fish

Examination and direct inoculation of solid media before examining a fish internally, the external body surface, including gills, tail and fins should be examined for the presence of any lesions. Observations should always be recorded on paper. Samples from these sites can be taken by searing the surface with a hot scalpel blade followed by insertion of a sterile bacteriological loop or swab. Material from the loop/swab is then plated out onto suitable agar medium by the spread plate technique. Agar plates containing the streaked out samples should be incubated and examined daily for any evidence of growth. The majority of bacterial fish pathogens will grow on Tryptone Soya Agar (TSA) within seven days. Media such as Marine Agar or TSA plus 1.5% salt (NaCl) can be used for marine pathogens.

Control of bacterial diseases affecting in aquaculture by using *Bacillus thuringiensis*

Pathogenic viruses, bacteria, and parasites are key causes of mortality and economic losses in fish aquaculture (Naylor *et al*., 2021; Miccoli *et al*., 2021; Yildiz *et al*., 2019). In China, as the world's largest aquaculture producer, 15 to 20 % loss in production occurs per year due to diseases caused by bacteria (Khan *et al*., 2011). Disease prevention and control are challenges for aquaculture, and diseases are treated mostly by various chemicals and antibiotics. However, the overuse of antimicrobials in aquaculture has made the emergence of antimicrobial resistant bacteria more prominent (Cabello *et al*., 2013, Ljubojevic *et al*., 2016). Antimicrobial resistance also presents a serious threat to human populations worldwide (Hendriksen *et al*., 2019). Although, there are strict regulations and recommendations regarding the use of antimicrobials in aquaculture, there is still extensive usage in many countries.

The antagonistic activities of aforesaid biopesticides were tested against the isolated fish pathogens

The antagonistic organisms and fish pathogens were grown individually in sterile broth medium for about 7 days at 37^0C with intermittent shaking and the titer inoculum was maintained around 10^8 cfu ml^{-1}. The standard agar cup method was used for studying the interaction of antagonistic organisms with the fish pathogens. A basal layer of nutrient agar (6 mm) was prepared in a 9 cm petriplates. After solidification, this layer was super-layered with a second layer of nutrient agar seeded with heavy suspension of the fish pathogen. The wells were made in the center with the help of cork borer of 10 mm diameter and were filled with 0.2 ml broth culture of the antagonistic organisms in triplicate. In control plates, the wells were filled with sterile nutrient broth. In case of fungal pathogens, potato dextrose agar was used. After pre-diffusion time of 30 min. petriplates were incubated for 48 hrs at 37°C. At the end of incubation, the diameter of the zone of inhibition was measured in mm with the help of zone reader, the averages were calculated.

RESULTS AND DISCUSSION

In this study, for all fish samples isolated the ranged between 1 x 10^6 and 20 x 10^6cfu ml^{-1} are shown in Table 1. Out of the 40 fish samples analysed, for the skin had the highest number of bacteria with 19.88 x 10^6cfu ml^{-1} in *Channa marulias*. The intestine had the lowest isolation with 1.06 x 10^6 cfu ml^{-1} in *C. marulias*. The *Pseudomonas spp.* was highest in *Channa marulias* 19.88 x 10^6cfu ml^{-1} on skin.

Table 1. Count of bacteria present at different parts of examined sample fishes

Fish	**Parts** (cfu ml^{-1})	***Coliforms*** (cfu ml^{-1})	***E. coli*** (cfu ml^{-1})	***S. aureus*** (cfu ml^{-1})	***P. aeruginosa*** (cfu ml^{-1})	***V. cholerae*** (cfu ml^{-1})	***S.typhi*** (cfu ml^{-1})	***S. dysenteriae*** (cfu ml^{-1})
	106	**106**	**106**	**106**	**106**	**106**	**106**	
Channa marulius	**Intestine**	8.56	14.50	6.18	17.20	8.19	5.17	1.06
	Gill	10.50	12.04	3.84	19.49	-	4.18	3.64
	Skin	13.60	9.08	5.46	19.88	-	1.20	1.20
	Mouth	16.68	15.20	2.48	16.80	2.48	4.10	3.10

Table1. Revealed the isolation of *Pseudomonas spp.* with the skin having the highest number in *C. marulias*. The *S. dysenteriae*. isolated had the lowest count of 1.06x 10^6cfu ml^{-1}from the intestine of*C.marulias* as compared with the skin of fish samples.

Figure1. Graphical representation of bacterial count of different parts of *Channamarulius*

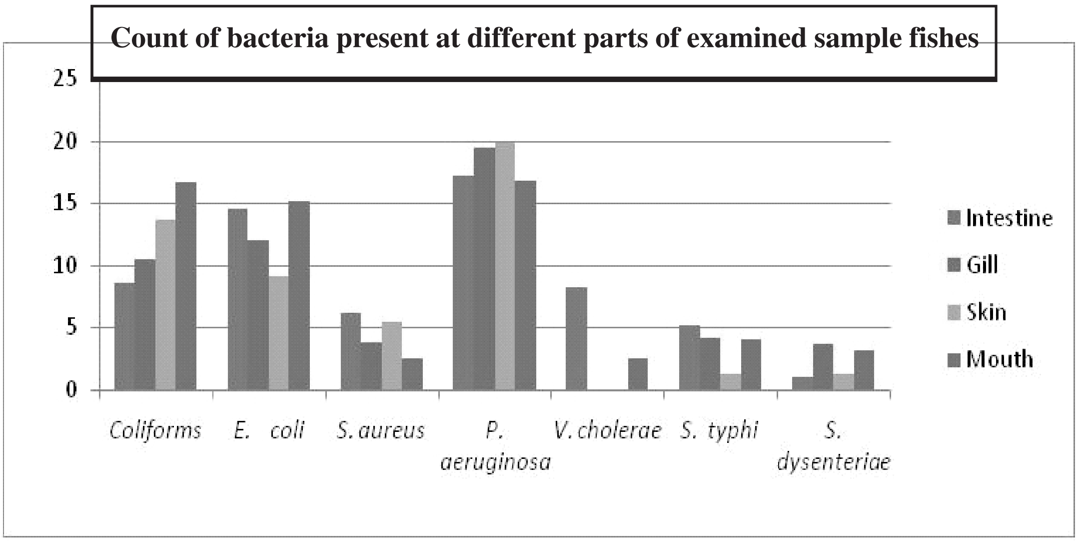

Table 2. Antagonistic activity of *Bacillus thuringiensis*against fish pathogen

Antagonistic Organism	**Inhibitionzone–mm(averageof3replication)**							
	Pseudomonas sp.	***Salmonella sp.***	***E. coli***	***Proteus vulgaris***	***Saprolegnia sp***	***Klebsiella sp.***	***Fusarium sp.***	***Aspergillus hydrophila***
Bacillus thuringiensis (Strain-I)	42	31	26	27	ND	41	38	36
B.t (Strain-II)	17	27	23	26	26	53	ND	ND
Bacillus thuringiensis (Strain-III)	26	ND	18	ND	ND	ND	ND	ND

These protocols, based on the diffusion of antimicrobial substances in culture media (Moraes et al., 2010). Many studies show that the trend of antibiotic resistance is changing depending on the country of origin of the seafood and antibiotic usage in a specific country for aquaculture practices etc. (Murugadas and Ezhil, 2017).

The inhibitory activities of isolates of *Bacillus thuringiensis* (Strain-I), (Strain-II) and (Strain-III) *Staphylococcus sp., E. coli., Pseudomonas sp., Proteus vulgaris and Klebsiella sp.* in addition to filamentous fungi *Fusarium sp a*nd *Aspergillus sp.* are shown in Table 2. In case of *Klebsiella sp.*maximum growth was inhibited by *Bacillus thuringiensis* Strain-II followed by *Bacillus thuringiensis* Strain-I. On the other hand, in case of *Pseudomonas sp.* maximum growth was inhibited by *Bacillus thuringiensis* Strain-I. The inhibition efficacy of *Bacillus thuringiensis* Strain-II was found to be superior followed by *Bacillus thuringiensis* Strain-I and *Bacillus thuringiensis* Strain-III. No antagonistic action of *Bacillus thuringiensis* Strain-III was observed against *Salmonella sp., Proteus vulgaris, Klebsiella sp., Aspergillus niger* and *Aspergillus flavus.* Maximum inhibition of both *Pseudomonas sp.* and *Klebsiella sp.* was observed with *Bacillus thuringiensis* Strain-I and Strain-II. However, for fungal pathogens i.e. *Fusarium sp.* and *Aspergillus sp.,* only *Bacillus thuringiensis* Strain-Iwas found to be effective.

On the other hand, the production of ?-exotoxin in strains of *Bacillus thuringiensis* has been reported associated with clusters of genes housed in megaplasmids (He *et al.*, 2011) which also express Cry proteins (Espinasse *et al.*, 2002).

From the above study following results were observed; *Bacillus thuringiensis* may be safely used for the control of insect pests of agricultural crops and forests. *Bacillus thuringiensis* -strain a potential applicant to be developed as bio-insecticide and be used in the biological control of pests of agricultural interest, avoiding possible risks of resistance by having six different types of toxins (Ferre and Van Rie, 2002).

Establishment of *Clarias batrachus* and *Channa morulias* in several continents and its popularity as a freshwater culturable fish species among consumers made the species suitable for meticulous reviews with respect to various parameters. Accordingly the demand for above mentioned fishes throughout the world is increasing and their several beneficial aspects remain as a hit among the asians in particular.

World Health Organization (WHO) and the Environmental Protection Agency (EPA) requirement for formulations of *Bacillus thuringiensis* strains with potential for use in the biological control of pests in european and north american countries (Hansen and Salamitou, 2000).

However, it should be noted that vegetative *Bacillus thuringiensis* has the potential for the production of toxins, the significance of which as a cause of human disease is not known. Thus, from the above observation it is found that *Bacillus thuringiensis* could be used as a potent and safe biological control agent against the microbial diseases of fishes like *Channa morulias*. We conclude that farmers are using *Bacillus thuringiensis* as a biological controller in addition to aquaculture has created large and sustainable benefits, which contribute to positive economic and social development in India.

However, use of antibiotics and disinfectants often results in the problems such as residual accumulation in tissues, development of antibiotic resistance and immunosuppression. Besides the risk of favoring microbial antibiotic resistance, antibiotic residues can be absorbed by plants, interfering with physiological processes and causing potential ecotoxicological effects. In order to highlight the negative effects, numerous chronic and acute toxicity tests have been performed, which revealed the impact of antibiotics on photosynthesis (chloroplasts gene expression, and cell proliferation) and mitochondria (oxidative stress response in plants), probably explained by the bacterial origins of chloroplasts and mitochondria (Wang *et al.*, 2015). Also, the concentrations of antibiotics which are found in agricultural soils could delay germination or reduce biomass, and consequently may negatively affect yield in farmland fertilized with contaminated manure (Minden *et al.*, 2010).

Moreover, antibiotic residues can alter the human micro biome and cause health disturbances, such as allergic reactions, chronic toxic effects after prolonged exposure, and disruption of digestive system functions (Ben *et al.*, 2019). The species *Bacillus thuringiensis* produces proteins that are toxic and highly specific against a variety of pests such as protozoa, insects, helminths, and mites of agricultural and veterinary importance (Bravo *et al.*, 2006) and they are harmless against non-target insects and vertebrates. Thus, commercial bioinsecticides have been developed based on this species (Bravo and Soberon 2008) which makes *Bacillus thuringiensis* a suitable control alternative. With the use of *Bacillus thuringiensis* it is found to be a cost effective affair for fish farmers in intensive and semi -intensive fish farming which is found to be economical as well as high amount of fish production year on year basis. It helps in improving not only the aquaculture practices but also financial status of fish farmers.

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Rec. on 28.06.2022 & Acc. on 08.07.2022