EDWARDSIELLA TARDA BIOFILM FORMATION AND INHIBITION BY SECONDARY METABOLITES OF ACTINOMYCETES

K.Sivasankari[1], M. Ganesh[2], P. Bothammal[3] & Dr. K. Natarajaseenivasan[4]
[1,2,3 & 4]Department of Microbiology, Centre for Excellence in Life Sciences,
Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India.

Received: August 25, 2018 Accepted: October 14, 2018

ABSTRACT *Edwardsiellatardais a most virulent fish pathogen that causes extensive economic losses in the aquaculture industry worldwide. The antibiotic resistance status of E.tardais high, especially in the biofilm status; however, the mechanisms underlying its resistance remain largely unknown.New broad spectrum antimicrobial agents are urgently needed to combat frequently emerging multidrug resistant pathogens. In this study, the 40 commensal actinomycetes was isolated and primary screening was done using cross-streak method in which A76 isolate showed a good activity and its secondary metabolites was used against the E.tardabiofilm inhibition. The results of the present study revealed that the commensal Actinomycetes have immense potential activity against the Edwardsiella biofilm at 100μg.*

Keywords: *Edwardsiellatarda, Biofilm, Actinomycetes.*

Introduction

*Edwardsiellatarda*is a Gram-negative bacteria pathogen with a broad host range including fish and humans (Jandaand Abbott 1993a). *E. Tarda* infection is responsible for tremendous economic loss of a variety of cultured fish in Asia, especially Japan and India, and also in the United States (Herman and Bullock 1986; Castro *et al.,*2006; Lima *et al.,* 2008). In India, edwardsiellosis has been found to affect many fish species, such as *Anabas testudineus* (Bloch) (Sahoo*et al.,*2000), *Clariasbatrachus* (L.) (Sahoo, Mukherjee &Sahoo 1998) and *Channapunctata* (Kumar *et al.,* 2007).*Edwardsiellatarda*was also isolated from Indian major carp rohu, *Labeorohita* Hamilton (Acharya et al. 2007), and it causes mass mortality in stocks of Asian catfish, *Clariasbatrachus* (L.) during the observation of hatchery-rearing operations (Sahoo*et al.,* 1998; Mohanty&Sahoo 2007). *Edwardsiellatarda*is widely distributed in nature and has been found to affect reptiles, birds and mammals, including humans (Bullock & Herman 1985).Antibiotic resistance has been referred to as “the silent tsunami facing modern medicine” (Cox, 2015). We now take for granted that any infectious disease is curable by antibiotic therapy. Antibiotics are manufactured at an estimated scale of about 100,000 tons annually worldwide, and their use had a profound impact on the life of bacteria on earth. More strains of pathogens have become antibiotic resistant, and some have become resistant to many antibiotics and chemotherapeutic agents, the phenomenon of multidrug resistance. Many types of microorganisms cause infection in humans and animals, so disease prevention and treatment strategies must be adapted to reflect infection risk factors and available treatment options. Over the past decades, most pathogenic species have developed resistance to one or more antimicrobials.. Some reports have observed that resistance emerge Antibiotics resistance has been reported in shrimps (Tjahjadi*et al.,* 1994), eels (Alcaide*et al.,*2005), and aquaculture environments (Chelossi*et al.,*2006) (Kim*et al.,* 2004) within few years of treating infections with antibacterial drugs (Sorum H 1999) and this is a factor, which limits their value in the control of bacterial fish diseases (Smith*et al.,* 1994). Apart from any public health concerns, recognition of the resistance issue has led to calls for intensified surveillance of antibiotic use and antibiotic resistance (Aoki T 1992) .Actinomycetes from the genera Actinoplane, Streptomyces and Actinopolyspora have been reported to produce a number of broad- spectrum antibiotics. Apart from production of antibiotics, actinomycetes have been looked upon as potential sources of bioactive compounds and they are the richest sources of secondary metabolites. Actinomycetes population has been identified as one of the major group of soil population, which may vary with soil type. Apart from soil, they are found in marine and terrestrial environments and also exist in symbiotic association with plants and other living organisms. The important genera of actinomycetes are Streptomycetes, Nocardia, Micromonospora, Thermomonospora, Actinoplanes, Microbispora, Streptosporangium, Actinomadura, Actinosynnema, Dactylosporangium, Rhodococcus, ActinosynnemaKitasatospora, Gordona, Intrasporangium and Streptoalloteichus. Actinobacteria from terrestrial origin produce hundreds of antibiotics which are widely used at present.

Some differences could be expected among organisms existing in marine and terrestrial environments due to variation in the physical, chemical and biological factors. Actinomycetes are present as commensal organisms in healthy humans and are considered as opportunistic pathogens generally have low virulence and are part of the normal flora. Actinomyces species are members of the endogenous mucous membrane flora in the oral cavity, lower gastrointestinal tract, bronchi, and female genital tract (Westhoff, 2007). The use of an intrauterine contraceptive device is linked to the development of actinomycosis of the female genital tract (Shanmughapriya*et al.,* 2012). The present work was undertaken to isolate potent actinobacteria from healthy individuals to elucidate their antibiofilm activity against the fish pathogen *Edwardsiellatarda.*

Materials and Methods

Samples

Disease infected fishes (*Labeorohita*) were collected from the commercial fish farms of Tiruchirappalli district during January – March 2015. The infected fishes were caught by the help of seint net. All the fishes collected were having symptoms like small hemorrhages and necrosis lesions externally. The infected fish samples were collected and brought to the Medical Microbiology Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli.

Bacterial strains and media

Isolation of bacteria was carried out from liver of the diseased fish. Liver of the fish were taken out aseptically and homogenized separately in sterile physiological saline. Aliquots of 0.1ml were inoculated on RimlerShotts Agar (RS Medium; Himedia, India) by spread plate method for their presumptive identification and incubated at 37°C for 24h. Well differentiated single bacterial colony was further streaked onto tryptone soya agar (TSA; HiMedia, India) for obtaining pure culture.

Multi drug resistance (MDR) strains

In vitro sensitivity of the E. tarda isolates to different commercial antibiotics was determined by disc diffusion method as described by Rahman*et al.,* 2010. The antibiotics and concentration ranges tested were erythromycin (10μg), kanamycin (30μg), amoxyclav (30 μg) doxycycline hydrochloride (30 μg), novobiocin (30 μg), cephalothin (30 μg). Briefly, a young culture (16–18 h) of each isolate grown in tryptone soy broth was used to prepare a lawn on MHA plates. After gentle drying for 10 min in a laminar flow cabinet, antibiotic discs were placed on the lawn culture plates. The plates were incubated at 30 °C for 24 h and the zone of inhibition was recorded.

Screening of potent biofilm former

Biofilm assay

The biofilm formation of the isolates was determined by periodically in different time intervals by using crystal violet assay. Biofilm formation on polystyrene surface was determined as described previously. Briefly, cells were cultured in LB medium to exponential phase and transferred into a 96-well polystyrene plate. After incubating at 28°C for 48 h, the plate was washed with PBS and the attached cells were stained with 0.4% crystal violet.

Quantitative assessment of biofilms

The assay was conducted as previously described (Schaber *et al.,* 2004) with minor modifications. TMX coverslips were carefully removed from each well of the 24-well plates (static biofilm formation), rinsed gently with double distilled water, and placed into the corresponding wells of a new 24-well plate containing 1 ml of 4.0% crystal violet solution (wt/vol) incubated at room temperature for 30 minutes. After the incubation the crystal violet solution was discarded and the coverslips rinsed, and placed in the corresponding wells containing 1 ml of 95% ethanol. The plates were incubated at room temperature for 1 hour and the extracted crystal violet, which indicates the strength of the biofilm, was measured at an absorbance of 595 nm.

Isolation of Actinomycetes

The cervix of each patient was exposed by sterile bivalve speculum and endocervical swabs were obtain with sterile cotton swabs. These swabs were transported to the laboratory on ice for further processing on the same day to Medical Microbiology Laboratory (MML), Department of Microbiology, Bharathidasan University, Tiruchirappalli. This study has been approved by human ethical committee (DM/2011/101/19) of Bharathidasan University. The swab was directly streaked on Hichorme Candida agar media and Actinomycetes isolation agar. The plates were incubated for 3 days at 27˚C.

Preliminary screening for antimicrobial activity

The isolated actinomycetes were cross streaked against microbial pathogens by using cross streak method (Oskay, 2009). Muller–Hinton agar plates were prepared, inoculated with the actinomycetes isolates by a single streak in the centre and incubated at 28°C for 10–14 days (Kumar *et al.,* 2014). The plates were then inoculated with the microbial pathogens by a single streak at 90 angles and the plates were incubated overnight at 3–5 days at 30°C. (Kumar *et al.,* 2012)

Extraction of secondary metabolites

Fermentation process

For production of antimicrobial metabolites 500ml of production media was prepared in two sets and was inoculated with 5ml of 24-hour old grown broth of the isolate and selected *Actinomycetes* strain. Flask was incubated in a shaking incubator at 28°C/120 rpm for four days. Fermented broth was transferred into centrifuge tubes and spun at 5000 rpm for 10 minutes. Supernatant was treated for solvent extraction.

Solvent extraction

Ethyl acetate was added to the supernatant in the ratio 1:1 (v/v) and shaken vigorously for 24 h at 28°C and kept stationary phase by using separating funnel for another 15 min to separate the organic phase from the aqueous phase. The organic phase was collected and concentrated in rotary vacuum evaporator.

Anti-biofilm assay

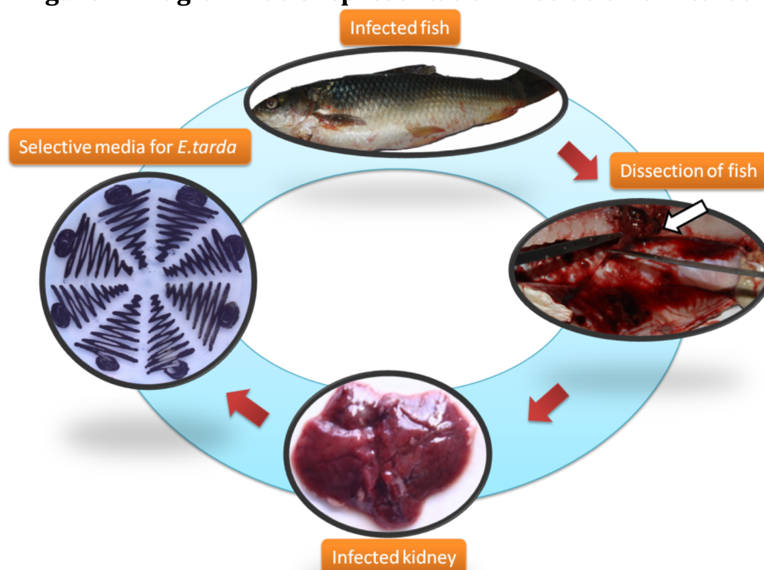
The effect of crude ethyl acetate extract used to treat against *candida* biofilm was examined for their anti-biofilm activity. An overnight culture of strong biofilm forming candida cultures with OD600 at 0.2-0.3nm was inoculated into the wells. 100µl (1000µg) of crude extracted compound was added into the wells. The control corresponding an identical volume of ethyl acetate and empty broth was used as negative control Fluconazole was used as antibiotic control. The plates were incubated at 27°C for 5 days. After incubation biofilm formation was quantified by the crystal violet assay.

Result

Isolation from Infected Fish

The visceral organs (kidney & liver) excised from the diseased fishes were aseptically, homogenized, serially diluted and plated on RS agar plate (Figure 1). The plates were incubated at 30°C for 24h, and small colonies with black centres on RS agar were further processed.

Figure 1. Diagrammatic representation – isolation of *E.tarda*



Antibiotic susceptibility and resistance

All *Edwarsiellae* isolates were sensitive to kanamycin, amoxyclav, novobiocin, and resistant to cephalothin, doxycycline hydrochloride, erythromycin. All the twelve isolates have multidrug resistance towards the broad spectrum antibiotics. The pathogen which shows resistance to more than or equal to three antibiotics were considered as multidrug resistance organisms. All the twelve isolates were showed a multidrug resistance against broad spectrum antibiotics which shown in figure 2

Figure 2. Resistance profiles of *E.tarda*isolates collected in this study

KAN AMO NOV CEP DOX ERY MDR

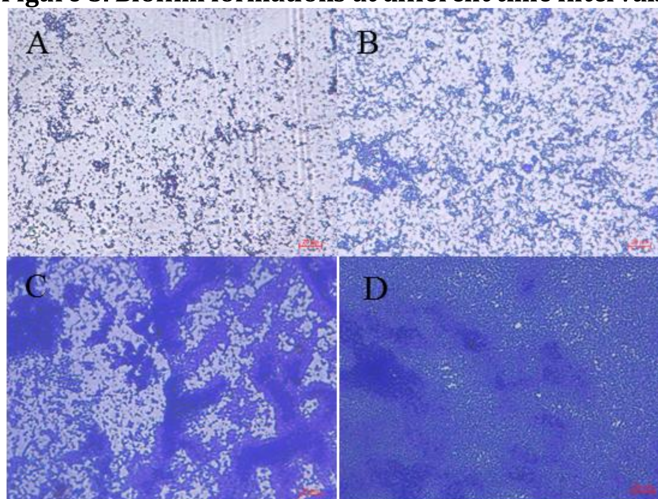
ETBDU1
ETBDU2
ETBDU3
ETBDU4
ETBDU5
ETBDU6
ETBDU7
ETBDU8
ETBDU9
ETBDU10
ETBDU11
ETBDU12

Gray – Susceptible, Black – Multiple Drug resistance, Orange – Intermediate resistance, Red – Complete resistance (3 or more antibiotics)

Biofilm formation assay

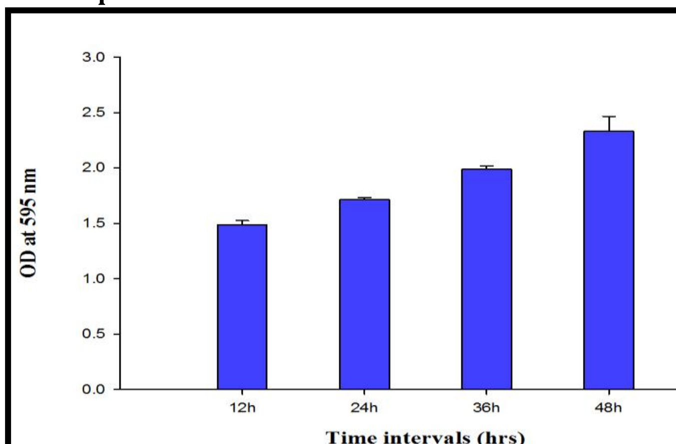
Biofilmformation of the isolates was characterized by observing at different time intervals periodically by crystal violet assay (Figure 3). The biofilm formation was good at the time interval of 48 hours (Hu *et al.,*2010). The observance was read 590nm it is represented in figure 4.

Figure 3. Biofilm formations at different time intervals



A-12hrs; B- 24hrs; C-36hrs; D-48hrs

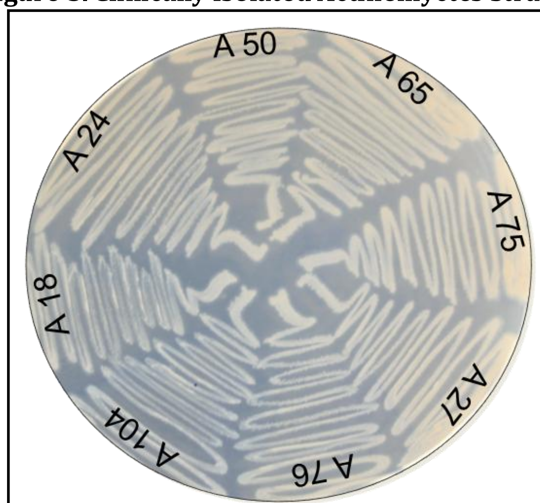
Figure4. Graphical representation of Biofilm formations at different time intervals



Isolation of Actinomycetes strain

Healthy cervical swab samples were collected and directly streaked on actinomycetes isolation agar and incubated at 37ºC for 3 weeks. Out of 100 samples nearly 41 strains were isolated with the help of actinomycetes isolation agar and the colony showed yellow colour, mucoid colonies (figure 5).

Figure 5. Clinically Isolated Actinomyctes Strains

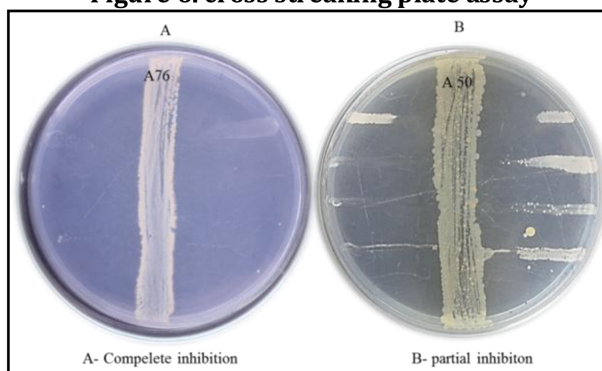


Representative image of clinically isolated from healthy vaginal samples

Preliminary screening for antimicrobial activity

All the 41 cultures were cross streaked against bacteria, and fungal pathogens. Only 8 (A18, 24, 27, 50, 65, 75, 76 and 104) (20%) cultures showed activity (Figure 6). From 8 cultures, 1 (A76) isolates with best activity were selected for secondary screening against the *E.tarda.*

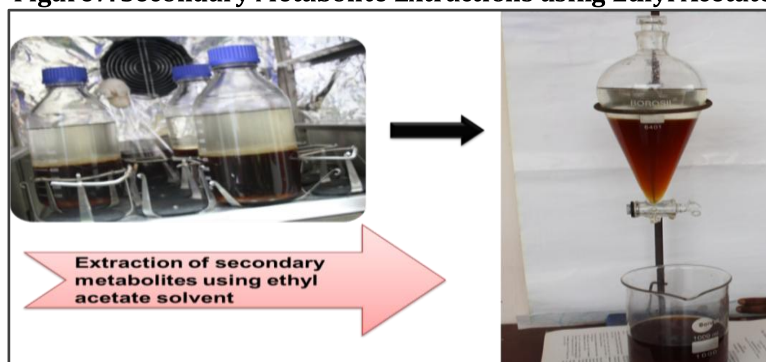
Figure 6. cross streaking plate assay



Extraction of secondary Metabolites

The media was prepared by using the optimized condition and the secondary metabolites were extracted by ethyl acetate extraction which is shown in figure 7.

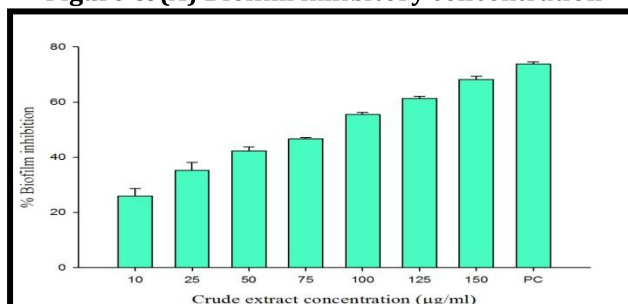
Figure7. Secondary Metabolite Extractions using Ethyl Acetate



Biofilm inhibition assay

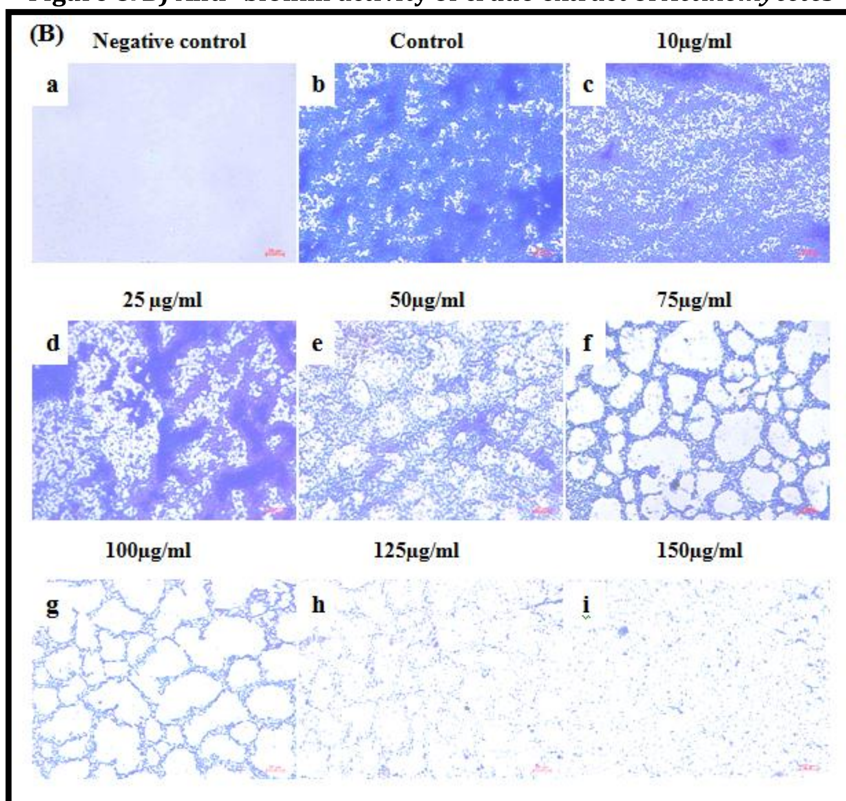
Light microscopic analysis was performed to visualize the antibiofilm activity of crude extract against *E. tarda*biofilms which were grown on TSB medium. The TSB medium enhances the biofilm formation of *E. tarda*. The BIC of crude extract of *Actinomycetes* (100µg/ml) exhibited good antibiofilm activity as there was reduced aggregation of cells when compared to the control and lower concentrations of crude extract (Figure 8 A and B).

Figure 8. (A) Biofilm inhibitory concentration



Pc- positive control (kanamycin 30µg/ml)

Figure 8. B) Anti- biofilm activity of crude extract of *Actinomycetes*



Summary

*Edwardsiellatarda*is one of the serious fish pathogens. Research on edwardsiellosis has revealed that *E. tarda* has a broad host range and geographic distribution. This poses a great risk to fish, vertebrates with potential to cause significant mortality and morbidity in human population due to the development of pathogenic organisms and its formation of biofilm activity. The present study is investigated the antibiofilm activity of multi drug resistant *Edwardsiellatarda*using the crude extract from commensal Actinomycetes. These *Edwardsiellatarda*already isolated from infected fish among the isolated *Edwardsiella* strain the biofilm formation was identified by crystal violet assay it showed good biofilm formation at 48 hours. The potentially active Actinomycetes were identified and secondary metabolites were extracted. The crude extract was subjected *Edwardsiella* biofilm inhibition. Interesting BIC biofilm inhibitory concentration is very low compare 100µg. Further analysis of the crude extract by HPLC, GCMS, and NMR may result in the identification of good antibiofilm compound.This study also gives basic information for those who are

interested in studying the role of actinomycetes present in the normal flora and its antimicrobial activity, with many opening for further studies in this aspect.

Acknowledgement
This work was supported by Department of Biotechnology (DBT Grant No BT/PR12133/AAQ/3/707/2014)

Reference:

1. Alcaide, A., Blasco, M.D. and Esteve, C. 2005. Occurrence of Drug-Resistant Bacteria in Two European Eel Farms. Journal of Applied Environmental Microbiology, 71, 3348-3350.
2. Aoki, T. 1992. Present and Future Problems Concerning the Development of Resistance in Aquaculture. In: Michel, C. and Alderman, D., Eds., Chemotherapy in Aquaculture: From Theory to Reality, Office International des Epizooties, Paris, 254-262.
3. Bullock, G.L., Herman, R.L., 1985. Edwardsiella infections of fishes. US Fish & Wildlife Publications Paper 132.
4. Castro, N., Toranzo, A.E., Barja, J.L., Nunez, S. and Magarinos, B. 2006. Characterization of Edwardsiellatardastrains isolated from turbot, Psetta maxima (L.). J Fish Dis 29, 541–547.
5. Chelossi, E., Vezzulli, L., Milano, A., Branzoni, M., Fabiano, M., Ricarrdi, G. and Banat, I.M. 2003. Antibiotic Resistance of Benthic Bacteria in Fish Farm and Control Sediments of Western Mediterranean. Journal of Aquaculture, 219, 83-97.
6. Cox D. Antibiotic resistance: the race to stop the silent tsunamifacing modern medicine. TheGuardian. 2015 Aug 21
7. Herman, R.L. and Bullock, G.L. 1986. Pathology caused by the bacterium Edwardsiellatardain striped bass. Trans Am Fish Soc 115, 232–235.
8. Janda, J. M. & Abbott, S. L. 1993a. Infections associated with the genus Edwardsiella: the role of Edwardsiellatardain human disease. Clin Infect Dis 17, 742–748.
9. Kim, S.R., Nonaka, L. and Suzuki, S. 2004. Occurrence of Tetracycline Resistance Genes tet (M) and tet (S) in Bacteria from Marine Aquaculture Sites. FEMS Microbiology Letter, 237, 147-156.
10. Kumar, A., B. Sharma and R.S. Pandey. 2007.Cypermethrin and lcyhalothrin induced alterations in nucleic acids and protein contents in a freshwater fish, Channapunctatus. Fish Physiol. Biochem., 34, 331-338
11. Lima, L.C., Fernandes, A.A., CostaII, A.A.P., Velasco, F.O.,Leite, R.C. and Hackett, J.L. 2008. Isolation and characterization of EdwardsiellatardafrompacuMyleusmicans. Arq Bras Med Vet Zootec 60, 275–277.
12. Rahman MM., and Hossain MN., Antibiotic and herbal sensitivity of some Aeromonas sp. collected from diseased carp fish. Progress Agric. 2010. 21(1&2):117-129.
13. Sahoo, P. K. 2000. Comparative evaluation of various immunomodulators in aflatoxintreated fish. Ph.D Thesis, Utkal University, Bhubaneswar, India. 114 pp.
14. Sahoo, P. K., Sahu, B. B., Mohanty, J., Murjani, G. and Mukherjee, S. C. 1998. Ulcer diseases in catfish, Heteropneustesfossilis. Proceedings of the National Symposium on Technological Advancements in Fisheries and its impact on Rural Development. 5-7 December, 1995. pp. 147-152. School of Industrial Fisheries, Cochin University of Science and Technology, Cochin.
15. Shanmughapriya, S., Lency Francis, A., Kavitha, S., and Natarajaseenivasan, K. 2012. In vitro actinomycete biofilm development and inhibition by the polyene antibiotic, nystatin, on IUD copper surfaces. Biofouling 28: 929–935.
16. Smith, P., Hiney, M. and Samuelson, O. 1994. Bacterial Resistance to Antimicrobial Agents Used in Fish Farming: A Critical Evaluation of Method and Meaning. Annual Review Fish Disease, 4, 273-313.
17. Sorum, H. 1999. Antibiotic Resistance in Aquaculture. ActaVeterinariaScandinavica. Supplementum, 92, 29-36.
18. Tjahjadi, M.R., Angka, S.L. and Suwanto, A. 1994. Isolation and Evaluation of Marine Bacteria for Bio-control of luminous Bacterial Disease in Tiger Shrimp Larvae (Penaeusmonodon, Fab.). Asian-Pacific Journal of Molecular Biology and Biotechnology, 2, 347-352.
19. Westhoff, C. 2007. IUDs and colonization or infection with Actinomyces. Contraception75.