BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2013. Vol. **10**(1), 365-370

Enhancement of Antibiotic Production in Marine Bacteria

T. Prem Anand[1]*, C. Chellaram[1], R. Sivakumar[2] and C. Felicia Shanthini[3]

[1]Department of Biomedical Engineering, [2]Department of Electronics and Communication Engineering, Vel Tech Multitech Engineering College, Chennai- 600 062, India.
[3]Department of Marine Science and Coastal Resource Management, Madras Christian College, Chennai, India.

(Received: 11 April 2013; accepted: 17 May 2013)

Marine microbes use a variety of strategies to colonize both animate as well as inanimate surfaces in marine environment. The competition among microbes to colonize surfaces acts as a potent selective force. One of the important strategies these microbes use is the secretion of antimicrobial compounds, which helps to inhibit the growth of the competitors. Bacterium - bacterium antagonistic interactions involving antibiotics are well documented in soils but work relating to this in marine environment is scanty. In this present study 75 antibiotic producing bacterial strains were challenged to enhance the production of antibiotics by these strains. Enhancement of antibiotic production by the producer strain was carried out by inducing the producer strains with heat killed and live *Escherichia coli* and *Pseudomonas aeruginosa* cells. Out of the 75 induced strains 21 strains were found to enhance their antibiotic production. In the search for novel antibiotics from marine bacteria, these strategy can be used to enhance production of the potent compounds to overcome the problem of low yield to a certain degree.

Key words: Marine bacteria, Antibiotics, Secondary metabolites, Enhancement, Epibiosis.

The recent definition given by Bennett and Bentley (1989)[1] for secondary metabolite is," A metabolic intermediate or product found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism". They further elaborated that secondary metabolites are often materials of low molecular weight and more frequently accumulated in large quantities after the cessation of growth in families of related compounds (congeners). Bacteria, fungi or plants, interspecific, produce most secondary metabolites and intraspecific variation in secondary metabolism is the rule. The best-known secondary metabolites exhibit pharmacological activity as drugs, toxins etc., but the function of secondary metabolites in the production organism is not always obvious.

Antagonistic interactions among bacteria involving antibiotics are well documented in soils. In situ production of antibiotics in soil has been detected only in association with organically rich microenvironments like seeds, rhizospheres and straw fragments in soil[2-5].

Competition amongst microbes for space and nutrients in the marine environment is a powerful selective force, which has led to the evolution of a variety of effective strategies for colonizing and growing on surfaces[6]. Many marine free living and sediment inhabiting marine bacteria have been shown to produce secondary metabolites that display antibacterial properties[7]. In addition bacteria in biofilms on the surfaces of marine organisms have been documented to

* To whom all correspondence should be addressed.
E-mail: prem68.a@gmail.com.

contain a higher proportion of antibiotic producing bacteria than some other marine environments[8]. Marine epiphytic bacteria associated with nutrient rich algal surfaces and invertebrates have also been shown to produce antibacterial secondary metabolites, which inhibit the settlement of potential competitors[9-10]. Unlike symbiotic bacteria living inside the tissue of marine invertebrates, which are difficult to culture, the surface associated bacteria are less difficult to culture. This abundance of inhibitory bacteria on surfaces of marine invertebrates is a potential source for natural product research[11-15].

Spragg *et al.,* (1998)[16] have reported that certain marine bacteria can be induced to produce antibiotics. These works have been carried out by inducing the strains with live, heat killed or broth supernatant of terrestrial bacteria. These inducements appear to be in response to chemical signals received from potential competitor strains, which elicit an antagonistic response. Such a response is inducible rather that constitutive has been observed in other species of bacteria (Patterson and Bolis, 1997)[17] but remains a little studied phenomenon.

In this present study marine bacterial strains were induced for enhancement in antibiotic production. This type of study may help to find more novel structures and screening can be widened to include non-producers also.

MATERIALS AND METHODS

Inducement and enhancement study

A total of 75 producer strains were chosen for the antibiotic enhancement study. Clinical isolates of *E.coli* and *P. aeruginos* were the terrestrial strains used for the assay and the study was carried out following the method of Spragg *et al.,* (1998)[17].

Five days cultures of *E. coli* and *P. aeruginosa* in nutrient broth were used to induce production of antimicrobial activity in the marine strains either as living or heat killed cells (121 °C, 15min).

Marine strains were inoculated separately in triplicate into three 30ml glass tubes containing 5 ml of marine broth 2216E (Himedia, Bombay, India). Heat killed or live *E.coli* cells (1ml) were placed inside a dialysis tubing and placed in contact with each marine strain in the test tubes. The control bottle contained only marine broth, marine producer strain and 1ml nutrient broth. Similar set-up and experiment was carried out for *P. aeruginosa.*

Antibiotic activity screening

Antibiotic activity was screened in duplicate employing the standard disc diffusion assay. *E. coli* and *P. aeruginosa* were used as the test organisms. Paper discs (Whatman 6mm) were saturated with centrifuged (5000 rpm 10 mins) supernatant fluid (200 ul) from the test tubes and placed onto nutrient agar plates inoculated with the test organism. Plates were then incubated overnight at room temperature. Production of antimicrobial compounds was determined by measuring the inhibition zones from the edge of the disc to the edge of the clear zone.

RESULTS

In the present study, out of the 75 producers, 21 strains responded. Notable increase of inhibition zone was noted in producer strains such as A14, AF3, GM3, CB9, BFC3, ASA2, CE5 and GE11 (Fig.1- 4). The strains A14, AA2, AC3, AH4, AK4, AF3, AH3 were isolated from seaweeds. The strain A14 exhibited activity only against *P. aeruginosa* in control and after inducement it exhibited activity against *E. coli* also. There was an increase in inhibition zone from 5 (Control) to 8 mm (inducement by heat killed) against *E. coli.* The strain AA2 exhibited activity only against *E. coli* in control, but exhibited activity against P. *aeruginosa* after inducement with Heat killed (4mm) and Live cells (7 mm) of P. *aeruginosa.* Against *E. coli* the activity was induced from 1 (control) to 6 mm (heat killed). The strain AC3 exhibited activity against *E. coli* and P. *aeruginosa* in control and inducement was from 2 (control) to 7mm (heat killed) against *E.coli* and 4 (control) to 6 mm (Heat killed) against P. *aeruginosa.* The strain AH4 exhibited activity in control against both the pathogens and the inducement was from 6 to 9 mm (Live cells) and 4 to 6 mm (Heat killed) in *E. coli* and P. *aeruginosa* respectively. The strain AK4 exhibited activity only against *E.coli* in control and the inducement was from 3 to 5 mm (Heat killed and Live cells) for *E. coli* and 0 to 7mm (Live cells) for P. *aeruginosa.* The strain AF3 exhibited activity against both the

pathogens in control and the inducement was from 6 to 8.5mm (Heat killed and Live cells) and 4 to 7 (Live cells) for *E. coli* and P. *aeruginosa* respectively. The strain AH3 exhibited activity only against *E. coli* in control and inducement was from 3 to 5 mm (Heat killed and Live cells) and 0 to 7.5 (Live cells) for *E. coli* and P. *aeruginosa* respectively.

The strain SCU2 (sea cucumber isolate) also exhibited activity only against *E. coli* in control. The inducement was from 4 to 7 (Heat killed and Live cells) and from 0 to 6 (Live cells) for *E. coli* and P. *aeruginosa* respectively. The strain SCR 3 (sea urchin isolate) exhibited activity only against *E. coli* in control and the inducement was from 5 to 7.5mm (Live cells) for *E. coli* and 0 to 6.5 mm (Live cells and Heat killed cells) P. *aeruginosa.* The strains GMA3 and GMC 9 were isolated from gastropod gut and GMA3 exhibited activity only against *E.coli* in control. The inducement was from 6 to 9.5mm (Live cells) for *E. coli* and 0 to 6.5 (Heat killed cells) for *P. aeruginosa.* The inducement was from 0 to 6.5mm (Heat killed and Live cells) and 2 to 5mm (Heat killed and Live cells) for *E.coli* and P. *aeruginosa* in the strain GMC9. The strains CA4, CB3, CB9 and CC6 were isolated from crabs. The strain CA4 exhibited activity only against P. *aeruginosa* in control and inducement was from 0 to 7 (Heat killed) for *E. coli* and 5 to 8mm (Heat killed and Live cells) for P.*aeruginosa.* For the

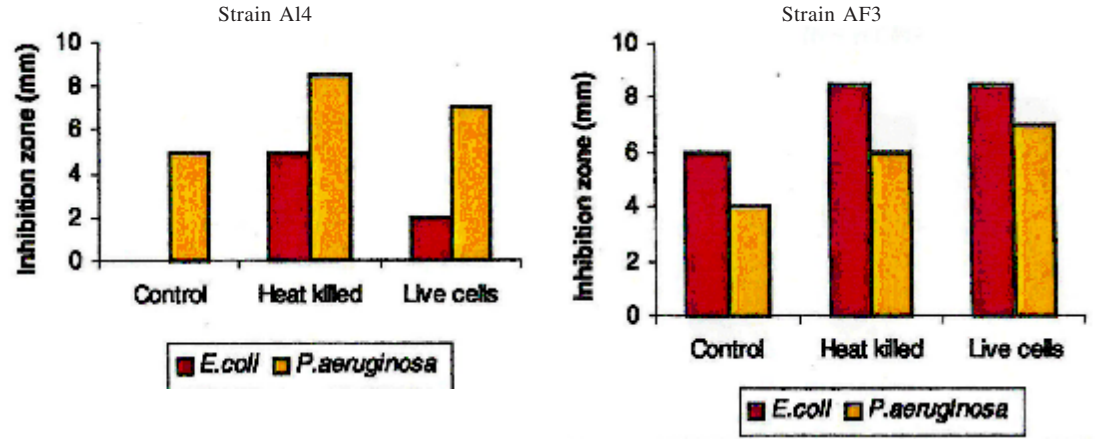

Fig. 1. Antibiotic inducement of producer strains AI4 & AF3 against *E.coli* and *P.aeruginosa* using heat killed and live cells of *E.coli* and *P.aeruginosa*

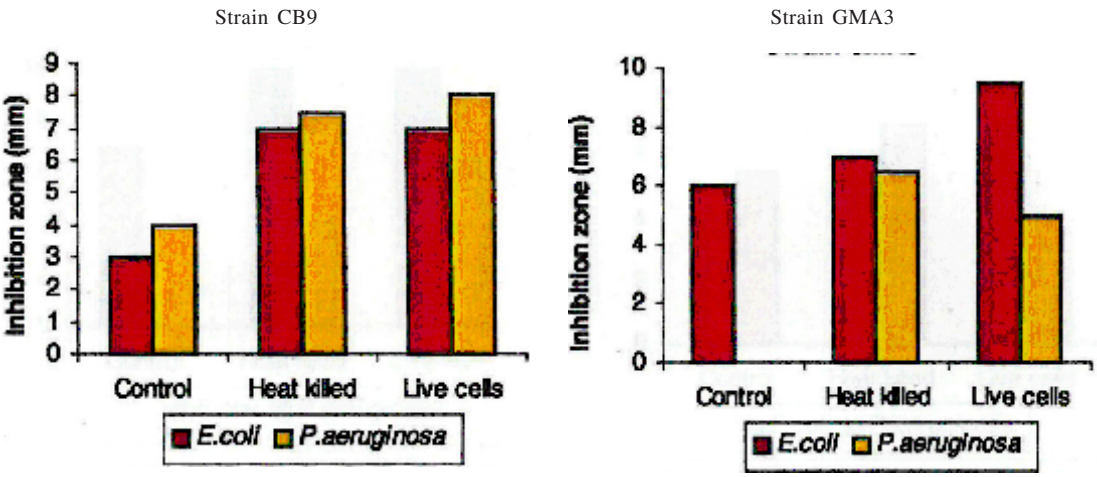

Fig. 2. Antibiotic inducement of producer strains CB9 & GMA3 against *E.coli* and *P.aeruginosa* using heat killed and live cells of *E.coli* and *P.aeruginosa*

strain CB3 the inducement was from 0 to 5mm (Heat killed and live cells) for *E. coli* and 2 to 6.5mm (Live cells) for P. *aeruginosa* . The inducement of CB9, which exhibited activity against both the pathogens in control, was from 3 to 7mm (Heat killed and Live cells) and 4 to 7.5mm (Heat killed) for *E. coli* and P.*aeruginosa* respectively.

The strain CC6 exhibited activity only against P.*aeruginosa* in control and the inducement was from 0 to 5mm (Heat killed and Live cells) and 3 to 7.5mm (Live cells) for *E. coli* and P. *aeruginosa.* The strains BFA7, BFA18, BFC3 were isolated from biofilm and the strain BFA7 exhibited activity only against P.*aeruginosa* in control and exhibited inducement from 0 to 4.5mm (Heat killed and Live cells) for P. *aeruginosa* and inducement of *E.coli* was also noted. The strain BFA18 was induced from 3 to 6.5 (Heat killed and Live cells) for *E. coli* and 0 to 7.5 (Heat killed and Live cells) P. *aeruginosa.* The strain BFC3 exhibited activity only against P. *aeruginosa* in control and the inducement was from 0 to 7 (Live cells) against *E. coli* and 5.5 to 9.5 (Live cells) against P. *aeruginosa.* The strains ASA2 and ASB2 were ascidian derived and the ASA2 strain was induced from 3 to 6 (Heat killed and Live cells) against *E. coli* and 4.5 to 7 (Live cells) against P. *aeruginosa* In the strain ASB2 the inducement was from 7 to 10 (Heat killed and Live cells) and 0 to 6.5 (Live cells) against *E. coli* and P. *aeruginosa* respectively. The strains CE5 and GE11 were isolated from crab egg and gastropod egg respectively. The strain CE5 exhibited activity only

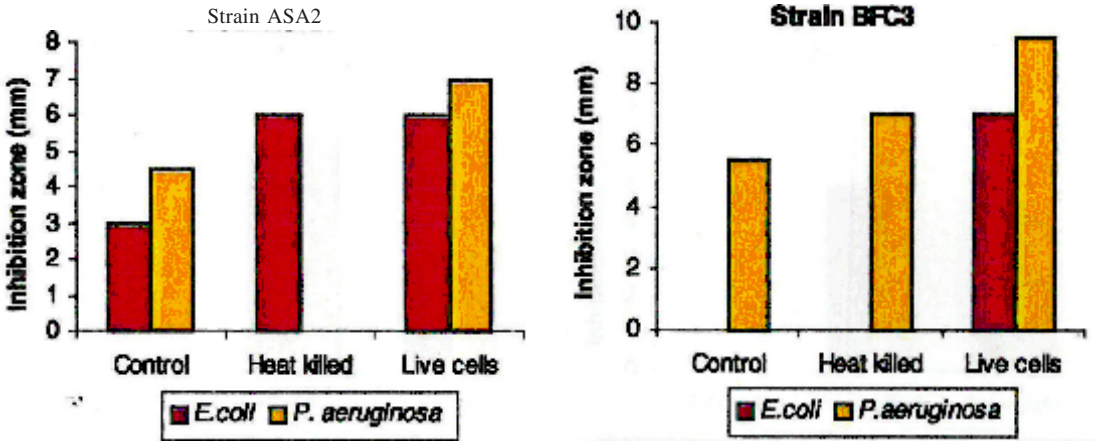

Fig. 3. Antibiotic inducement of producer strains ASA2 & BFC3 against *E.coli* and *P.aeruginosa* using heat killed and live cells of *E.coli* and *P.aeruginosa*

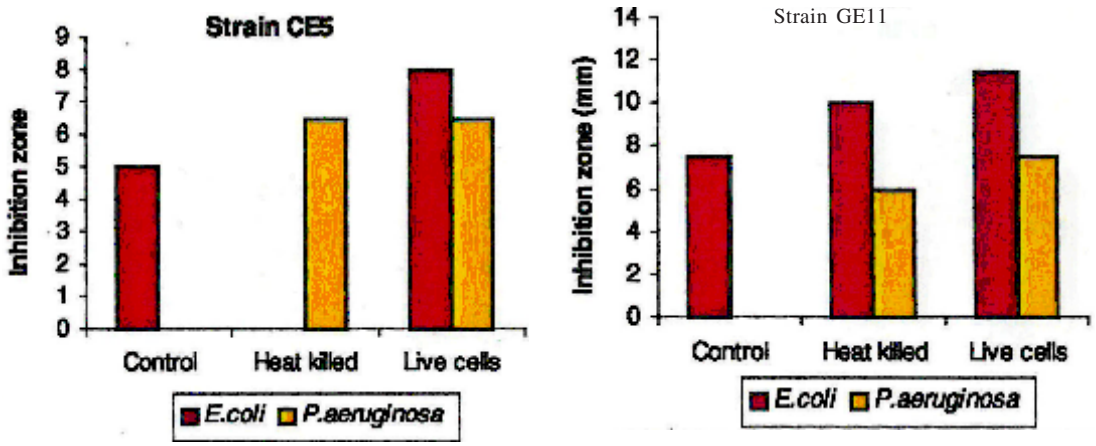

Fig.4. Antibiotic inducement of producer strains CE5 & GE11 against *E.coli* and *P.aeruginosa* using heat killed and live cells of *E.coli* and *P.aeruginosa*

against *E.coli* in control and the inducement was from 5 to 8 (Live cells) against *E. coli* and 0 to 6.5 (Heat killed and Live cells) against P.*aeruginosa.* The GE11 was induced from 7.5 to 11.5 (Live cells) against *E. coli* and 0 to 7.5 (Live cells) against P. *aeruginosa.* The strain BCL5 was isolated from a brain coral and the strain BCL5 exhibited activity against both the pathogens in control and inducement was from 5mm to 7.5 mm (Heat killed and Live cells) and 3 to 6.5 mm (Live cells) against *E. coli* and P. *aeruginosa* respectively.

DISCUSSION

Epibiosis is a common phenomenon in the marine environment. The tendency of aquatic bacterial populations to colonize surfaces was recognized as early as in the 20th century. In natural aquatic ecosystems, surface-associated microorganisms vastly outnumber organisms in suspension. This is suggested to confer a strong survival and selective advantage for surface dwellers over their free-ranging counterparts[18]. Mostly the production of antimicrobial compounds by marine bacteria is usually assayed under straightforward growth conditions and only strains[16]. However few attempts have been made to study such chemical communication between different bacterial species or how this might affect the secretion of antimicrobial compounds[16].

In the present study, out of the 75 producers, 21 strains responded. Notable increase of inhibition zone was noted in producer strains such as A14, AF3, GM3, CB9, BFC3, ASA2, CE5 and GE11. Strains, which do not produce antibiotics against E.coli in control were also found to produce antibiotic after inducement (A14, GM9, CA4, CB3, CC6, BFA7 and BFC3) and strains, which exhibited no activity against *P.aeroginosa,* were also found to produce antibiotics against this pathogen after inducement (AA2, AK4, AH13, SCU2, SUR3, GMA3, BFA18, ASB2, CE5 and GE11). Similar study was carried out by Spragg *et al.,,* (1998), using heat killed and live cells of *Staphylococcus aureus* and live cells of *P.aeroginosa* and *E.coli* to induce activity against Methicillin susceptible *Staphylococcus aureus* (MSSA) and Methicillin resistant *Staphylococcus aureus* (MRSA). They reported 5 out of 12 strains showed increased antimicrobial production to heat killed cells of *Staphylococcus aureus,* and in both live and heat killed conditions two strain exhibited activity. In this study all the 21 strains responded and exhibited activity in both heat killed and live conditions but the activity varied in some strains, exhibiting activity only against *E.coli* or *P.aeroginosa* (BFC3, ASA2 and CE5) Spragg *et al.,* 1998 also reported that *P.aeroginosa* cells (Live) are able to induce and enhance antimicrobial production in 3 out of 7 strains tested.

Burgess *et al.,* (1999) reported inducement of antibiotic production by strains, which did not normally produce antibiotics. They used cell free supernatants to enhance antimicrobial production as well as for inducement of antibiotic production. These type of studies were scanty and their importance was stressed by both Spragg *et al.,* (1998)[16] and Burgess *et al.,* (1999)[19]. Long and Azam (2001)[20] reported production of inhibitory compounds by attached and free living bacteria and found significantly greater percentage of attached bacteria than free living bacteria produced inhibitory compounds (66.7 and 40.7 %), but the actual antibiotic-producing bacteria may be higher if inducement considerations are taken into account. The finding of this study corroborates with the study of Long and Azam (2001)[20].

It is important here to once again highlight the point emphasized by Spragg *et al.,* 1998 and Anbuselvi et al., 2009 [21] "in order to find more novel structures, new way of screening for these compounds must be applied" and inducement studies can thus become part of a screening program.

ACKNOWLEDGMENTS

Authors thank sincerely the Dr Patterson Edward, Director, Suganthi Devadason Marine Research Institute, Tuticorin, for the sample collection and laboratory support and Chairman and Director, Vel Tech Multi Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Chennai, for their unremitting encouragement.

REFERENCES

1. Bennett, J.W., Bentley, R., What's in a name? - Microbial secondary metabolism. *Adv. Appl. Microbiol.*, 1989; **34**:1-28.

2. Bruehl, G., Mittar, R., Confer, B. Significance of antibiotic production by *Lephasporium gramineum* to its saprophytic survival. *J. Plant Sc*i., 1969; **49**: 235-246.
3. Thomashow, L.S., Weller, D.M., Bonsall, R.F., and Pierson, L.S. Production of the antibiotic phenazine - 1 - carboxylic acid by fluorescent *Pseudomonas sp.* in the rhizosphere of wheat. *Appl. Environ. Microbiol*., 1990; **56**: 968-912.
4. Wright, J. The production of antibiotics in soil III, production of glizeotin in wheatstraw buried in soil. *Ann. Appl. Biol*., 1956a; 44,461-466.
5. Wright, J. The production of antibiotics in soil, IV. Production of antibiotics in coats of seeds sown in soil, *Ann. Appl. Biol*., 1956b; **44**: 561-566.
6. Burgess, J.G., Jordan, E.M., Bregu, M., Spragg, A.M., and Boyal, K.G. Microbial antagonism, a neglected avenue of natural products research. *J. Biotechnol*., 1999; **70** : 27-32.
7. Burgess, J. G., Miyashita, H., Sudo, H., and Matsunaga . Antibiotic production by the marine photosynthetic bacterium *Chromatium purpuratum* NKPB 031704 localization of activity to the chromatophores. *FEMS Microbiol. Lett*., 1991; **84**: 301-306.
8. Lemos, M. L.., Toranzo, A. E., and Barja, J. L. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microb. Ecol*., 1985; **11**:149-163.
9. Jensen, P. R., and Fenical, W. Strategies for the discovery of secondary metabolites from marine bacteria: Ecological perspectives. *Annv. Rev. Microbiol*., 1994; **48**: 559 - 584.
10. Bernan, V.S. Mining marine microorganisms as a source of new natural products. Book of Abstracts: International Symposium on Natural Products from Marine microorganisms, Germany, 2002; pp-10.
11. T. Prem Anand, C.Chellaram and Felicia Shanthini. Isolation and Screening of Marine Bacteria Producing Antibiotics against Human Pathogens. *International Journal of Pharma and Bio Science*, 2011; **2**: 1-15.
12. Prem Anand, T., Chellaram, C., Felicia Shanthini, C., Screening for Herbicidal and Growth promotor activity of marine bacteria, *International Journal of Pharma and Biosciences,* 2012; **3**(2): 359-368.
13. Chellaram,C., Prem Anand, T., Kumaran, S., Sreenivasan, R.S., Antagonistic bacteria from live corals, Tuticorin coastal waters, southeastern India. *Pakistan Journal of Pharmaceutical Science*s, 2011; **24**(2): 153-158.
14. Chellaram, C., Prem Anand, T., Kesavan, D., Chandrika, M. Gladis, C., Priya, G., 2012. Antagonistic effect of hard coral associated bacteria from Tuticorin coastal waters. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2012a; **4**(1):580-583.
15. Chellaram, C., Raja, P., Alex John, A., Krithika, S. Antagonistic effect of epiphytic bacteria from marine algae, Southeastern India. *Pakistan Journal of Biological Sciences*, 2013; **16**(9): 431-434.
16. Spragg, A.M., Brega, M., Boyd, K.G., and Burgess, J.G. Cross - species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates after exposure to terrestrial bacteria. Lett. Appl., Microbiol, 1998; **27**: 142-146.
17. Patterson, G.L., and Bolis, CM. Fungal cell wall polysaccharides elicit an antifungal secondary metabolite (phytoalexin) in the cyanobacterium *Seytonema ocellatum.* J. Phycol., 1997; **33**: 54-60.
18. Zobell C. E. The effect of solid surfaces on bacterial activity. *J Bacteriol*, 1943; **46**: 39-56
19. Burgess, J.G., Jordan, E.M., Bregu, M., Spragg, A.M., and Boyal, K.G. Microbial antagonism, a neglected avenue of natural products research. *J. Biotechnol.,* 1999; **70**: 27-32.
20. Long, R.A., and Azam, F. Antagonistic interactions among marine pelagic bacteria, *Appl. Environ. Microbiol.,* 2001; **67** (11): 4975 – 498
21. Anbuselvi, S., Chellaram,C., Jonesh, S., Jayanthi, L., Edward, J.K.P. Bioactive potential of coral associated gastropod, Trochus tentorium of Gulf of Mannar, Southeastern India. *Journal of Medical Sciences*, 2009; **9**(5): pp. 240-244.