

FACTORS AFFECTING BIOFILM FORMATION BY
Vibrio harveyi

ANILKUMAR, H. A., *B.F.Sc*

DEPARTMENT OF FISHERY MICROBIOLOGY
COLLEGE OF FISHERIES, MANGALORE
KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCE
UNIVERSITY, BIDAR

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ANILKUMAR, H. A., B.F.Sc

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**DEPARTMENT OF FISHERY MICROBIOLOGY
KARNATAKA VETERINARY, ANIMAL AND FISHERIES
SCIENCE UNIVERSITY, BIDAR**

CERTIFICATE

This is to certify that the thesis entitled “**Factors affecting biofilm formation by *V. harveyi***” submitted by Mr. AnilKumar for the award of Master of Fisheries Science in Fishery Microbiology of the Karnataka Veterinary, Animal and Fisheries Science University, Bidar is a record of research done by him during the period of study in the University under my supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship or other similar titles.



(Dr. M. N. VENUGOPAL)
Associate Professor
Department of Fishery Microbiology

MANGALORE
JUNE 2006

APPROVED BY:

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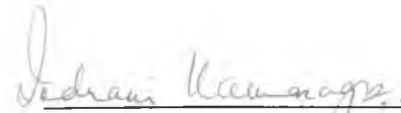


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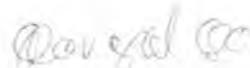
Members:



(I. KARUNASAGAR)



Dr. (Mrs.) I. KARUNASAGAR



(Shri. M. M. MARAGAL)

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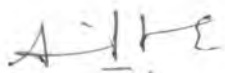
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Introduction

1. Introduction

Microorganisms have been primarily characterized as planktonic, free suspended cells on the basis of their luxurious growth in nutritionally rich environment. In the nature, nearly all bacteria live with, and depend on other microorganisms for energy, carbon and other nutrients. Thus, most of the bacteria live in the micro ecosystems filled with hundreds of other microorganisms. It has now been well recognized that in nature, more than 99% of all bacteria exist as biofilms (Costerton *et al.*, 1987).

Biofilms are ubiquitous and have been observed in virtually all-natural, medical and industrial settings where bacteria exist (Costerton *et al.*, 1995). They can form in almost any hydrated environment that has the proper nutrient conditions, and can develop on a variety of abiotic hydrophobic and hydrophilic surfaces, including glass, metals and plastics (Miller and Ahearn, 1987). Biofilms also readily form on biotic surfaces including human skin and epithelial cells. The transition from the planktonic, free swimming mode of existence to a biofilm state is a regulated developmental process that leads to a complex surface attached bacterial community (O'Toole *et al.*, 2000). This biofilm community has a number of distinct characteristics including the production of exopolysaccharides, chemical and pH gradients, a marked degree of structural heterogeneity and the development of high level of resistance to a wide variety of biocides (Hoyle *et al.*, 1991).

Formation of biofilms can have profound negative and positive impact in the environments. Biofilms determine water quality by influencing dissolved oxygen content, involve in nutrient cycling and act as sink for toxic or hazardous materials. Biofilms formed on the inert surfaces consist of mixed population of microorganisms and provide highly conducive environment for the treatment of both organic and inorganic wastewater contaminants from domestic and industrial units (Bryers and Characklis, 1990). Biofilms formed in food processing environments have the potential to act as a chronic source of microbial contamination that may lead to food spoilage or transmission of diseases. Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation (Bower and Daeschel, 1999; Joseph *et al.*, 2001).

Biofilm development is a multistep process. Bacteria approach the surface, attach and then become immobilized on the surface. They then move along the surface and

associate with one another to form microcolonies resulting in a three-dimensional structure. This structure is composed of pillars of bacteria surrounded by water channels that allow nutrients to reach biofilm associated bacteria and allow toxic metabolites to diffuse out of the biofilm (Costerton *et al.*, 1995). Surface adhesion and exopolysaccharide production by bacteria have been shown to depend on the inorganic and organic composition of the bathing medium. In particular, concentrations of Na^+ and Ca^{2+} in growth media have been shown to enhance biofilm development by diverse bacteria (Kim *et al.*, 1999). The carbohydrate composition of the growth medium influences exopolysaccharide production and biofilm development by many bacterial species (Allison *et al.*, 1994). Biofilms and microbial mats are thus important microbial communities in most aquatic ecosystems. Interestingly, the first known fossils of single microbes and microbial communities share almost identical structural characteristics found in recent biofilms and microbial mats (Schopf and Klein, 1992).

In the commercial shrimp (*Penaeus monodon*) hatcheries, larval mortalities due to luminous *Vibrio harveyi* have been reported from a number of countries (Lightner, 1983; Sunaryanto and Mariam, 1986; Tansutapanit and Ruangpan, 1987; Lightner, 1988; Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). Among *Vibrio* species, *V. harveyi* is the most important opportunistic pathogen associated with bacterial disease in shrimps. *V. harveyi* is a gram-negative bacterium naturally associated with tropical marine environment and intestinal tracts of marine fauna. This organism is recognized as an autochthonous flora of the marine environment (Baumann and Baumann, 1981) and the source of infection is suspected to be near shore waters (Lightner *et al.*, 1992). To combat this problem, most shrimp hatcheries use extensive water treatment such as sand filtration and chlorination. In spite of it, *V. harveyi* can be found in larval rearing tanks in considerable numbers (Karunasagar *et al.*, 1994).

The role of bacterial biofilms in the survival and persistence of *V. harveyi* and its possible role in perpetuating infection in shrimp hatcheries is a cause for concern (Karunasagar *et al.*, 1996). Cell surface hydrophobicity, presence of fimbriae and flagella and production of extracellular polysaccharides influence the rate and extent of attachment of microbial cells. Biofilm formation is one of the survival strategies of bacteria in adverse conditions where it undertakes a sessile mode of life. *V. harveyi* biofilm formation on different substrates such as cement, plastic (HDPE) and steel has been studied and it was

demonstrated that *V. harveyi* biofilms are resistant to sanitizers and antibiotics in contrast to planktonic cells of *V. harveyi*, which were found to be sensitive (Karunasagar *et al.*, 1996). Since *V. harveyi* biofilm is of great significance in hatcheries, it is important to study the factors involved in its formation especially the role of salt and organic substances that are an inherent part of seawater in hatchery environment.

Against this background, objectives of the present investigation were

1. To study the role of salts such as CaCl_2 , NaCl , KCl , MgCl_2 etc. in biofilm formation.
2. To study the effect of organic nutrient levels on formation of biofilm.

Review of literature

2. REVIEW OF LITERATURE

2.1 Definition of biofilm

According to McFeters *et al.* (1984), a biofilm consists of microorganisms and extracellular substances in association with a substratum. Characklis and Marshall (1990) define a biofilm as cells immobilized on a substratum and frequently embedded in an organic polymer matrix of microbial origin. Matthyse (1992) in the encyclopedia of microbiology defined biofilm as a group of microorganisms growing as a layer on a surface. According to Lappin-Scott *et al.* (1992) biofilm is a complex association of microorganisms and microbial products attached to a surface. A biofilm is a community of microbes embedded in an organic polymer matrix, adhesive to surface (Carpentier and Cerf, 1993, Costerton *et al.*, 1994). Biofilms are also defined as an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002).

2.2 Historical perspectives

The evidence of biofilm formation exists in the fossil record of hydrothermal environments. Microcolonies of biofilm have been identified by morphology in the 3.3-3.4 billion year old South African Kornberg formation (Westall, 2001) and filamentous biofilms in the 3.2 billion year old deepsea hydrothermal rocks of the Pilbara Craton, Australia (Rasmussen, 2000).

The first classical study of biofilms was by Zobell (1943), who suggested a two-step process for microbial colonization, an initial reversible step followed by an irreversible binding of the cell to the surface. Progress in biofilm research was relatively slow until the early 1970's, but subsequently researchers in various fields became aware of the universal association of microorganisms with surfaces or with each other (Marshall, 1976). Antoni Van Leeuwenhoek, using his simple microscope, first observed 'animalcule' on tooth surfaces and can be credited with the discovery of microbial biofilms (Costerton *et al.*, 1999). The bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach (Heukelekian and Heller, 1940). Characklis (1973) studied microbial slime in industrial water systems and showed that they were not only tenacious but also highly resistant to disinfectants such as chlorine. Costerton (1978) reported

the importance of biofilms and coined the term biofilm. He put forth a theory for biofilm formation that explained the mechanisms whereby microorganisms adhere to living and non-living materials. Microbial colonization of surfaces has been studied in a wide range of environments (Zobell, 1943; Gibbons and Van Houte, 1980; Zoltai *et al.*, 1981).

Research by Beech *et al.* (1991) showed that *Desulfovibrio desulfuricans* produced more polysaccharide when coupons made of mild stainless steel were incorporated in the growth medium. Over the last several years, biofilms have been recognized as a potential source of concern in food processing environments (Joseph *et al.*, 2001; Jeyasekaran *et al.*, 2000). Zoltai *et al.* (1981) used scanning electron microscopy to demonstrate the adherence of food borne microorganisms to food contact surfaces. The role of biofilm as source of pathogens in aquaculture has also been documented (Karunasagar *et al.*, 1996). Extensive work has been carried out on the role of bacterial attachment in ship hull fouling (Lewin, 1984), plaque formation and dental caries, infections from implanted medical prosthesis (Dankert *et al.*, 1986; Costerton *et al.*, 1987; Rosen *et al.*, 1998; Amita, 2004), fouling of heat exchangers (Lewin, 1984; Lehmann *et al.*, 1992) and fouling of water distribution systems (Ridgeway and Olson, 1981; Lechevallier *et al.*, 1987).

2.3 Biofilms in nature

Biofilms in nature can have a high level of organization, as they may exist in single or multiple species communities, form a single layer or three dimensional structures, or take the form of aggregates such as flocks or granules (Allison *et al.*, 1987; Bryers, 1987; Bagge *et al.*, 2001). In nature, biofilms offer a protective strategy that allows bacteria to survive in hostile environments. Bacterial biofilms colonize any humid surface. Some common biofilms include plaque on the teeth, slippery slime on river stones, gel like film on the inside of a vase which held flowers for a week, and infected tissue (Costerton *et al.*, 1999).

Biofilms as slime sites thrive wherever there is water; in the kitchen, on contact lenses, in the gut linings of animals etc. Until recently, the slimy conglomerations of bacteria were recognized for their propensity to coat and corrode pipes, clogging water filters and harbouring bacteria that contaminate drinking water (Donlan, 2001). Biofilms have become a problem of great concern in medical, industrial and environmental settings because these communities express biofilm-specific properties such as increased resistance to antibiotics, UV light and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production (Costerton *et al.*, 2000).

2.4 Heterogeneity of biofilm matrix

Tolker-Nielsen and Molin (2000) noted that every microbial biofilm community is unique although some structural attributes can generally be considered universal. A logical assumption is that any given cell within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus be growing at a different rate. Gradient of nutrients, water products and signalling factors contribute to this heterogeneity in biofilm. Heterogeneity has also been shown for protein synthesis and respiration activity as DNA content remains relatively constant throughout the biofilm (Xu *et al.*, 2000).

Biofilms are heterogeneous and components such as water, polysaccharides and other macromolecules contribute not only for the heterogeneity of the matrix but also for its multicellular function. Microcolonies of bacterial cells encased in an EPS (Extracellular Polymeric Substances) matrix are separated from each other by interstitial voids (water channels) (Lewandowski, 2000). Liquid flow occurs in water channels, allowing diffusion of nutrients, oxygen and even antimicrobial agents. This concept of heterogeneity is descriptive not only for mixed-culture biofilms, but also for pure-culture biofilms common on medical devices and those associated with infectious diseases. The matrix will change considerably as equilibrium between the species is established and a balance between competition and commensalism is achieved within the microbial community. Bacteriocins, microcins and bacteriophages also provide specific tools for the selective attack of bacterial cells within the mixed biofilms (Sutherland, 2001). Microscopic observations indicate that biofilms are not flat and the distribution of microorganisms is not uniform. Instead, multispecies biofilms were observed with complex structures containing "voids," channels, cavities, pores and filaments, and with cells arranged in clusters or layers. Such complex structures were found in a wide variety of biofilms such as methanogenic films from fixed-bed reactors (Robinson *et al.*, 1984), aerobic films from wastewater plants (Mack *et al.*, 1975; Eighmy *et al.*, 1983), nitrifying biofilms (Kugaprasatham *et al.*, 1992), and pure culture biofilms of *Vibrio parahaemolyticus* (Lawrence *et al.*, 1991) and *Pseudomonas aeruginosa* (Stewart *et al.*, 1993).

2.5 Biofilm Structure

Biofilm structure refers to the spatial arrangement of bacteria, cell clusters, EPS and particulates, which are known to influence the transport resistance, and hence it is a significant determinant in the activity of the biofilm. Various conceptual and mathematical models have been proposed to describe the structure and function of biofilms (Wanner and Gujer, 1986; Characklis, 1990a; Rittmann and Manem, 1992). Mathematical models describing transport, conversion, cell growth and biofilm development are based on conceptual models. Biofilms were initially considered as planar structures, impermeable and with homogeneous cell distribution. Mass transfer through the mass boundary layer and within the biofilm was assumed to be diffusional and perpendicular to the surface to which it was attached (the substratum).

Biofilms and mats are matrices of cells and extracellular polymeric substances (EPS). The EPS is produced by the cells, and consists of polysaccharides, polyuronic acids, proteins, nucleic acids and lipids (Decho, 1990; Decho and Lopez, 1993; Schmidt and Ahring, 1994). The conceptual model for biofilm structure proposed at the 1988 Dahlem conference (Wilderer and Characklis, 1989), divides the biofilm system into specific compartments; the substratum, the biofilm, the bulk liquid and a possible headspace. The biofilm compartment was further subdivided into a base film and a surface film. Although the model recognized a certain degree of biofilm roughness, it was essentially a planar-layered model.

2.6 Process of biofilms formation

2.6.1 Attachment

The first step in biofilm formation involves the adhesion of bacterial cells to a surface or interface which is a process that is dependent both on chance (i.e. whether or not the bacterium comes in direct contact with the surface) and on favourable cell-surface interactions to overcome the repulsive forces generated between the two surfaces (Geesey, 2001). A bacterial biofilm begins to form when individual cells initially attach to a surface (Costerton, 1995; O'Toole and Kolter, 1998). The ability of a cell to perform this initial attachment event is controlled by both environmental factors (including nutrient levels,

temperature and pH) and genetic factors (including the presence of genes encoding motility functions, environmental sensors, adhesins etc.) (Costerton, 1995; O'Toole *et al.*, 2000). The bacterium approaches the surface so closely that its motility is slowed and it forms a transient association with the surface and other microbes previously attached to the surface (Prakash *et al.*, 2003). Many extracellular appendages are involved in surface attachment by other bacterial species (O'Toole *et al.*, 2000).

The solid-liquid interface between a surface and an aqueous medium (e.g. water, blood) provides an ideal environment for the attachment and growth of microorganisms (Costerton *et al.*, 1999). Chamberlain (1992) investigated contact of various extracts of animal products with surfaces like stainless steel, polypropylene and polyethylene which differed widely in free energy and measured similar contact angles for each of these surfaces. Kjellberg *et al.* (1982) have observed a generation time of 57 min for *Vibrio* species growing at the liquid-solid interface whereas, the same organism in the liquid phase showed no detectable growth. In study involving *Pseudomonas* spp, 73% of the adhering bacteria attached irreversibly in 4hrs (Marshall, 1992).

In general, attachment occurs most readily on surfaces that are rough, more hydrophobic and coated by surface 'conditioning' films. An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels. Properties of the cell surface, such as presence of fimbriae, flagella and surface associated polysaccharides or proteins, are also important and may possibly provide a competitive advantage for one organism where a mixed community is involved (Donlan, 2002). In marine *Pseudomonas* species studied, one of the extracellular polysaccharides is partly released at the end of the exponential growth phase associated with the increased bacterial adhesion to hydrophobic surface (Fletcher and Loeb, 1979; Christensen *et al.* (1985). Shea *et al.* (1991) showed that temperature also has an impact on the degree of adhesion of a microorganism.

Cell surface properties such as flagella, pili, adhesin protein, capsules and surface charge influence attachment (Kumar *et al.*, 1998). The extent of microbial colonization appears to increase as the surface roughness increases (Characklis *et al.*, 1990). Mittelman (1996) noted that a number of host-produced conditioning films such as blood, tears, urine, saliva, intervascular fluid and respiratory secretions influence the attachment of bacteria to biomaterials. Marshall *et al.* (1971) provided evidence based on SEM (Scanning Electron

Microscopy) that attached bacteria were associated with the surface via fine extracellular polymeric fibrils. Korber *et al.* (1989) used motile and non-motile strains of *P. fluorescens* to show that motile cells attach in greater numbers and attach against the flow more rapidly than do non-motile strains.

2.6.2 Microcolony formation

After the bacteria adhere to the inert surface or living tissue, the association becomes stable for microcolony formation. Microcolonies, a community of bacterial cells three to five layers deep, develop following the adhesion of bacterial cells to a surface (Davey and O'Toole, 2000). Clonal growth and stable cell-cell interactions are needed to form and hold the microcolony together (Reisner *et al.*, 2003).

Microcolony formation will proceed after irreversible attachment, given appropriate growth conditions. Microcolony formation results from simultaneous aggregation and growth of microorganisms and is accompanied by the production of EPS. The bacteria begin to multiply while emitting chemical signals that “intercommunicate” among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated (Costerton, *et al.*, 1999). In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony (McKenney *et al.*, 1998).

P. aeruginosa, *Escherichia coli*, and *V. cholerae* lose their flagella and increase their EPS production upon attachment to a surface (Davey and O'Toole, 2000). Cooksey (1992) suggested that adhesion itself stimulates the synthesis of the EPS which enable build up of a biofilm. The type IV pilus of *P. aeruginosa* has multiple effects on biofilm formation, one of which is to enable microcolony formation under some conditions (O'toole and Kolter, 1998; Heydorn *et al.*, 2002).

In *Staphylococcus aureus* and *S. epidermidis*, microcolony formation is mediated by the production of polysaccharide intracellular adhesin (PIA). Several environmental factors influence the production of PIA, including glucose, anaerobiosis, high osmolarity, high temperature, ethanol and iron limitation (Gotz, 2002). Consistent with sigma -B affecting microcolony formation, a strain of *S. aureus* over expressing sigma-B has enhanced microcolony development (Bateman *et al.*, 2001). In *Bacillus subtilis*, the transcription factor

SpoOA regulates the transition from surface attached cells into microcolonies (Hamon and Lazazzera, 2001).

2.6.3 Formation of three- dimensional structure and maturation

Under conditions suitable for sufficient growth and agglomeration, biofilm in nature may develop an organized structure. This process is called maturation. After the initial stages of surface attachment and microcolony formation, mature biofilm structure can form. These structures are encased in an extracellular polymeric matrix. Biofilms can be thick, homogeneous mats of cells, or they can be complex structures composed of pillars with water channels that have been proposed to allow for nutrient influx and waste efflux (Davey and O'Toole, 2000). The structure formed by a bacterial species can vary depending on processes that are not regulated by bacteria, such as physical shear forces. However, the structure can also vary as a result of processes that are regulated by bacteria. Regulatory mutants have been identified that affect either the overall depth of the mature biofilm or the architecture of the biofilm.

The mature biofilm may consist of a single layer of cells in porous extracellular polymer or multilayered loosely packed microcolonies held together with EPS and interspersed with water channels. Lawrence and others (1991) observed the spatial redistribution of cells after microcolony formation to produce the mature biofilm structure by using confocal laser microscopy.

2.6.4 Detachment

Bacteria from the biofilm are released due to mechanical shear force or stoppage of EPS production. Biofilm cells may be dispersed either by shedding of daughter cells from actively-growing cells, or detachment as a result of nutrient levels or quorum- sensing, or shearing of biofilm aggregates because of flow effects (Baselga, *et al.*, 1994). The biofilm once formed does not increase in size indefinitely as there are factors that bring about the removal of sessile cells or sections of the biofilm by sloughing or shearing depending on thickness (Characklis, 1990; Wimpenny *et al.*, 2000). As the thickness of the EPS increases, anaerobic conditions develop within the biofilm with loci of the biofilm consisting of anaerobic bacteria. Because of film thickness and the activity of anaerobic species, the film

detaches and sloughs-off from the surface of the substrate (Howell, *et al.*, 1976). Bryers (1987) suggests that the anaerobic condition results in an increase in acid and insoluble gas accumulation by the activity of anaerobic bacteria that weakens the biofilm structure, resulting in sloughing. Brading *et al.* (1995) have emphasized the importance of physical forces like erosion or shearing (continuous removal of small portions of the biofilm), sloughing (rapid and massive removal) and abrasion (detachment due to collision of particles from the fluid with the biofilm) in detachment. Bryers (1987) states that after biofilm reaches a certain thickness, the rate of biofilm removal increases under constant laminar flow. Polysaccharide enzymes such as alginate lyase specific for alginate, major component of EPS of *P. aeruginosa* may also contribute to detachment (Boyd and Chakrabarty, 1994). Detachment of cells or biofilm aggregates may result in blood stream or urinary tract infections leading to the production of emboli (Donlan, 2002). Sequence of detachment includes release of planktonic bacterial cells from biofilms, an natural pattern of programmed detachment of these cells, which can colonize on other surfaces or individuals to form new microcolonies (Baselga, 1994).

2.7 BACTERIAL GLYCOCALYX AND EPS

Bacterial glycocalyx is defined as those polysaccharide-containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of gram-positive cells. The glycocalyx may be composed of fibrous polysaccharide or globular glycoproteins. The role of bacterial glycocalyx in adhesion to sister cells and to inert tissue substrata to form adherent microcolonies that are the predominant form of bacterial growth in nature and disease has been established (Costerton *et al.*, 1981). Glycocalyx is involved in the non specific adhesion of bacteria to inert surfaces in aquatic ecosystems and these anionic EPS molecules are typical of many fibrous molecules that initiate and cement cell surface interactions in many areas of aquatic biology (Matthysse, 1992).

The bacterial glycocalyx mediates the formation of microcolonies, which constitutes the predominant mode of growth in many natural and pathogenic systems. These microcolonies may adhere to inert or tissue surfaces by the interaction of these glycocalyxes and/or by their pili with surface components. Autochthonous bacterial populations that contribute both physiological and protective benefits to animal tissue grow in glycocalyx

enclosed microcolonies to facilitate their adhesion and persistence on the colonized tissue and some of these autochthonous organisms use the same mechanism to colonize normally sterile tissue of compromised hosts and to initiate slow developing cryptic infections in which they persist in spite of vigorous immune response (Costerton *et al.*, 1981). The bacterial glycocalyx increases resistance of bacteria to surfactants, which form the first line of defense against bacterial colonization. In many animal tissues glycocalyx enclosed cells are less efficiently phagocytized by various animal cells that are activated in response to bacterial presence (Costerton *et al.*, 1981).

The theory of phenotypic plasticity states that without genomic mutations, bacteria can respond to alternations in this microenvironment by profound changes in their structure and metabolic processes. Production of an elaborative glycocalyx in a competitive multispecies environment and their subsequent loss during culture in non-competitive single species culture is a typical example for this (Matthysse, 1992).

Biofilms are composed primarily of microbial cells and EPS. This EPS account for 50% to 90% of the total organic carbon of biofilms (Flemming *et al.*, 2000). The EPS synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties. Some are neutral macromolecules, but the majority is polyanionic due to the presence of either uronic acids (D-glucuronic acid being the commonest, others being D-galacturonic and D-mannuronic acids) or ketal-linked pyruvate. Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status (Sutherland, 1990). Some EPS may even be polycationic, as in case of adhesive polymer obtained from strains of staphylococci that consists of teichoic acid mixed with small quantities of proteins (Hussain *et al.*, 1993; Mack *et al.*, 1996). EPS may contain polysaccharides, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances hydrated to 85% to 95% water (Costerton *et al.*, 1981; Sutherland, 1983). EPS provides protection to the biofilm inhabitants by concentrating nutrients, preventing access of biocides, sequestering metals and toxins, and preventing desiccation (Carpentier and Cerf, 1993). Many bacterial EPS possess backbone structures composed of sequence of 1,3-or 1,4-beta linked hexose residues. When such sequences are present, the polymers tend to be much more rigid in structure, less deformable and in the case of polysaccharides such as mutan or those from some strains of *Enterobacter agglomerans*, either poorly soluble or effectively insoluble. These EPS molecules may be very robust. The long chains of stiff macromolecules may be present as gels due to the

entanglements found within the long chains and also, in some polymers, to the ionic environment (Ross-Murphy and Shatwell, 1993; Ross-Murphy, 1995).

Though it is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms (Allison and Sutherland, 1987; Watnick and Kolter, 1999), they may be still attach to surfaces and form microcolonies to a limited extent. However, when the bacteria are components of mixed biofilms, the presence of one species producing copious amounts of EPS may enhance the stability of other cell types even if they do not themselves synthesize EPS. Such stabilizing effects were commensal interactions. As pointed out by Skillman *et al.* (1991), the proportions of different EPS in mixed biofilms do not necessarily reflect the proportions of the cells present, nor do the EPS contribute equally to the structure and properties of the resulting biofilms. Leriche *et al.* (2000) showed that different organisms produce differing amount of EPS and that the amount of EPS increases with age of the biofilm. Flemming *et al.* (2000) reported that EPS might associate with metal ions, divalent cations, and other macromolecules such as proteins, DNA, lipids and even humic substances. Chemically EPS is colonic acid in *E.coli* and alginate in *P. aeruginosa*. So EPS is important for the development of typical biofilm architecture (Davey and O'Toole, 2000).

EPS can mask the original surface properties of the cells and render hydrophobic surfaces to hydrophilic. The phenomenon of flotation of anaerobic aggregates occurs by attachment of gas bubbles to the hydrophobic aggregate surface. Aggregates with low amounts of EPS showed a strong tendency to float, leading to severe biomass losses from the reactors. The presence of carbohydrates in the feed increases the amount of EPS, especially on the aggregate surface, inhibiting attachment of gas bubbles and preventing flotation (De Beer, 1996; Neu and Lawrence, 1997).

2.8 Regulation of biofilm formation

2.8.1 Surface

The surface may have several characteristics that are important in the attachment process. Characklis *et al.* (1990) noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because of less shear forces and more surface area on rougher surfaces. Most of the microorganisms attach more rapidly to

hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Fletcher and Loeb, 1979). The surface could be a dead or living tissue, or any inert surface. The attachment of microorganisms to the surface is a complex process, with many variables affecting the outcome (Pratt and Kolter, 1998).

A material surface exposed in an aqueous medium becomes conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment. Conditioning films are formed on surfaces exposed in seawater. These are organic in nature, formed within minutes of exposure, and continue to grow for several hours (Loeb and Neihof, 1975). A prime example may be the proteinaceous conditioning film called 'acquired pellicle', which develops on tooth enamel surfaces in the oral cavity.

2.8.2 Nutrients

Nutrient availability has a major influence on biofilm structure and the composition of the microbial community. Studies showing the effect of nutrient on pure culture of *Listeria* biofilm development (Kim *et al.*, 1995) have suggested that low levels of phosphate initially stimulate biofilm development, but after several days the effect was reduced.

Increase in nutrient concentration correlated with an increase in the number of attached bacterial cells (Cowan *et al.*, 1991). However, nutrient concentrations too low to measure are sufficient for biofilm growth. Biofilm bacteria acquire nutrients by concentrating trace organics on surfaces by the extracellular polymer, using the waste product from their neighbours and secondary colonizers, and by pooling their biochemical resources with different enzymes to breakdown food supplies. Because the biofilm matrix is often negatively charged, many nutrients (particularly cations) are attracted to the biofilm surface. Besides, nutrients with negative charge can exchange with ions on the surface.

2.8.3 Environmental cues

Other characteristics of the aqueous medium, such as pH, nutrient levels, iron, oxygen, ionic strength and temperature, may also play a role in the rate of microbial attachment to a substratum (O'Toole *et al.*, 2000). Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems. (Donlan *et al.*, 1994). This effect may be due to water temperature or other unmeasured, seasonally

affected parameters. Fletcher (1998) found that an increase in the concentration of several cations (Na^+ , Ca^{2+} , La^{3+} , Fe^{3+}) affected the attachment of *P. fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces.

2.9 Biofilms and antimicrobial resistance

The bacteria enclosed within the biofilm are extremely resistant to antibiotic treatment. The EPS secreted by biofilm bacteria, act as a physical/chemical barrier, thus preventing penetration by antibodies or many antibiotics (Thien and O'Toole, 2001). Moreover, EPS is negatively charged and functions as an ion-exchange resin, which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells. Embedded biofilm bacteria are generally not actively engaged in cell division, smaller in size and less permeable to antibiotics. Virtually all antimicrobials are more effective in killing rapidly growing cells. Further, transition from exponential to slow/no growth is generally accompanied by expression of antibiotic-resistant factors (Wentland *et al.*, 1996).

Some of the antibiotic degrading enzymes such as (β -lactamase) may also be immobilized in the EPS matrix, so that the incoming antibiotic molecules can be inactivated effectively. It is interesting to note that biofilm cells of the *P. aeruginosa* have been shown to produce 32 fold more β -lactamase than cells of the same strain grown planktonically (Potera, 1999). Up to 40% of the cell-wall protein composition of bacteria in biofilm is altered from that of its planktonic brethren (Potera, 1999; O'Toole *et al.*, 2000). The membranes of biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage, or even antibiotics targets may disappear.

The antimicrobial agent is deactivated faster than it diffuses in the outer layers of the biofilm. This is true for reactive oxidant such as hypochlorite and H_2O_2 (De Beer *et al.*, 1994; Thien and O'Toole, 2001). These antimicrobial oxidants are product of the oxidative burst of phagocytic cells and poor penetration of these may partially account for the inability of phagocytic cells to destroy biofilm microorganisms.

Biofilms also provide an ideal niche for the exchange of extra chromosomal DNA responsible for antibiotic resistance, virulence factors and environmental survival capabilities at accelerated rates, making it a perfect milieu for emergence of drug resistance pathogens (Ghigo, 2001; Donlan, 2002). Plasmid carrying strains have also been shown to transfer plasmids to recipient organisms, resulting in biofilm formation; without plasmids, the same organisms produce only microcolonies without any further development. The probable reason for enhanced conjugation is that biofilm environment provides minimal shear and closer cell-to-cell contact. Since plasmids may encode for resistance to multiple antimicrobial agent, biofilm association also provides a mechanism for selecting and promoting the spread of bacterial resistance to antimicrobial agents.

2.10 Methods for Studying Biofilms

A variety of experimental observational methods have been developed for the study of bacterial attachment and colonization. One of the most widely used reactors for biofilm study is the flow cell. A simple system consists of two microscopic slides and a spacer (Caldwell and Lawrence, 1988; Lawrence *et al.*, 1991; Wolfaardt *et al.*, 1995), which is ideal for microscopic monitoring of biofilms. This holds a small volume, hence a one-way medium supply can be used. It can also be easily sterilized and used for pure cultures. A more complicated flow cell is needed when the biofilm is monitored with microsensors. Either a closed flow cell with openings for introduction of the sensors (De Beer *et al.*, 1994) or an open flow cell (Horn and Hempel, 1997) can be used. Since these flow cells are larger, a recycle is needed to obtain good mixing of the water phase. A special type of flow cell is the Robbins device (Whiteley *et al.*, 1997; Sly *et al.*, 1990), essentially a pipe with sample holders, with the surface flush with the lining of the pipe. The sample holders are removable, allowing multiple biofilm sampling. Also, flow cells with observation windows for microscopy have been equipped with pressure and flowmeters to study the interaction of hydrodynamics and biofilm accumulation (Stoodley *et al.*, 1998).

A special flow cell was developed for immobilization of marine snow flocs (Ploug and Jorgensen, 1998). The device (called net-jet) consists of a cylinder with a fine stocking separating the top and bottom part. The hydrodynamics are not well understood, but this simple device allows fixation of flocs in an upward water stream. The flocs can be penetrated by microsensors under settling conditions, resembling the normal hydraulic regime. In this

flow cell, also activated sludge flocs with a diameter of 0.2–1.5 mm were investigated (De Beer, 1998). If flow cells are well designed, the hydrodynamics can be well characterized (in contrast with many other types of bioreactor) allowing the influence of fluid shear and mass transfer on biofilm processes such as adhesion, detachment and biotransformation rates.

The annular reactor is essentially a chemostat consisting of a cylinder rotating in an outer cylinder with the reactor content between the outer and inner cylinder (Characklis, 1988). The outer cylinder can be equipped with removable sample plates, that are flush with the surface. The inner cylinder rotates at variable speed, thus allowing adjustment of the hydraulic regime over a wide range, independent of the residence time of the medium. With this device, many studies have been done on initial biofilm formation (Escher and Characklis, 1990), the effect of biofilms on shear stress (Characklis, 1990), and the effect of hydraulics on biofilm formation (Gjaltema *et al.*, 1994). It is difficult to maintain a pure culture in these reactors. The hydraulics are not well described and not uniform (Gjaltema *et al.*, 1994). Therefore, this device cannot easily be used for quantitative studies.

Hydrodynamics are important for the attachment of cells and development of biofilm. The Fowler cell (Fowler, 1988) is a radial flow cell, consisting of two plates mounted parallelly. The inlet is mounted in the center so that flow occurs radially, from the center to the periphery. The flow velocity is the highest in the center and decreases with increasing radial distance. The shear forces can be calculated assuming a flat geometry.

The modified robbins device (MRD) consists of a square or rectangular channel in a polycarbonate block in which 25 sampling-port studs are inserted along the length of the MRD (Hall-Stoodley *et al.*, 1999). The studs can be fitted with different materials to investigate biofilm formation on different surfaces. The MRD is usually sterilized with ethylene oxide and the studs can be removed aseptically. The advantage of the system is that several samples can be taken simultaneously at different times to study biofilm development.

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), normal light microscopy (LM), fluorescence microscopy (FM) and confocal scanning laser microscopy (CSLM) all have been used to study biofilms (Surman *et al.*, 1996). Most microscopic methods involve some preparation of the sample, including staining, fixation, freezing, dehydration, embedding, and sectioning. For this reason, it is important to realize

that biofilms are soft and mostly consists of water (<95%) (Christensen and Characklis, 1990). Preparations for microscopy may significantly change the matrix structure by shrinking and deformation (Stewart *et al.*, 1995), and the resulting artifacts have influenced the concept of biofilm structure for years. Most relevant is the underestimation of the spatial heterogeneity, as several steps in the preparation may level the soft biofilm structures. Then, EPS appears as strands connecting the cells. EPS morphology changes by dehydration: diffuse polymeric matter is condensed to strands leading to overestimation of the pore-size. From SEM images, the pore-size appears to be in the order of 1 μm . Good TEM preparations show a pore size of ca. 100 nm (Beeftink and Staugaard, 1986).

Lawrence *et al.* (1991) published an excellent description of confocal microscopy techniques for biofilm research. Scanning electron microscopy, environmental scanning electron microscopy (ESEM) and atomic force microscopy (AFM) can be used for surface scanning, while the other techniques to some extent allow observation below the surface.

Specific staining is an important tool to unravel the spatial distribution of different biofilm components, most importantly in cells, EPS and voids. For viewing cells, stains nonspecific for DNA, such as acridine orange, diamidino-phenylindole (DAPI), ethidium bromide, and hexidium iodide are most useful. These dyes can be combined with confocal microscopy, thus giving an image of cell distributions in undisturbed biofilms or mats. Species-specific staining by oligonucleotide probes or antibodies will be treated elsewhere.

Much less attention has been paid to visualization of EPS. Staining of EPS for fluorescent microscopy or CSLM is possible for proteins (fluorescein isothiocyanate), polyuronic acids and polysaccharides (lectin conjugates, calcofluor). Calcofluor stains most polysaccharides (attaching to β -1, 4 and β -1, 3 polysaccharides; Haigler *et al.*, 1980), while lectins are more specific. EPS dyes will stain cells that become visible as discrete points, whereas EPS appears as a continuous sheet. *The Handbook of Fluorescent Probes and Research Chemicals* (a catalogue of molecular probes) is a highly valuable source of information about dyes and staining techniques (Haugland, 1996). Also, EPS can be stained by ruthenium red for TEM, or observed directly by SEM. Voids can be made visible with negative staining using fluorescein, that is quenched by the presence of biomass. Using CSLM, voids appear as bright fluorescent areas, while biomass remains dark (Lawrence *et al.*, 1991; De Beer *et al.*, 1994). Also fluorescent microbeads can be used, that penetrate the voids, but not the cell clusters (Stoodley, 1998).

A microtiter plate procedure is also one of the important method for estimation of bacteria *insitu* and can be modified for various biofilm assays (An and Friedman, 2000). O'Toole and Kolter (1998) used this assay with *P. fluorescens* to evaluate the impact of various growth conditions and environmental signals and as a genetic screen for biofilm-deficient mutants. Stepanovic *et al.* (2000) modified microtiter plate to test for quantification of staphylococcal biofilm formation. Djordjevic *et al.* (2002) standardized microtiter plate assay to compare the ability of *L. monocytogenes* to form biofilms and they showed that this method can be used as a rapid, simple method to screen for differences in biofilm formation between strains or growth conditions prior to performing labor-intensive microscopic analysis.

2.11 Relevance of bacterial biofilms

Microbial biofilms are attracting attention of scientists in different areas such as the medical field, aquatic environment, food processing industries etc. Microbial biofilms are detrimental and undesirable in food processing premises. Biofilms by pathogenic bacteria such as *Salmonella* (Somers *et al.*, 1994; Dhir and Dodd, 1995; Humphery *et al.*, 1995; Jones and Bradshaw, 1996), *Klebsiella* (Jones and Bradshaw, 1996; Morin *et al.*, 1996), *Pseudomonas* (Brown *et al.*, 1995), *Campylobacter* and enterohaemorrhagic *E. coli* (Somers *et al.*, 1994) and *Listeria* (Mafu *et al.*, 1990; Ren and Frank, 1993) have been reported. Such biofilms could be a continuous source of contamination to foods coming in contact with them when formed on contact surfaces. Increased resistances of these biofilms to antibacterials have also been reported (Costerton *et al.*, 1987; Carpenter and Cerf, 1993; Ren and Frank, 1993).

Bacterial biofilms cause 'biofouling' in a wide variety of industrial settings. Biofilms grow inside pipelines transporting a myriad of substances, including potable water, oil, chemicals and fire extinguishing agent. In marine settings, biofilms reduce the hydrodynamic efficiency of ships and propellers. Fire protection systems represent a particularly complex challenge for biological fouling prevention and control (Mittelman, 2001). The biofilms that build up in ships hulls consist essentially of diatoms, single celled algae and bacteria. A biofilm on the hull of a ship slows it down and increases fuel consumption (Cooksey and Wigglesworth, 1992). Microbial colonization also lowers the performance levels of periscopes and submarine probes. In food industry, the formation of biofilm by pathogenic

bacteria on cutting boards and other processing equipments cannot be easily removed or killed by disinfectant or normal cleaning processes (Ben Embarck, 1994). Similarly, the bacterial biofilms of pathogens associated with food-borne diseases are of great concern in the food processing industry. Development of biofilm by sulphate reducing or acid producing bacteria, it can cause corrosion. These microorganisms create anodes and cathodes on metal surfaces with unequal distribution of ions resulting in a metal loss (Costerton and Lappin-Scott, 1989). The output of power station heat exchanges, which can have up to 100 km of tubing is affected by biofilms that form on the surface in contact with the river water used for cooling (Bott, 1992). Fungal biofilms associated with drinking water distribution systems have received recent attention due to health related problems posed by them (Doggett, 2000). In filtration systems, Flemming *et al.* (1992) observed biofilms up to 500 μM thick. They greatly reduce the permeability of the membranes.

The human and animal organs represent surface structures for the colonization of a number of pathogenic bacteria such as *P. aeruginosa* (lungs), *Ureaplasma urealiticum* (urogenital tract), *S. aureas* (nasal mucosal cells) and *E. coli* (ileal cells), which are associated with variety of diseases (Marshall, 1991; Glass *et al.*, 2000; Singh *et al.*, 2000). Biofilms can also form on catheters and implants (Ory *et al.*, 1987; Croize *et al.*, 1989; Sheretz *et al.*, 1990). Biofilms on urinary catheters may contain organisms that have the ability to hydrolyze urea in the urine to form free ammonia through the action of urease. The ammonia may then raise the pH at the biofilm liquid interface, resulting in the precipitation of minerals such as calcium phosphate (hydroxyapatite) and magnesium ammonium phosphate (struvite) (Tunney and Jones, 1999). It is also well established that the microorganisms colonizing the tooth surface are of serious health concern in the dentistry. The biofilm of *Streptococcus mutans* and *S. sanguis* were associated with tooth surface (Gibbons and Van Houte, 1980). Biofilms formed on indwelling medical devices serve as a reservoir of bacteria that can be shed into the body, leading to a chronic systemic infection. Indeed, upto 82% of nosocomial bacteremias are the result of bacterial contamination of intravascular catheterizations (Archibald and Gaynes, 1997).

Aquaculture systems especially hatcheries (fish and shrimp) are another area which face a threat from bacterial biofilms. Bacterial shrimp pathogen (*V. harveyi*) formed antibiotic and chlorine resistant biofilms on HDPE and cement, which are common substrates, encountered in hatcheries (Karunasagar *et al.*, 1996). This being the case, the role of bacterial

biofilms in causing large scale mortalities and disease out breaks in aquaculture systems is even more convincing. *V. alginolyticus* was found to cause gill disease leading to progressive low level mortalities among juvenile turbot, *Scophthalmus maximus* (Austin *et al.*, 1990). Epidemic mortalities of Chinook salmon alevins (*Oncorhynchus tshawytscha*) occurs due to harborage of a bacterial biofilm on the gills has been reported (Newbound *et al.*, 1993).

2.12 Prevention and control of biofilms

Biofouling is the detrimental development of biofilms in engineered systems, such as industrial process equipment, drinking water distribution systems, and ship hulls. Biofilms can decrease heat transfer in heat exchangers, increase the pressure drop in pipelines, enhance corrosion, and may be a source of bacterial contamination of drinking water (McCoy, 1987; Characklis, 1990; Camper, 1994). Biofilms are a nuisance in these systems and control of their development may be necessary to maintain process efficiency and safety. Biofilm control is often performed with biocides, of which the most commonly used is chlorine, a strong oxidizing agent and disinfectant. Biocides are much less effective against biofilms than suspended cells (LeChevallier *et al.*, 1988; Nichols, 1988; Chen *et al.*, 1993).

Biological control by phage and its associated specific polysaccharide depolymerases could be used to selectively eliminate particular species of bacteria from mixed community biofilms (Huges *et al.*, 1998). Huges *et al.* (1998) reported a wide distribution of bacteriophages active on biofilm of gram-negative bacteria in nature. The combination of polysaccharide hydrolyzing enzymes and oxidoreductases are known to cause both removal and inactivation of bacterial biofilms (Johansen *et al.*, 1997). Possibility of biofilm elimination due to biofilm self-destruction has also been observed. As the oxygen gets depleted by the growing biofilm mass, a specific exopolysaccharide lyase is induced which digests the biofilm matrix, liberating the cells (Allison *et al.*, 1998).

Czechowski (1991) suggested that chlorinated alkaline detergents are the most effective for destructing the biofilm, followed by non- chlorinated alkaline detergents. Acid products are less effective than hot water. Lewis *et al.* (1989) showed that removal of an *Acinetobacter* sp increased with pH (about 100 times as many bacteria are detached at pH 12 as at pH 2) and, to lesser extent, with temperature (about 10 times as many bacteria are detached at 80 °C as at 10 °C). Nutrient and water limitation, equipment design, and temperature control are important in biofilm control. Since it is often not possible to reduce

water availability, improve equipment design, or reduce operating temperatures, the biofilm control efforts are most often aimed at effective cleaning of potential growth sites (Frank, 2000).

Augustin *et al.* (2004) concluded that the choice of disinfectant or cleaning agent along with the optimum concentration and the time of action is very important when destroying microbes present in the Biofilms. They also suggested that the resistance of microbes to different disinfectants and cleaning agents to be taken into account when planning the cleaning process. The extent to which the biofilms is removed also depends on the bacterial strain concerned (Holah *et al.*, 1989). It has been shown that exposing biofilms to electric currents or to ultrasound in the presence of antibiotics has a synergetic effect that can achieve killing of all the organisms in the biofilm (Wellman *et al.*, 1996; Rediske *et al.*, 2000). Some substances are known to improve performance in detaching biofilms. Cations like Ca^{++} play important role in bonding polymer molecules in the Biofilms and absence of these ions or their chelation by compounds like EDTA can lead to detachment of Biofilms. (Turakhia *et al.*, 1983).

Material and Methods

3. MATERIALS AND METHODS

3.1 Bacterial isolates

A total of 56 *V. harveyi* isolates maintained by keeping at -80°C in the Department of Fishery Microbiology, College of Fisheries, Mangalore were used for the study. These cultures were maintained in sterile TSAS slants and subjected to a battery of biochemical tests for the confirmation purpose.

Tryptone Soya Agar with 1% NaCl (TSAS)

Tryptone	1.7%
Soy peptone	0.3%
Sodium chloride	1.0%
K ₂ HPO ₄	0.25%
Agar	1.5%
pH	7.3 \pm 0.2

The ingredients were dissolved in 100 ml of distilled water, sterilized at 121°C for 15 min and poured in sterile petriplates.

3.2 Bacterial identification

A series of biochemical tests (Mac Faddin, 1980) were performed to identify bacteria. Identification scheme of West and Colwell (1984) was used for identification of *V. harveyi*.

3.2.1 Gram's staining

Hucker's modification of gram staining procedure was followed. After staining, slides were observed under oil immersion objective to record the Gram reaction of each isolate.

3.2.2 Motility test

16 to 18 h cultures of bacteria, grown in TSB containing 1 % NaCl were used for observing motility by hanging drop technique using cavity slide.

Tryptone Soya broth with 1% NaCl (TSBS)

Tryptone	1.7%
Peptone	0.3%
NaCl	1.0%
K ₂ HPO ₄	0.25%
pH	7.3 \pm 0.2

These ingredients were dissolved in 100 ml distilled water and sterilized by autoclaving at 121⁰C for 15 min.

3.2.3 Oxidation fermentation test (O/F test)

Hugh and Leifson's O/F medium was used to test whether the organism was fermentative, oxidative or inert. About 2.5 ml each of media were poured into a set of two tubes and autoclaved. Organisms were stabbed into the butt and one of the tubes was overlaid with liquid paraffin. Fermentative organisms produced acid in both tubes and oxidative organisms produced acid in tube that is not over laid liquid paraffin. Inert organisms failed to produce acid and hence medium colour was unchanged.

Hugh Leifson O/F medium

Peptone	0.2%
Yeast extract	0.5%
Sodium chloride	1.0%
Glucose	1.0%
Bromocresol purple	0.0015%
Agar	0.3% - 0.4%
pH	7.2 ± 0.1

The ingredients were added to 100 ml of distilled water and boiled to dissolve the agar. The medium was then distributed in 2.5 ml volume in test tubes and sterilized at 110⁰C for 15 min.

Liquid paraffin

This was sterilized at 160-180⁰ C for 1¹/₂ - 2 h in a hot air oven. Liquid paraffin was used to create anaerobic environment in the fermentative tubes.

3.2.4 Oxidase test

Cytochrome oxidase test was performed using pre-moistened filter paper strips soaked with 1% oxidase reagent. Young colonies of bacteria were spotted on the oxidase paper using sterile toothpicks. Development of dark purple colour within 10 sec indicated positive reaction.

Oxidase reagent

Oxidase reagent (N,N,N',N' - tetramethyl p-phenylene diamine dihydrochloride)	1.0 g
Distilled water	100 ml

Whatman filter paper No.1 was cut into strips of 2.5 x 1.0 cm, sterilized in hot air oven at 140°C for 1h. The strips were later dipped in pre prepared oxidase reagent, allowed to absorb and then dried. The strips were stored in dark bottle at 4°C.

3.2.5 Sensitivity to O/129 compound

A lawn culture of the test organism was prepared on TSAS plates and O/129 disc (150 µg) placed at the center of the lawn. Development of a clear zone of inhibition around the disc was recorded as 'sensitive' and the absence of clear zone of inhibition indicated 'resistance' to the pteridine compound.

O/129 reagent

O/129 (2-4, diamino 6-7 diisopropyl pteridine)	15 mg
Acetone	1 ml

O/129 compound was dissolved in acetone and 100 discs of 6.5 mm diameter each (pre-sterilized at 140°C for 1 h) were soaked in the solution, dried and stored at 4°C. Each disc contained 150 µg of the pteridine compound.

3.2.6 Amino acid decarboxylase test

Ability of the microorganism to decarboxylate aminoacids lysine, ornithine and arginine was tested by inoculating test cultures into media containing amino acids in separate tubes and to a tube with only basal medium which served as a control. After overlaying with liquid paraffin, all the tubes were incubated at 30°C. The change in the colour of the indicator from purple to yellow and back to purple was considered as positive for decarboxylation.

Basal medium for amino acid decarboxylase test

Peptone	0.5%
Yeast extract	0.3%
Sodium chloride	1.0%
Glucose	0.1%
Bromocresol purple	0.0016%
pH	7.2 ± 0.2

The basal medium was divided into four parts and aminoacid lysine, ornithine and arginine were added individually to each quarter at a concentration of 0.5, 0.5 and 0.4% respectively. The last quarter served as control. The media was distributed into tubes in 4 ml volumes and sterilized at 110°C for 15 min.

3.2.7 Sugar fermentation test

Different sugars viz. glucose, sucrose, arabinose, mannose and inositol were used as substrates to test the ability of the bacterial cultures to utilize them with resultant production of acid. Cultures were inoculated into pre-sterilized individual sugar media in tubes and colour change from purple to yellow and collection of gas in Durham's tube indicated fermentation of the sugar.

Sugar fermentation medium

Peptone	1.0%
Sodium chloride	1.0%
Sugars	1.0%
Bromocresol purple	0.0016%
pH	7.1 ± 0.2

The ingredients were dissolved in distilled water and then dispensed in 4 ml volume into test tubes containing Durham's tubes and sterilized by autoclaving at 110°C for 10 min.

3.2.8 Indole test

The cultures were grown in tryptophan broth for 24-48 h and then a few drops of Kovac's reagent were added. Formation of a pink indole ring at the surface of culture was recorded as a positive reaction.

Tryptophan broth

Tryptophan	1.0 %
Sodium chloride	1.0 %
pH	7.1 ± 0.2

The ingredients were dissolved in 100 ml distilled water. This was distributed in 5 ml volumes into test tubes and autoclaved at 121°C for 15 min.

Kovac's reagent

p-Dimethyl aminobenzaldehyde (DMAB)	5.0 g
Amyl alcohol	75 ml
Conc. Hydrochloric acid	25 ml

DMAB was dissolved in amyl alcohol and then the conc. HCl was added slowly. The solution was stored at 4°C in a dark bottle and used to perform the test whenever required.

3.2.9 Salt tolerance test

This test was carried out to determine the ability of bacteria to grow at varying concentrations of salt. Cultures were inoculated to tryptone broth containing 0%, 3%, 6%, 8% and 11% sodium chloride and incubated at room temperature for 18-24 h.

Tryptone broth

Tryptone	1.0%
Yeast extract	0.3%
pH	7.2

The ingredients were dissolved in 100 ml distilled water. To this basal medium, sodium chloride was added to give final concentrations as mentioned above.

3.3 Quantification of biofilm formation by microtiter plate method

The method described by Stepanovic *et al.* (2000) for Staphylococcal biofilms was followed with some modification. Each *V. harveyi* isolate was grown in 5ml of tryptone soya broth containing 1% NaCl (TSBS), at 30⁰C for 18h. Biofilm production assays were performed with TSBS with and w/o dextrose, brain heart infusion broth, luminescent broth containing 1% NaCl, Farghaly medium (a minimal medium) and TSB diluted in natural and defined seawater. The wells of a sterile 96-well-flat-bottomed polystyrene microtiter plate (Tarsons, radiation sterilized) were filled with 230µl of appropriate medium. A 20µl aliquot of *V. harveyi* grown overnight in TSBS was added into each well. Each isolate was tested in triplicate. The control wells contained broth only. After inoculation, the plates were incubated for 24h at 30⁰C, following which the contents of the plate were poured off and the wells were washed three times with 300µl of sterile PBS to remove loosely adhering bacteria. The remaining attached bacteria were fixed by adding 250µl of methanol to each well, and after 15 min the contents of microplates were emptied and air dried. The microplates were stained with 250µl of 1% crystal violet solution in water for 5 min. After staining, the wells were washed with sterile PBS to remove the excess stain. At this point, biofilms were visible as purple rings formed on the sides of each well. After the plates were air dried, the dye bound to adherent cells was resolubilized with 250µl of 33% glacial acetic acid per well. The optical density (O.D.) of each well was measured at 630 nm using microplate reader (EL_X 800, Bio-Teck instrument). Effect of calcium, magnesium, and potassium on biofilm formation was tested using TSB diluted in

defined seawater and luminescent broth with and without calcium (0.14%), magnesium and potassium chloride as medium.

Based on the O. D. produced by bacterial films, strains were classified into the following categories: no biofilm producers, weak, moderate, or strong biofilm producers. Briefly, the cut off O. D. (O. D. c) was defined as three standard deviations above the mean O. D. of the negative control. Strains were classified as follows: O. D. \leq O. D. c = no biofilm producer,

O. D. c < O. D. \leq (2 \times O. D. c) = weak biofilm producer,

(2 \times O. D. c) < O. D. \leq (4 \times O. D. c) = moderate biofilm producer and

(4 \times O. D. c) < O. D. = strong biofilm producer.

All tests were carried out in triplicate and the results were averaged.

Tryptone Soya broth with or w/o dextrose (HiMedia, Mumbai) (supplemented with 1% NaCl)

Casein enzymatic hydrolysate	1.7%
Papaic digest of soyabean meal	0.3%
Sodium chloride	1.0%
Dipotassium phosphate	0.25%
Dextrose	0.25%
pH	7.3 \pm 0.2

The medium was prepared according to manufacturer's instructions and sterilized by autoclaving at 110 $^{\circ}$ C for 15 min.

Brain Heart Infusion Broth (HiMedia, Mumbai) (supplemented with 1% NaCl)

Peptic digest of animal tissue	1.0%
Calf brain, infusion (solids)	1.25%
Beef heart infusion (solids)	0.5%
Dextrose	0.2%
Sodium chloride	1.0%
Disodium phosphate	0.25%
pH	7.4 \pm 0.2

The medium was prepared according to manufacturer's instructions and sterilized by autoclaving at 110 $^{\circ}$ C for 15 min.

Luminescent Broth (supplemented with 1% NaCl, with and w/o CaCl₂)

Pancreatic digest of casein	1.75%
Pancreatic digest of soymeal	0.3%
Dibasic potassium phosphate	0.25%
Sodium chloride	1%
Magnesium chloride	0.4%
Potassium chloride	0.1%

The ingredients were dissolved in 100ml-distilled water (with and w/o calcium, magnesium, potassium chloride) and by autoclaving at 121⁰C fo15 min.

Farghaly medium (A minimal medium)

NaCl	3.0%
Na ₂ HPO ₄ .7H ₂ O	0.7%
KH ₂ PO ₄	0.1%
(NH ₄) ₂ HPO ₄	0.05%
MgSO ₄	0.01%
Glycerol	0.3ml

The ingredients were dissolved in 100ml of distilled water and sterilized at 110⁰C for 15 min.

Tryptone Soya broth (TSB) diluted in seawater / defined seawater (with and w/o calcium, magnesium and potassium chloride)

Two gram of TSB dehydrated powder (w/o dextrose) (HiMedia, Mumbai) was dissolved in 10ml distilled water and sterilized by autoclaving at 121⁰C for 15 min. 1 ml aliquot of this medium was added to 99 ml pre-sterilized seawater or defined seawater (with and w/o calcium, magnesium and potassium chloride).

Defined seawater (Instant Ocean: Aquarium systems)

NaCl	468	mM
MgSO ₄ .7H ₂ O	55	mM
NaHCO ₃	3	mM
CaCl ₂ .2H ₂ O	9.9	mM
KCl	10.3	mM
Na ₂ B ₄ O ₇ .10H ₂ O	0.14	mM
SrCl ₂ .6H ₂ O	0.1	mM
NaBr	0.03	mM
NaI	0.002	mM
LiCl	0.026	mM

Salt mixtures were dissolved in 1000 ml of distilled water and sterilized by autoclaving at 121⁰C for 15 min.

Phosphate buffered saline (Sambrook *et al.*, 1989)

NaCl	0.8%
KCl	0.02%
Na ₂ HPO ₄	0.144%
KH ₂ PO ₄	0.024%

The above ingredients were dissolved in 80 ml distilled water and pH was adjusted to 7.4 with 1N HCl and the volume was made upto 100ml. Sterilized by autoclaving for 20 min at 15 lb/inch² and stored at room temperature.

3.4 Ruthenium red microplate assay for EPS quantification

For the quantification of EPS production by test bacteria, the method of Borucki *et al.* (2003) was followed with some modifications. Each *V. harveyi* isolate was grown in 5 ml of TSBS, at 30⁰C for 18h. These cultures were diluted (1:40) in TSB (diluted in defined seawater) and vortexed for 5 sec. 250 µl of this diluted culture was transferred to seven wells of a microtiter plate and 250 µl sterile TSB (diluted in defined seawater) was added to the outer well of each row of the microtiter plate as blank. The plates were incubated for 24 h at 30⁰C, and unattached cells were removed aseptically by pipetting and washed 3 times with 250µl of cacodylyte buffer. 250 µl of an aqueous solution of ruthenium red (0.1%) was added to each well of the plate, and biofilm was stained for 45 min at room temperature. The liquid from each well was then transferred to another new microtiter plate and the OD₆₃₀ was measured in a microplate reader (EL_x 800, Bio-Teck instrument). The amount of dye bound by the biofilm in each well was determined by subtracting the OD₆₃₀ of the well from the average of the 12 blank wells.

Experimental Results

4. EXPERIMENTAL RESULTS

4.1 Bacteriological analysis

A total of 56 *V. harveyi* isolates taken from the culture stock maintained in the Department of Fishery Microbiology were used in the study laboratory stock. These isolates were further subjected to a battery of biochemical tests for the confirmation purpose. All these isolates exhibited typical biochemical reactions as shown in the (Table 1). All the isolates were identical with respect of their biochemical reactions.

4.2 Quantification of biofilm formation by microtiter plate assay

The biofilm forming ability of 56 *V. harveyi* isolates was tested using different media namely, TSBS with and without dextrose, luminescent broth, brain heart infusion broth containing 1% NaCl, Farghaly medium (a minimal media) and TSB diluted in seawater. The results of the evaluation of biofilm formation on plastic surfaces (microtitre plate) by *V. harveyi* strains cultivated in six different media revealed that all tested *V. harveyi* strains produced biofilm in suitable medium. The nutrient content of the medium significantly influenced the quantity of biofilm produced by *V. harveyi* strains. The production of biofilm for each strain was tested in six media, and every strain produced the highest quantity of biofilm in one medium. Based on the optical densities of highest quantity of produced biofilm, isolates were classified as strong, moderate or weak biofilm producers (Table 2).

TSB diluted in seawater was the most effective in promoting biofilm production by tested *V. harveyi* strains (average O. D of strong, moderate, weak biofilm producer strains \pm S.D. was 0.334 ± 0.002), followed by BHI broth (0.309

± 0.015), TSB with dextrose (0.303 ± 0.011), luminescent broth (0.256 ± 0.018), and TSB without dextrose (0.119 ± 0.007) (Table 3,4,5,6,7, 8; Fig. 1).

4.3 Effect of calcium on biofilm formation

A total of 56 isolates were grown in diluted TSB prepared in defined seawater and Luminescent broth with or without calcium (Table 9). In this, diluted TSB prepared in defined sea water with calcium was the most effective in promoting biofilm production by tested *V. harveyi* strains (average O. D. of strong, moderate, weak biofilm producer strain was 0.371 ± 0.002) and diluted TSB prepared in defined sea water without calcium was the least effective in promoting biofilm production (0.30 ± 0.006) (Table 10 and 11). Similarly, luminescent broth with calcium was the more effective in biofilm production (0.419 ± 0.012) than luminescent broth without calcium (0.256 ± 0.018) (Table 12 and 6, Fig. 2).

4.4 Effect of magnesium on biofilm formation

The same 56 *V. harveyi* isolates were grown in diluted TSB prepared in define seawater and luminescent Broth with or without magnesium (Table 13). Diluted TSB prepared in defined sea water with magnesium was the most effective in promoting biofilm production by tested *V. harveyi* strains (0.371 ± 0.002), while diluted TSB prepared in defined sea water without magnesium was the least effective in promoting biofilm formation (0.248 ± 0.014) (Table 10 and 14). Whereas, luminescent broth with magnesium was the most effective in promoting biofilm production by tested *V. harveyi* strains (0.256 ± 0.018) than luminescent broth without magnesium (0.20 ± 0.008) (Table 6 and 15, Fig. 3).

4.5 Effect of potassium on biofilm formation

All the 56 *V. harveyi* isolates were grown in diluted TSB prepared in defined sea water and luminescent broth with or without potassium chloride (Table 16), where some strains produced the highest quantity of biofilm in diluted TSB prepared in defined sea water with potassium chloride (average O. D. \pm S.D. was 0.371 ± 0.002) and some produced lowest quantity of biofilm in diluted TSB prepared in defined sea water without potassium (0.291 ± 0.004) (Table 10 and 17). Similarly, luminescent broth containing potassium chloride was the most effective in promoting biofilm formation by all 56 *V. harveyi* strains tested (0.256 ± 0.018) but the luminescent broth without potassium chloride was less effective in promoting biofilm formation (0.209 ± 0.027) (Table 6 and 18, Fig. 4).

4.6 EPS production

Microtiter plate assay was employed to test the isolates for EPS production using ruthenium red, a carbohydrate binding dye. Three sets of isolates were chosen for the assay; first, 5 strong biofilm producer strains, second, 5 weak biofilm producer strains and third, 5 no biofilm producer strains in the microtiter assay. The uptake of ruthenium red was measured for these isolates. EPS production was quantified by measuring the dye bound to the cell layer. The results were consistent with the ability of the isolates to form biofilms in microtiter plate assay using diluted TSB prepared in defined seawater. Strong biofilm producer strains showed EPS production more than O.D. 0.124, while weak and no biofilm producer strains showed less than the blank value i.e. zero (Table 19).

Table 1. Biochemical characteristics of *V. harveyi*

Characteristic	Reaction as per West and Colwell	Reaction of isolates tested
Gram's reaction	+	+
Cytochrome oxidase	+	+
Nitrate reduction	+	+
O/129 sensitivity (150µg)	+	+
Motility	+	+
Luminescence	v	+
Thornley's arginine dihydrolase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Growth at 0% NaCl	-	-
Growth at 3% NaCl	+	+
Growth at 8% NaCl	+	+
Growth at 10% NaCl	v	v
Gas from glucose	-	-
Fermentation to acid;		
L-arabinose	-	-
m-inositol	-	-
D-mannose	+	+
Sucrose	v	+
Indole	+	+
*O/F	F	F

*O/F – Oxidation fermentation test

Table 2. Biofilm formation by *V. harveyi* isolates in different media

Number of strains that produced highest quantities of biofilm (%)

<i>V. harveyi</i> strains	TSB w/o dextrose	TSB with dextrose	BHI broth	luminescent broth	Farghaly medium	TSB diluted in sea water
Strong biofilm producer	–	3 (5.3)	3 (5.3)	1(1.78)	2 (3.5)	18 (32.1)
Moderate biofilm producer	4 (7.1)	2 (3.5)	2 (3.5)	3 (5.3)	3 (5.3)	9 (16.0)
Weak biofilm producer	7 (12.5)	9 (16.0)	5 (8.9)	18 (32.1)	8 (14.2)	14 (25.0)
No biofilm producer	45(80.3)	42(75)	46(82.1)	34(60.7)	43(76.7)	15(26.7)
Total	56	56	56	56	56	56

Table 3. Biofilm formation by different *V. harveyi* isolates in TSB diluted in sea water (OD₆₃₀)

Isolate No	TSB diluted in sea water	Nature of biofilm
2	0.738	Strong biofilm producer
3	0.366	
12	0.571	
13	0.401	
14	0.304	
20	0.404	
21	0.53	
22	0.864	
23	0.357	
29	0.625	
31	0.663	
32	0.613	
33	0.497	
34	0.730	
35	0.718	
49	0.378	
101	0.797	
104	0.438	
7	0.259	Moderate biofilm producer
36	0.242	
37	0.265	
40	0.283	
41	0.246	
43	0.178	
59	0.250	
106	0.231	
107	0.285	
1	0.08	
4	0.092	
10	0.107	
19	0.109	
24	0.092	
25	0.120	
42	0.09	
44	0.140	
45	0.086	
46	0.085	
55	0.146	
60	0.099	
105	0.132	

Table 4. Biofilm formation by different *V. harveyi* isolates in Brain Heart Infusion broth containing 1% NaCl (OD₆₃₀)

Isolate No	BHI containing 1% NaCl	Nature of biofilm
2	0.470	Strong biofilm producer
31	0.483	
32	0.764	
29	0.282	Moderate biofilm producer
34	0.280	
7	0.179	Weak biofilm producer
14	0.164	
15	0.142	
45	0.157	
102	0.176	

Table 5. Biofilm formation by different *V. harveyi* isolates in TSB with dextrose containing 1% NaCl (OD₆₃₀)

Isolate No	TSB with dextrose containing 1% NaCl	Nature of biofilm
21	0.576	Strong biofilm formation
32	0.838	
107	0.716	
7	0.243	Moderate biofilm formation
34	0.377	
14	0.169	Weak biofilm formation
29	0.180	
31	0.182	
35	0.164	
38	0.17	
40	0.204	
45	0.172	
49	0.145	
102	0.111	

Table 6. Biofilm formation by different *V. harveyi* isolates in luminescent broth containing 1% NaCl (OD₆₃₀)

Isolate No	luminescent broth containing 1% NaCl	Nature of biofilm
34	0.653	Strong biofilm producer
7	0.401	Moderate biofilm producer
31	0.283	
32	0.506	Weak biofilm producer
1	0.165	
4	0.230	
11	0.149	
13	0.161	
16	0.236	
19	0.201	
20	0.141	
21	0.142	
22	0.187	
23	0.223	
29	0.257	
33	0.159	
41	0.256	
42	0.207	
44	0.304	
49	0.265	
101	0.209	
102	0.181	

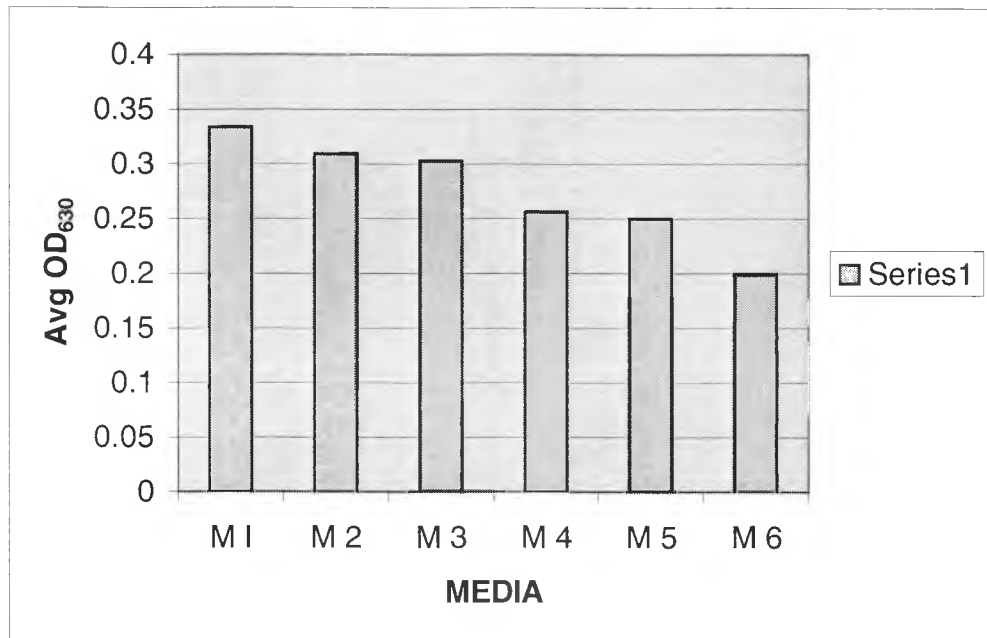
Table 7. Biofilm formation by different *V. harveyi* isolates in Farghaly medium containing 3% NaCl (OD₆₃₀)

Isolate No	Farghaly medium containing 3 % NaCl	Nature of biofilm
2	0.621	Strong biofilm producer
34	0.543	
32	0.250	Moderate biofilm producer
45	0.167	
104	0.259	
23	0.100	Weak biofilm producer
29	0.146	
31	0.141	
33	0.121	
35	0.150	
36	0.116	
101	0.151	
103	0.114	

Table 8. Biofilm formation by different *V. harveyi* isolates in TSB without dextrose containing 1% NaCl (OD₆₃₀)

Isolate No	TSB w/o dextrose containing 1% NaCl	Nature of biofilm
-	-	Strong biofilm producer
2	0.314	Moderate biofilm producer
32	0.313	
45	0.311	
55	0.343	
20	0.127	Weak biofilm producer
28	0.136	
29	0.125	
35	0.119	
41	0.128	
44	0.142	
102	0.135	

Fig. 1. Biofilm formation by *V. harveyi* in different media



M 1: TSB diluted in seawater

M 2: BHI

M 3: TSB with dextrose

M 4: luminescent broth

M 5: Farghaly medium

M 6: TSB w/o dextrose

**Table 9. Effect of calcium on biofilm formation by *V. harveyi* strains in respective media
Number of strains that produced highest
quantities of biofilm (%)**

<i>V. harveyi</i> strains	TSB diluted in defined sea water with CaCl ₂	TSB diluted in defined sea water w/o CaCl ₂	luminescent broth with CaCl ₂	luminescent broth w/o CaCl ₂
Strong biofilm Producer	13 (23.2)	5 (8.9)	14 (25)	1 (1.7)
Moderate biofilm producer	5 (8.9)	3 (5.3)	13 (23.2)	3 (5.3)
Weak biofilm producer	10 (17.8)	1 (1.7)	10 (17.8)	18 (32.1)
No biofilm producer	28(50)	47(83.9)	19(33.9)	34(60.7)
Total	56	56	56	56

Table 10. Biofilm formation by different *V. harveyi* isolates in TSB diluted in defined sea water with calcium, magnesium and potassium chloride (OD₆₃₀)

Isolate No	TSB diluted in defined sea water	Nature of biofilm
12	0.584	Strong biofilm producer
19	0.389	
20	0.466	
21	0.686	
22	0.788	
29	0.909	
31	0.704	
32	0.976	
34	0.545	
35	0.931	
37	0.351	
49	0.306	
101	0.47	
13	0.225	
23	0.174	
33	0.265	
104	0.201	
107	0.241	Weak biofilm producer
2	0.145	
7	0.104	
14	0.116	
36	0.103	
40	0.137	
42	0.100	
45	0.129	
46	0.137	
59	0.117	
102	0.09	

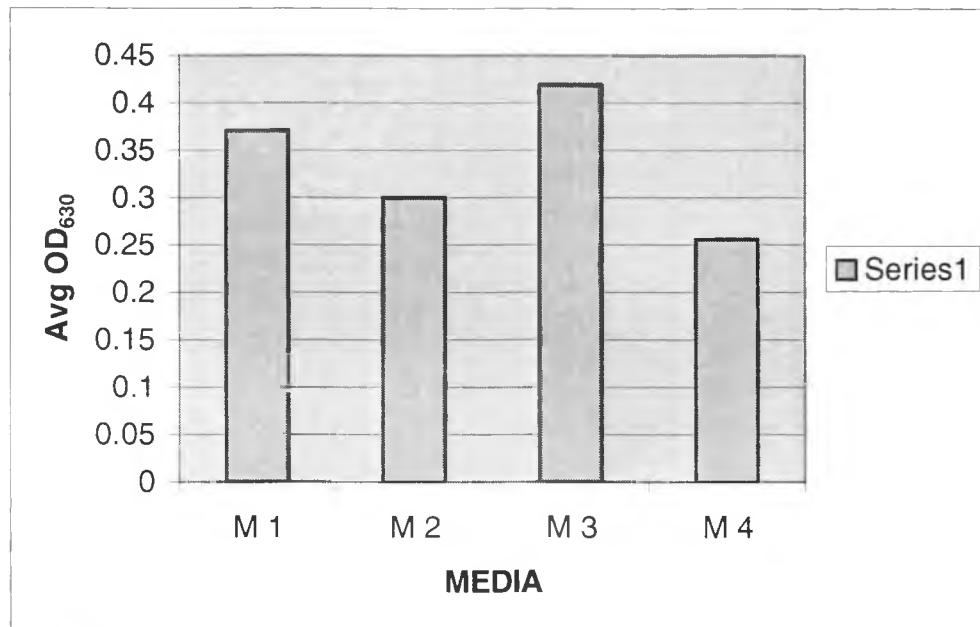
Table 11. Effect of calcium on biofilm formation by *V. harveyi* (OD₆₃₀)

Isolate No	TSB diluted in defined sea water w/o CaCl₂	Nature of biofilm
2	0.38	Strong biofilm producer
19	0.35	
22	0.398	
29	0.36	
32	0.481	
31	0.245	Moderate biofilm producer
34	0.175	
101	0.192	
20	0.132	Weak biofilm producer

Table 12. Effect of calcium on biofilm formation by *V. harveyi* (OD₆₃₀)

Isolate No	luminescent broth with CaCl₂	Nature of biofilm
6	0.674	Strong biofilm producer
7	0.591	
13	0.553	
19	0.589	
20	0.565	
21	0.651	
23	0.760	
29	0.727	
31	0.676	
32	0.680	
4	0.322	Moderate biofilm producer
8	0.399	
10	0.426	
12	0.430	
16	0.365	
22	0.362	
33	0.674	
34	0.615	
35	0.337	
37	0.454	
40	0.356	
143	0.352	
101	0.427	
2	0.171	Weak biofilm producer
9	0.133	
11	0.155	
17	0.156	
25	0.164	
27	0.19	
41	0.28	
44	0.266	
48	0.170	
49	0.811	
104	0.286	
105	0.196	
106	0.334	
107	0.212	

Fig. 2. Effect of calcium on biofilm formation by *V. harveyi*



M 1: TSB diluted in defined seawater with calcium

M 2: TSB diluted in defined seawater w/o calcium

M 3: luminescent broth with calcium

M 4: luminescent broth w/o calcium

Table 13. Effect of magnesium on biofilm formation by *V. harveyi* strains in different growth media

Number of strains that produced highest quantities of biofilm (%)

<i>V. harveyi</i> strains	TSB diluted in defined sea water with MgCl ₂	TSB diluted in defined sea water w/o MgCl ₂	luminescent broth with MgCl ₂	luminescent broth w/o MgCl ₂
Strong biofilm Producer	13 (23.2)	1 (1.7)	1 (1.7)	–
Moderate biofilm producer	5 (8.9)	6 (10.7)	3 (5.3)	1 (1.7)
Weak biofilm producer	10 (17.8)	13 (23.2)	18 (32.1)	2 (3.5)
No biofilm producer	28(50)	36(64.2)	34(60.7)	53(94.6)
Total	56	56	56	56

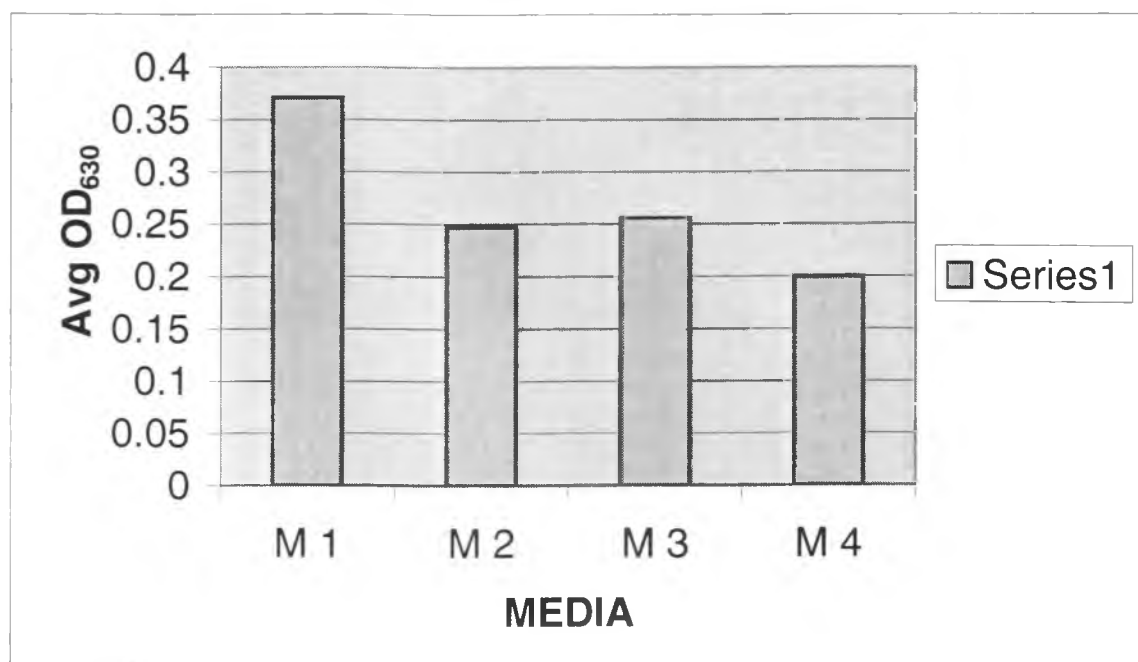
Table 14. Effect of magnesium on biofilm formation by *V. harveyi* (OD₆₃₀)

Isolate No	TSB diluted in defined sea water w/o MgCl ₂ (MgSO ₄)	Nature of biofilm
35	0.538	Strong biofilm producer Moderate biofilm producer
22	0.478	
29	0.510	
32	0.360	
34	0.452	
49	0.299	
101	0.314	
6	0.175	
12	0.200	
31	0.307	
33	0.139	
36	0.21	
37	0.228	
39	0.137	
40	0.72	
46	0.183	
48	0.192	
104	0.161	
106	0.215	
107	0.234	

Table 15. Effect of magnesium on biofilm formation by *V. harveyi* (OD₆₃₀)

Isolate No	luminescent broth w/o MgCl ₂	Nature of biofilm
-	-	Strong biofilm producer
2	0.295	Moderate biofilm producer
3	0.165	Weak biofilm producer
11	0.140	

Fig. 3. Effect of magnesium on biofilm formation by *V. harveyi*



- M 1: TSB diluted in defined seawater with magnesium
- M 2: TSB diluted in defined seawater without magnesium
- M 3: luminescent broth with magnesium
- M 4: luminescent broth without magnesium

Table 16. Effect of potassium on biofilm formation by *V. harveyi* strains in different growth media

Number of strains that produced highest quantities of biofilm (%)

<i>V. harveyi</i> strains	TSB diluted in defined sea water with KCl	TSB diluted in defined sea water w/o KCl	luminescent broth with KCl	luminescent broth w/o KCl
Strong biofilm Producer	13 (23.2)	5 (8.9)	1 (1.7)	1 (1.7)
Moderate biofilm producer	5 (8.9)	7 (12.5)	3 (5.3)	5 (8.9)
Weak biofilm producer	10 (17.8)	14 (25)	18 (32.1)	15 (26.7)
No biofilm producer	28(50)	30(53.5)	34(60.7)	35(62.50)
Total	56	56	56	56

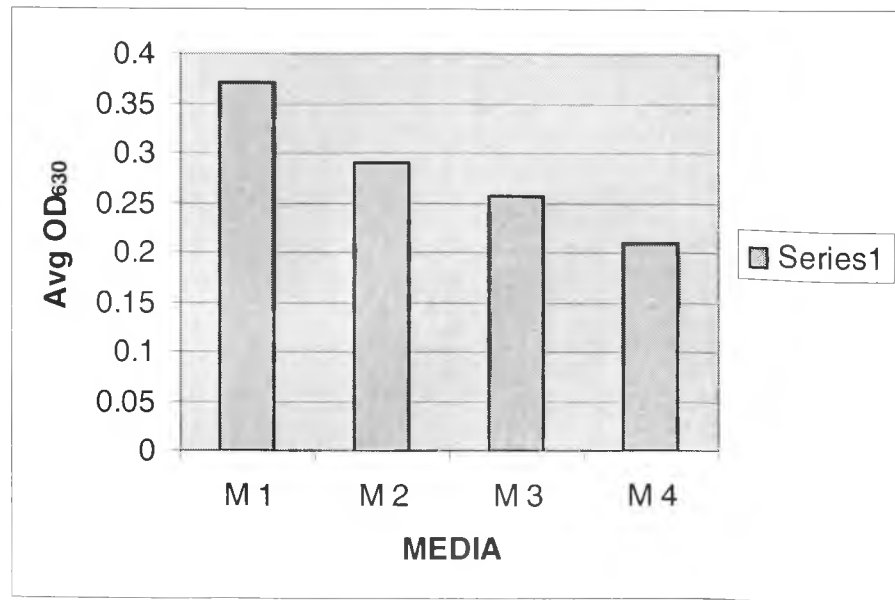
Table 17. Effect of potassium on biofilm formation by *V. harveyi* (OD₆₃₀) grown in diluted TSB without KCl

Isolate No	TSB diluted in defined sea water w/o KCl	Nature of biofilm
29	1.016	Strong biofilm producer
31	0.481	
32	0.471	
34	0.631	
35	0.532	
13	0.248	Moderate biofilm producer
21	0.288	
22	0.348	
37	0.254	
49	0.304	
101	0.415	
107	0.297	
6	0.146	Weak biofilm producer
7	0.154	
10	0.115	
12	0.108	
23	0.175	
25	0.120	
33	0.173	
36	0.179	
39	0.132	
40	0.122	
46	0.232	
48	0.246	
104	0.175	
106	0.207	

Table 18. Effect of potassium on biofilm formation by *V. harveyi* (OD₆₃₀) grown in luminescent broth w/o KCl

Isolate No	luminescent broth w/o KCl	Nature of biofilm
34	0.546	Strong biofilm producer
35	0.289	Moderate biofilm producer
48	0.286	
49	0.246	
60	0.283	
101	0.252	
7	0.274	
19	0.233	
33	0.141	
36	0.133	
37	0.167	
38	0.203	
39	0.133	
41	0.238	
42	0.15	
43	0.127	
44	0.187	
59	0.166	
102	0.129	
105	0.207	
106	0.185	

Fig. 4. Effect of potassium on biofilm formation by *V. harveyi*



M 1: TSB diluted in defined seawater with potassium

M 2: TSB diluted in defined seawater w/o potassium

M 3: luminescent broth with potassium

M 4: luminescent broth w/o potassium

Table 19. EPS production by different *V. harveyi* strains

Isolate No	OD₆₃₀	Nature of biofilm
33	0.136	Strong biofilm producer
19	0.124	
32	0.209	
34	0.144	
35	0.051	
7	-0.097	Weak biofilm producer
14	-0.166	
36	-0.147	
42	-0.129	
59	-0.111	
1	-0.151	No biofilm producer
6	-0.024	
8	-0.136	
44	-0.089	
11	-0.095	

Discussion

5. DISCUSSION

Biofilms of pathogenic bacteria are of great significance both in aquaculture and food processing industry. Bacteria in aquatic environments are rarely found in the planktonic or free-swimming state (Costerton *et al.*, 1987). They are found in association with a solid surface. Biofilm formation could be a strategy of microorganisms to survive in systems such as hatcheries and aquaculture ponds (Karunasagar *et al.*, 1996) and such biofilm bacteria could be a source of infection for cultured animals. Bacteria in a food-processing environment may be exposed to different levels of nutrients, depending on the location in a plant (Djordjevic *et al.*, 2002). Biofilms formed in food processing environments are of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases. Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation (Bower and Daeschel, 1999; Jeyasekaran *et al.*, 2000; Venugopal *et al.*, 1999; Joseph *et al.*, 2001).

Luminescent vibriosis due to *V. harveyi* had been an important disease causing mass mortalities in hatchery reared larvae of *P. monodon* (Lavilla-Pitogo *et al.*, 1990). This important pathogen of cultured shrimp is capable of forming sanitizer resistant biofilms on various surfaces encountered in aquaculture systems (Karunasagar *et al.*, 1996). Since *V. harveyi* biofilm is of great significance in hatcheries factors involved in its formation are of paramount importance in order to undertake any interventional measures.

5.1 Quantification of *V. harveyi* from biofilms

Microtiter plate assay has the advantage of facilitating screening of large number of isolates for their ability to adhere to surfaces. This method was used for quantification of staphylococci and *Salmonella* biofilm formation (Stepanovic *et al.*, 2000). Same method has been used for studying biofilm formation by a number of organisms such as *Pseudomonas*, *Listeria* etc (O'Toole and Kolter, 1998; Djordjevic *et al.*, 2002). We followed this method to screen different *V. harveyi* isolates for their ability to form biofilm. Evaluation of biofilm formation by various *V. harveyi* isolates on polystyrene surfaces when grown in 6 different media revealed that many of the isolates possess a high capacity for biofilm formation on plastic surfaces. Further, it was seen that there are differences in the biofilm forming ability

BY REGD. ARCEL WITH ACK.DUE

KARNATAKA VETERINARY, ANIMAL & FISHERIES SCIENCES UNIVERSITY, BIDAR

No.FCM/AAU/1/2006-2007

College of Fisheries
Mangalore 575 CC2
Date: 07.09.2006

To

The Director of Instruction(PGS)
Karnataka Veterinary, Animal &
Fisheries Sciences University
Nandínagar, Post Box No.6
B I D A R - 585 401.

Sir,

Sub: Copy of the thesis of Mr. Anil Kumar, H.A.
ID No.PFK 311 .. Reg.

Ref: You letter No.DI(PGS)/PPR/ID No.MVHK 5C2/C6-C7/
2133 dated 20.7.2006.

....

With reference to the above, I am enclosing herewith a copy
of the thesis of Mr. Anilkumar, H.A., ID No.PFK 311 admitted to
Masters degree programme during the year 2003-2004 in Fishery
Microbiology as desired by you.

Yours faithfully,

Encl: As above.

Prashant
DEAN (Fisheries)

DEAN (FISHERIES)
COLLEGE OF FISHERIES, MANGALORE - 2

among the isolates. Similar results were observed by Stepanovic *et al.* (2003a) for *Salmonella* spp. and *L. monocytogenes*, and by Kalmokoff *et al.* (2001) for *L. monocytogenes*.

It has been reported that *Salmonella* spp. and *L. monocytogenes* isolates adhere in higher numbers to more hydrophobic materials (Donlan, 2002). As adhesion is the first step in the complex process of biofilm formation (Donlan, 2002), this could be one possible explanation for the ability of these bacteria to produce biofilm in high numbers in plastic surface. It is well known that many factors influence biofilm formation, including composition of the medium (Dewanti and Wong, 1995; Hood and Zottola, 1997). The 6 culture media in the study used to investigate biofilm production led to different levels of biofilm formation by the *V. harveyi* isolates. This suggests the importance of nutrients in aiding biofilm formation. Out of 6 media used, the most effective medium in promoting biofilm production in this study was TSB diluted in seawater whereas the other 5 media (BHI, TSB with dextrose, luminescent broth, Farghaly medium and TSB without dextrose) were less effective in promoting biofilm production. However, the composition of the medium did not have the same influence on all the *V. harveyi* isolates tested (Table 2, Fig.1). These results are significant and indicate that seawater constituents play an important role in facilitating biofilm formation by *V. harveyi*.

5.2 Effect of calcium on biofilm formation

Biofilm formation was dependent on the presence of Ca^{2+} and Mg^{2+} ions in water, with the maximal effect seen at a concentration of 1 μM (Carter *et al.*, 2003). Divalent cations such as calcium and magnesium, which are cross-linked with the polymer strands, provide a greater binding force in a developed biofilm (Flemming *et al.*, 2000).

Surface adhesion of bacteria has been shown to depend on the inorganic components like Na^+ and Ca^{2+} of the medium (Rose and Turner, 1998; Knobloch *et al.*, 2001). Calcium has been known to be important for the production of exopolysaccharides and for biofilm formation in *V. cholerae* (Kierek and Watnick, 2003a). Removal of calcium from the bathing medium resulted in dissolution of biofilm in a number of *Vibrio* spp such as *V. cholerae*, *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis* and *V. vulnificus* (Kierek and Watnick, 2003b). This study demonstrated that Ca^{2+} was essential for biofilm formation by *V. harveyi*. In the present study better biofilm formation occurred only in media prepared in seawater, and hence it was assumed that one or several components present in seawater are responsible for these differences in adhesion to surfaces. So, defined seawater was prepared using concentrations

reported by Instant Ocean: Aquarium systems with and without calcium. These strains were also tested on Luminescent broth with or without calcium. It was found that out of 56 *V. harveyi* isolates tested, 50% of the strains produced the highest quantities of biofilm in TSB diluted in defined seawater with calcium and 66% strains produced the highest quantities of biofilm in Luminescent broth with calcium, whereas in the absence of calcium, 16% strains produced the highest quantities of biofilm in TSB diluted in defined seawater and 39.2% strains produced the highest quantities of biofilm in Luminescent broth media. This clearly indicated that calcium is one of the main components responsible for biofilm formation.

5.3 Effects of magnesium on biofilm formation

The effects of calcium and magnesium concentrations on the growth of two mucoid *P. aeruginosa* strains of cystic fibrosis origin and on the synthesis of their extracellular polyuronic acids (EPA) were examined. Both strains required a minimum concentration of Mg^{2+} for growth but differed in their Mg^{2+} requirements to achieve maximum growth potential (Dunne and Buckmire, 1985).

Dunne and Burd (1992) demonstrated that increasing concentrations of Mg^{2+} enhanced biofilm production by *S. epidermidis* on a plastic surface. The addition of Mg^{2+} (as either $MgSO_4$ or $MgCl_2$) to the bacterial suspension in concentrations as low as 16 μM significantly enhanced adhesion of all test strains to plastic (Dunne and Burd, 1992). This study showed that Mg^{2+} is essential for biofilm formation by *V. harveyi*. Since more biofilm formation occurred in media prepared in seawater and in luminescent broth, where both contained magnesium salts. It was assumed that presence of one or several components in seawater and luminescent broth was responsible for these differences in adhesion to surfaces. Defined seawater was prepared using concentrations reported by Instant Ocean: Aquarium systems with or without magnesium. These strains were also tested by luminescent broth with or without magnesium. It was found that, out of 56 tested *V. harveyi* isolates, 50% and 39.2% of the strains produced the highest quantities of biofilm in TSB diluted in defined seawater with magnesium and luminescent broth with magnesium, respectively. Whereas, in the absence

5.4 Effects of potassium chloride on biofilm formation

EPS production is known to be affected by nutrient status of the growth medium; excess availability of carbon and limitation of nitrogen, potassium, or phosphate promoted EPS synthesis (Sutherland, 2001). More biofilm formation occurred by *V. harveyi* strains in TSB diluted in seawater and in luminescent broth media. It was found that one or several components present in seawater and luminescent broth were responsible for these differences in adhesion to surfaces.

A total of 56 *V. harveyi* isolates were grown in diluted TSB prepared in defined seawater and luminescent broth with or without potassium. Defined seawater was prepared using concentrations reported by Instant Ocean: Aquarium systems with and w/o KCl. It was found that in TSB diluted in defined seawater and supplemented with KCl, out of 56 tested *V. harveyi* isolates, 50% of the strains produced highest quantities of biofilm and in Luminescent broth media with KCl, 39.2% strains produced the highest quantities biofilm, whereas in the absence of potassium only 46.4% strains produced highest quantities of biofilm in TSB diluted in defined seawater and 37.5% strains in luminescent broth. This study indicates that KCl is not so important in the biofilm formation by *V. harveyi*.

5.5 EPS production

EPS influences the rate and extent of attachment of microbial cells, and hence required for the formation of biofilm. EPS production by strong, weak and no biofilm producing strains of *V. harveyi* was studied using a carbohydrate binding dye (ruthenium red). This experiments showed that the isolates producing strong biofilm (with crystal violet microtitre assay) also had most intense staining with the ruthenium red assay. So it was concluded that the strong biofilm producer strains produce more EPS and form biofilm whereas, weak and no biofilm producer isolates did not or produce very low amount of EPS.

Summary

6. SUMMARY

V. harveyi, the causative agent of luminous vibriosis is one of the most important pathogen in shrimp hatchery and grow out culture systems. This organism is capable of forming biofilms on various surfaces, which in many cases is responsible for perpetuating infection that is resistant to antibiotics and sanitizer treatments.

In this study, different strains of *V. harveyi* maintained in the laboratory stock at -80°C were taken to study the role of salts such as calcium chloride, sodium chloride, potassium chloride and magnesium chloride and effect of organic nutrient levels on formation of biofilm. This was carried out using microtitre plate assay. Biofilm formation by various *V. harveyi* isolates on polystyrene surfaces when grown in the presence of 6 media namely Tryptone soya broth (TSB) diluted with seawater, brain heart infusion broth (BHI), Tryptone soya broth with dextrose, luminescent broth, Farghaly medium, and Tryptone soya broth without dextrose. Tryptone soya broth diluted with seawater seemed most favourable for biofilm formation as seen from the results of 56 isolates tested. The effects on all the isolates grown in the presence of other 5 media did not show any marked difference.

In seawater and Luminescent broth one or several components were found responsible for biofilm formation. Calcium, one of the components of seawater and luminescent broth was found to be a major factor responsible for the biofilm formation followed by magnesium and potassium. Extracellular polymeric substances (EPS) production has a predominant role in adhesion of cells to the surface and this was studied in all the isolates. Isolates showing a strong biofilm forming ability showed higher EPS production when compared to weak biofilm formers or those that failed to form biofilm.

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7. REFERENCES

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