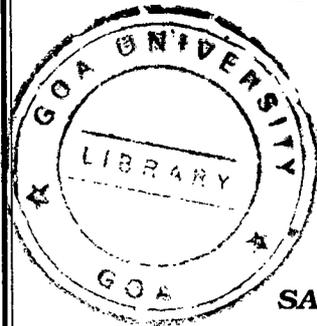


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STATEMENT

As required under the ordinance No. 19.8, I state that the present thesis entitled "Studies on marine fouling bacteria" is my original contribution and that the same or any part thereof has not been submitted elsewhere for the award of any degree to any other university on any previous occasion to the best of my knowledge.



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CHAPTER 1

General Introduction

1. INTRODUCTION

Microbial films are amongst the oldest witness to life on earth (Ehrlich, 1990; Meyer-Reil, 1994). Man has long been aware of microbial attachment to solid surfaces in sea and in fresh water. This is because the slime layers formed by attached microorganisms and their associated polymers are easily detected by touch. The formation of biofilms can be regarded as an early step in evolution towards organising cells in order to produce metabolic capacities which are much more powerful than those of single-celled organisms (Meyer-Reil, 1994).

Studies of the microbial adhesion to solid surfaces date back to the work of Zobell, who was among the first to recognize the significance of microbial adhesion to surfaces (Zobell and Anderson, 1936; Zobell, 1943). This was followed by others, especially pioneering studies by Stark *et al.*, (1938), Heukelekian and Heller (1940) and Zobell (1943) who demonstrated that bacterial growth was stimulated by attachment onto the surfaces. This "Surface effect" has since been repeatedly observed for surfaces immersed in seawater as well as in fresh water (Marshall *et al.*, 1971; Dawson *et al.*, 1981; Fletcher & Marshall, 1982; Characklis & Cooksey, 1983; Costlow & Tipper, 1984; Callow, 1993; Udhayakumar *et al.*, 1998).

Despite these earlier efforts, research on surface microbiology progressed very slowly. Over the past few years studies on bacterial attachment have received growing and enthusiastic attention, because

of the significance of bacterial interaction with the surfaces. It is now well known that nearly all surfaces are eventually colonized by microorganisms when immersed in an aquatic environment (Fletcher & Marshall, 1982) and biofilms are generally present on all wetted surfaces (Lock, 1993).

Attached microbial cells grow, reproduce and synthesize extracellular polymeric substances. Such attachment to and growth of microorganisms on surfaces is defined as biofilm. Biofilms may perform several beneficial activities in natural environments and in some man made systems like trickling filters. Conversely, the term “microfouling” is generally employed when biofilms are involved in economic losses to man.

Microfouling :

Formation of microfouling is an orderly process and appears to involve the following steps.

- i) Conditioning film or adsorption of organic molecules
- ii) Transport of microbial cells to wetted surface
- iii) Reversible adsorption of bacteria
- iv) Irreversible attachment of bacteria
- v) Development of a secondary microflora

i) Conditioning films

Immediately after their immersion in aquatic environment solid surfaces adsorb dissolved organic compounds onto their surfaces.

Such adsorption of organic matter onto surfaces is known as conditioning film or molecular film (Neihof & Loeb, 1972; Characklis, 1981a; Callow, 1993). Conditioning film influences surface free energy and surface charge (Carpentier & Cerf, 1993), and thus play an important role in subsequent colonization by microorganisms (Maroudas, 1975).

The chemical nature of conditioning film has been the subject of a number of studies (Baier, 1973; Loeb & Neihof, 1975; Baier, 1984; Little & Zolnay, 1985; Little & Jacobus, 1985; Chamberlain, 1992). Loeb & Neihof (1975) have reported that the adsorbed materials were humic compounds derived from degradation products of marine and terrestrial sources. Baier (1973) suggested that the conditioning film was composed primarily of glycoproteins. Surface charge, surface energy and wettability determine the chemistry of the conditioning layer and therefore the quantity and quality of nutrients available in the conditioning film. This in turn, is likely to influence the initial microbial colonization of the surface. Debates continue regarding the necessity of the conditioning film for the subsequent attachment of microbial cells.

ii) Transport of bacterial cells to surfaces

In very dilute suspension of bacteria (as in open ocean waters) transport is probably the rate - limiting step in the process of bacterial deposition at the surface. Bacterial cells are transported by a number of mechanisms. Following processes appear to play

important role in the transport of microorganisms to wetted surfaces.

Sedimentation

This process is only of significance in low-shear systems and with relatively large particles, such as very large bacteria or aggregates of normal bacteria.

Chemotaxis

Many bacteria are motile in the bulk aqueous phase as a result of the propulsive action of flagella. Motile bacteria are capable of displaying a positive chemotactic response to certain nutrient sources (Adler, 1969) and as such can respond to a nutrient concentration gradient that exists at a solid water interface. Thus, bacteria may actively swim towards a surface and ultimately be held by the attractive forces operative near the surface (Young & Mitchell, 1973). It is obvious that non-motile bacteria are incapable of chemotaxis and these bacteria must be transported to the surface by other mechanisms such as waves and currents (Young & Mitchell, 1973). Chemotactic responses are perhaps not of significance in turbulent flow conditions (Characklis, 1981b), but are probably important in transport through the viscous sublayer (Characklis, 1981a).

Brownian motion

The magnitude of displacement of a particle by Brownian motion is dependent upon particle size. Most bacteria have an

effective radius of less than $1.0\mu\text{m}$ and are capable of brownian displacement. Larger bacteria do not show this form of motion. Brownian motion probably contributes little to the transport of bacteria in turbulent flow, but should be a significant form of transport within the viscous sublayer.

Cell surface hydrophobicity

Mudd & Mudd (1924) demonstrated that bacteria vary in their cell surface hydrophobicity. This was confirmed by the recent studies on the relative hydrophobicity of bacterial surfaces (Rosenberg *et al.*, 1980). According to Marshall & Cruickshank (1973), hydrophobic bacteria are generally rejected from the aqueous phase and are attracted towards solid surfaces. Thus the hydrophobicity provide particular orientation in some bacteria which help them in getting attracted towards solid surfaces (Marshall & Cruickshank, 1973).

Fluid dynamic forces

Bacteria in turbulent flow systems are dispersed by eddy diffusion in turbulent core region, thus maintaining a uniform concentration in the bulk fluid. Eddy diffusion transport the bacteria to the region of viscous sublayer. If the bacteria are travelling faster than the fluid in the region of the wall a lift force directs the bacteria towards the wall (Characklis, 1981a). Apart from lift forces, another mechanism for directing particles of bacteria through the viscous sublayer to the wall is that of turbulent “downsweeps” (Lister, 1981). These spontaneous bursts of turbulence penetrate the

viscous sublayer and provide a significant fluid mechanical force to direct the bacteria to the solid surface.

iii) Reversible adsorption of bacteria

In reversible sorption, bacteria are weakly held to a surface by physical attractive forces such as Van Der Waals forces of mass attraction and electrostatic forces caused by ionic groups interacting on or around the approaching particles and the substratum surface (Dempsey, 1981; Kelly, 1981; Meyer-Reil, 1994). During this stage the bacteria reach a state of equilibrium between the attractive and the repulsive forces that surround them. This state of equilibrium allows bacteria to exhibit Brownian molecular motion. Due to the weak bonding bacteria can be removed from the substratum surface (Blenkinsopp & Costerton, 1991). After a few hours of contact with a substratum, however, the bacteria begin forming more secure bonds with the surface. This makes the beginning of irreversible sorption.

iv) Irreversible adsorption of bacteria

Irreversible sorption occurs once the bacteria start exuding acidic mucopolysaccharides or other cellular components. Extracellular polysaccharides (EPS) form bridges between the bacteria and substratum and thus firmly attach the bacteria to the substratum surface (Kelly, 1981; Blenkinsopp & Costerton, 1991). After irreversible attachment onto the substratum surface, bacteria grow and reproduce by binary fission with each half growing to an average size and then dividing again (Kelly, 1981). These dividing

bacteria spread and form extensive colonies and the slimy layer.

V) Development of a secondary microflora

The irreversible adsorption of bacteria i.e. the formation of slimy layer is followed by colonization by other microorganisms including microalgae, protozoans and fungi.

Invertebrate settlement

The mechanism by which the larvae “recognize” the proper substratum is not well understood. It is possible that lectins (proteins or glycoproteins) on the larvae bind specifically to microbial polysaccharides of biofilm formed on the surface. The polysaccharides may be produced by fouling bacteria, algae or other microfouling organisms. Larvae which settle on a wide range of surfaces may produce several lectins which recognize different polysaccharides. Alternatively, larvae may produce a single lectin with low affinity to a wide range of polymers (Mitchell & Kirchman, 1984).

It is generally believed that the biofilm may provide a favourable base for the adhesion and settlement, growth and development of larvae of many animals and algal cells (Zobell, 1939; Corpe, 1977). Meenakumari & Nair (1994) also reported that the slime films attract more settlement of cyprid larvae on the surfaces. Surfaces colonized by bacteria can serve as a source of regulatory signals for larval settlement (Young & Mitchell, 1973;

Bonar *et al.*, 1986; Maki *et al.*, 1990; Tsurumi & Fusetani, 1998; Wieczorek & Todd, 1998). The importance of the microbial films as a settlement cue for the larvae of some sessile marine macrofouling organisms has been well established (Fitt *et al.*, 1990; Johnson *et al.*, 1991; Szewzyk *et al.*, 1991; Holmstrom *et al.*, 1992; Maki *et al.*, 1992; Leitz & Wagner, 1993; Parsons *et al.*, 1993; Neal & Yule, 1994a,b; Todd & Jeough, 1994, Wieczorek *et al.*, 1995). Conversely, there are some reports which indicate the inhibition of larval settlement by bacterial biofilms (Maki *et al.*, 1990; 1992, Holmstrom *et al.*, 1992). For example, biofilm of the bacterium *Deleya marina* has been reported to stimulate attachment of spirobid polychaete larvae (Kirchman *et al.*, 1982) and inhibit attachment of bryozoan larvae (Maki *et al.*, 1989).

Mechanisms of bacterial adhesion

Two theories have been suggested to explain the adhesion of microorganisms to surfaces (Bellan-Fontaine & Cerf, 1991). First theory considers the electrostatic properties of the system (DLVO theory) and the second considers interfacial free energy of the system ("Wettability" Theory).

(a) DLVO theory :

The DLVO theory named after Derjaguin and Landau (1941) and Verwey and Overbeek (1948), involves Van der Waals and electrostatic forces of attraction. The two positions of attractions are called as the primary minimum (< 1nm) and the secondary minimum

(about 5 to 10nm). At a point between, repulsive forces are maximal. As the two solid bodies with similar charges approach one another, they experience attractive and repulsive forces that are additive in nature and vary independently with distance of separation. At long distances (secondary minimum) attractive forces are stronger than repulsive ones and the cell and the substratum mutually attract one another. This attraction is weak and is reversible by fluid shear forces. As the cell approaches closer to the surface, potential energy increases and the cell and the substratum repel one another. If the bacterium is able to overcome the forces of the potential energy maximum and get even closer to the surface (primary minimum), then attraction is once again the greater force and the cell becomes irreversibly attached.

It is unlikely that cells are able to approach a substratum sufficiently closely to overcome the repulsive peak which exists between the primary and secondary minima. The mathematical expression of DLVO theory of particle interaction includes the radius of particle. As the radius of the particle decreases, the repulsive energy barrier decreases. Thus, when cells are able to reduce their effective radii as in the production of filopodia (e.g. mammalian cells) or fimbriae and exopolymers (bacteria), they may overcome the repulsive forces and adhere at the primary attraction minima with the help of cell appendages and EPS (Weiss & Marlos, 1977; Rogers, 1979; Verhuet *et al.*, 1992). Further, EPS produced by the bacteria act as a cementing material by forming bridges across the repulsion zone.

Electrostatic interactions are not considered in this theory because in biological fluids as well as in marine waters these interactions are often negligible due to the presence of strong ionic groups.

(b) Interfacial free energy and adhesion

Theoretically, if the total free energy of a substratum containing a cell and an adjacent substratum is reduced by contact, then adhesion of the cells to the substratum takes place (Characklis & Cooksey, 1983). In many cases adhesion of cells has been related to the critical wetting tension (Dexter *et al.*, 1975; Baier, 1980). This parameter is in turn related to the contact angle between model liquids and the substrata being studied (Zisman, 1964).

In aquatic habitats bacterial adhesion to substrata is minimal when the critical surface tension is in the range of 20-30 mN/m (Baier, 1973). Such substrata are relatively hydrophobic and Baier (1973) has termed this range the minimally bioadhesive range. Several studies on a range of substrata immersed in seawater have shown minimal adhesion by bacteria on hydrophobic substrata, that is, in the minimally bioadhesive range predicted by Baier and maximal adhesion on high energy (hydrophilic) substrata (Dexter *et al.*, 1975; Dexter, 1979). On the other hand, others have indicated more rapid colonization of hydrophobic surfaces as compared to hydrophilic surfaces immersed in seawater (Loeb, 1977; Carson & Allisopp, 1980).

Fletcher & Loeb, (1979) reported that a marine pseudomonad exhibited maximal attachment to hydrophobic surfaces and relatively lower attachment to hydrophilic surfaces with a direct relationship between increasing adhesion and increased water contact angle (θ) of the substrata. For adhesion to occur between hydrated bacterial and solid surfaces water must be displaced as the two surfaces move together, and the greater work of adhesion between water and the surface (W_A) the more difficult will be this displacement of water (Pringle & Fletcher, 1983). The structure of water in the region near any surface is perturbed over distances of several molecular layers. Near a so called hydrophobic surface the water is less structured in terms of intermolecular hydrogen bonding between the water molecules, whilst near a hydrophilic surface, water is more structured. If two similar surfaces come together such that the two perturbed water layers overlap, then there must be displacement of perturbed water molecules into bulk water. This will lead to a decrease in free energy (i.e. an attraction) in the case of a hydrophobic surface, but to an increase in free energy (i.e. repulsion) in case of a hydrophilic surface. These structural interactions which clearly vary with distance of separation between the surfaces are an addition to the Van der Waals interactions between the particles concerned.

Temporary adhesion in gliding bacteria

Because gliding bacteria are capable of translational motion across a surface and are capable of breaking away from the

attachment surface, the mode of adhesion can be described as temporary (Crisp, 1973). A temporary adhesion holds surfaces together by the work done against viscosity when the surfaces are separated, yet allows the translational motion across the surface. This has been termed Stefens adhesion and describes the situation where the force required to separate two surfaces is very much greater than the horizontal drag.

Factors influencing microfouling

Any system in which microfouling takes place can be separated into the following three distinct phases.

- 1) Bulk fluid
- 2) Substratum
- 3) Biofilm.

Bulk Fluid

The bulk fluid serves as a source of thermal energy, pH ions, nutrients for the biofilm organisms and thus affect microbial adhesion (McEldowney & Fletcher, 1988). Attachment of a pathogen, *Pseudomonas aeruginosa* to stainless steel depended on the pH and ionic strength (Stanely, 1983). Stimulation of attached bacteria *Lactobacillus viridescens* by 10 to 20 gl^{-1} NaCl while inhibition using 30 and 40 gl^{-1} NaCl has been reported by Delaquis *et al.*; (1986, 1988). It has been observed that the rates and the extent of adhesion of microorganisms to polystyrene depend on sodium chloride concentration and the physiological status of the cells (Roller, 1991).

In fluid systems, there are two types of flow 1) Laminar i.e. smooth flow and 2) turbulent flow where movement within the flow may be irregular. When the flow is laminar eg. narrow pipelines, an increase in flow rate will increase the attachment of slowly grown cells, whereas a decrease in the attachment of bacteria growing at faster rate will be seen. This could be due to the effect on deposition of nutrients contained in the water. In turbulent systems, the attachment of bacteria to the surfaces increases with an increase in turbulence. This could be due to 1) preferential attachment by certain bacterial species 2) microbial response to environmental stress 3) removal of loosely bound water from the film by the fluid pressure force. Turbulence as well as shear forces in laminar flow also influence cell detachment from the biofilms.

Nutrients play a significant role in the adhesion of bacteria to surfaces (Corpe, 1974; Brown *et al.*, 1977). Yu *et al.* (1987) have shown that adhesion of *Vibrio furnissi* occurred only under nutritional conditions suitable for protein synthesis. Brown *et al.* (1977) provided experimental evidence to demonstrate that a nitrogen limited culture resulted in poor adhesion. In the case study of Madras Atomic Power Station, Venugopalan *et al.* (1992) concluded that there was massive biofouling accumulation owing to continuous supply of fresh food and oxygen and removal of metabolic wastes. Furthermore, sloughing of biofilms in trickling filters in response to both nutrient depletion and substrate loading has been reported (Characklis & Cooksey, 1983).

Substratum

Substratum provides shelter from otherwise adverse conditions. The substratum surface texture, topography and roughness can influence biofilm development (Pederson, 1990).

Rough surfaces are more prone to fouling than the smooth surfaces (Bott, 1993; Hunt & Parry, 1998). Hydrophobicity of the substratum also plays an important role in bacterial adhesion (Bidle *et al.*, 1993). The chemical nature of the substratum also influences attachment in the early phase of biofilm development (Wienczek & Fletcher, 1995). For example, some surfaces like copper are toxic to the bacterial cells and are inhibitory to the bacterial fouling (Roger *et al.*, 1994).

Role of bacterial polymers

Attachment of microorganisms to surfaces may stimulate the production of EPS (Vandevivere & Kirchman, 1993). EPS can make up to 5-90% of the dry-weight of the biofilm (Fleming *et al.*, 1992). These surface polymers include lipopolysaccharides, capsules, fimbriae, flagella and specialised structures such as appendages and prosticae. EPS condition the surface properties of microorganisms, thereby, influencing their adhesion to surfaces (Shea *et al.*, 1991).

Chemical nature of extracellular polysaccharides

Microbial EPS are formed from the condensation of monosaccharides units by eliminating water between C₁ hydroxyl

group of one unit and an available hydroxyl group of another monosaccharide. EPS are of two types; homopolysaccharides containing only one type of sugar moiety and heteropolysaccharides, with two or more types of sugar units. An example of homopolysaccharides is *cellulose*, which is a beta 1,4 D-glucan excreted by *Acetobacter* sp. However, most bacterial exo-polysaccharides studied are heteropolysaccharides. For example, *Xanthan* produced by *Xanthomonas campestris* is a branched anionic heteropolysaccharide. It contains D-glucose, D-mannose, D-glucuronate and variable amounts of acetate and pyruvate.

Quantity and composition of EPS vary with growth conditions (Bott, 1993). Carbon source and its concentration, nitrogen, phosphorus and growth phase of bacteria have pronounced effect on EPS production. D-glucose, D-galactose, D-mannose, fucose, ribose, arabinose, xylose, rhamnose have been reported as common monosaccharides in EPS. In addition to sugars, EPS contain non-sugar components. Amino sugars like D-glucosamine, D-galactosamine, D-mannosamine in N-acetylated form and sugar acids like glucuronic acid, galacturonic acid and mannuronic acid are important constituents of EPS. Non-sugar components may be either organic or inorganic. Other non-sugar components include organic compounds like formates, acetates or pyruvates and inorganic components such as sulphur, calcium, magnesium, phosphorus and potassium. The presence of non-sugar components gives negative charge to the EPS. EPS can therefore, react with positively charged

metal ions and can play a major role in biofouling and corrosion of various metals (Ford and Mitchell, 1990; Bhosle,1993).

Extracellular polymers play an important role in the adhesion of bacteria to surfaces by forming bridges across the repulsion barrier and thereby anchoring the cell to the surface. Zobell (1943) first suggested the participation of EPS in the adhesion of microbial cells to substrata. Since then considerable attention has been directed at the composition of EPS (Jones *et al.*, 1969; Corpe, 1970; Fletcher & Floodgate, 1973; Costerton *et al.*, 1978; Sutherland, 1980; Allison & Sutherland, 1987; Decho, 1990; Wrangstadh *et al.*, 1990; Beech *et al.*, 1991; Spenceley *et al.*, 1992; Cooksey, 1992; Hoagland *et al.*, 1993; Freeman & Lock, 1995; Titus *et al.*, 1995; Grobbsen *et al.*, 1998; Kimmel *et al.*, 1998; Lloret *et al.*, 1998; Stoderegger & Herndl, 1998). EPS appears to play important roles in biofilms including a) form cohesive forces within the biofilm, b) adsorb nutrients, c) adsorb toxic chemicals including metals and pesticides from the environment, d) serves as a buffering agent to maintain pH, e) serves as a means of intercellular communications within the biofilm, f) provide short term energy storage via the cell membrane potential and g) enhance intercellular transport of genetic material.

Role of fimbriae and flagella in attachment

Many aquatic bacteria possess fimbriae and/or flagella. According to Mayer (1971), the fimbriae disintegrate and form a

number of parallel fibers, which presumably help in increasing the strength of adhesion.

Flagella help periphytic bacteria to remain in close proximity to the surface until firm attachment can be accomplished by some other means.

Genetic control

It appears that some species have evolved complex genetic control to regulate their adhesion to surfaces. These Control mechanisms are classified as “responsive” and “variable” (Silverman *et al.*, 1984). If the bacterium senses some signal in the environment and responds to it by synthesis of adhesive substances, then it is defined as responsive control. Production of lateral flagella by marine *Vibrio parahaemolyticus* in contact with the surface is an example of “responsive” control. In this bacterium, adhesion triggers the expression of a ‘laf’ gene coding the synthesis of lateral flagella (Belas *et al.*, 1986). Dagosteno *et al.*, (1991) have also demonstrated that the adhesion triggers the expression of certain genes. Microorganisms such as *Agrobacterium* and *Rhizobium* adhere to plant surfaces by producing specific polysaccharide adhesins. After initial attachment of *Agrobacterium* and *Rhizobium*, they produce microfibrils which add to the stability of the bacterial adhesion to the surfaces. Synthesis of these microfibrils, which are composed of cellulose, appears to be a response to a signal resulting from the interaction between the bacteria and the plant surface (Silverman *et al.*, 1984).

Alternatively, when the clonal population of bacteria is heterogenous in its response to adhesive production the control is called as “variable”. A fraction of the cells is preadapted to adhere to a given surface and individual cells in the population are constantly interconverting to a variety of forms. In a population of bacteria growing on a surface, like fimbriated bacteria attached to epithelial cells, nonadherent variants arise, detach and can move on to colonize other habitats (Silverman *et al.*, 1984). The expression of type L fimbriae in *E. Coli* is an example of “variable” control. A population derived from a clone is heterogenous and is composed of fimbriate and non-fimbriate bacteria. Individual cells can switch from one form to the other. This switching is under transcriptional control (Eisenstein, 1981).

Neisseria gonorrhoeae also has specific adhesins on its surface. Clonal populations of cells contain both piliated and non-piliated bacteria and one form can convert to the other (Swanson *et al.*, 1971). The benefit derived from the variable control is that the culture is in effect preadapted to the changes in the ecosystem. The expression of adhesive function can be decided by the individual cells.

Advantages of surface attachment to bacteria

Attached organisms have some advantages over their free floating counterparts (Fig.1.1) (Blenkinsopp & Costerton, 1991). Firstly, they do not have to spend energy searching for food as

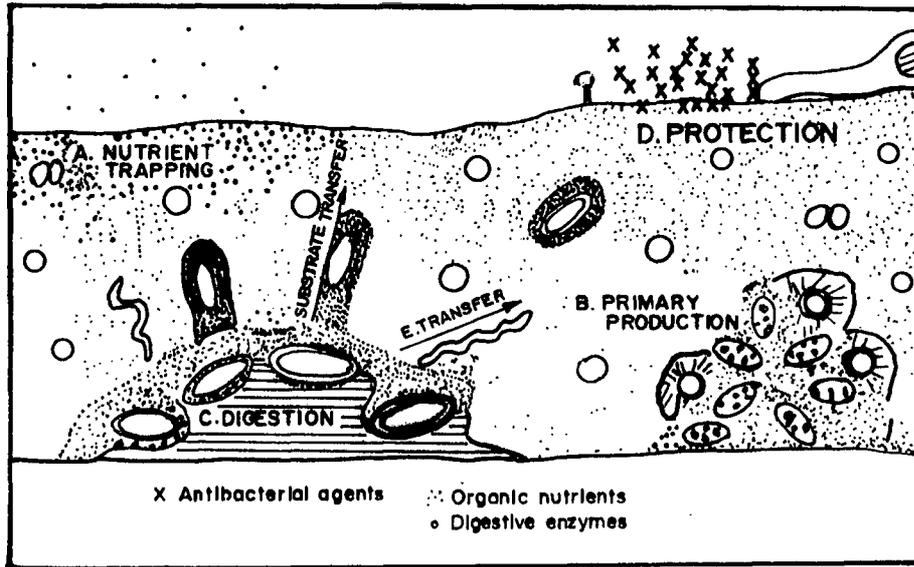


Fig.1-1. Advantages of surface attachment to the attached bacteria (from Costerton et al, 1987).

water with fresh nutrients flows over them and removes waste products at the same time. The high rates of biofilm development in oligotrophic environments strongly suggests that biofilms capture inorganic and organic molecules from the bulk liquid providing nutrients for cell growth within the biofilm (Carpentier & Cerf, 1993). Nutrients may also be available within the biofilm both as a result of cell death and because a metabolite of one organism may be a nutrient for another.

The second advantage occurs in particularly fast-flowing waters where bacteria would have been carried away by the water current if they were not provided with shelter by the substratum. Thirdly, the biofilm also protects the cells within it. Many conventional biocides are capable of killing planktonic cells, yet they do not kill sessile cells at the same biocide concentration and contact time (Keevil *et al.*, 1990; Page & Gaylarde, 1990; Pyle & Mc Feters, 1990; Wright *et al.*, 1991; Cerf & Carpentier, 1992). The sessile cells may be resistant due to physiological changes or as a result of living within a biofilm. Further, they may act as an effective barrier for the penetration and/or diffusion of the biocide. Moreover, biofilm glycocalyx matrix acts as a molecular sieve and as an ionic exchange matrix (Rodrigues & Bhosle, 1991).

In some cases the substratum is also the substrate, so that attachment or at least close association is a pre-requisite for assimilation of the substrate. For example, utilization of organic detritus by bacteria usually requires hydrolytic digestion by

extracellular enzymes. Thus, bacterial attachment to the substrate substratum allows bacteria to remain in close proximity to the nutrient source. Thereby, enzymes could be put to efficient use, if they remained at the substrate after production, either through adsorption or through retention of the enzymes into the surrounding medium (Fletcher & Marshall, 1982).

Effects of Microfouling/Biofilm

a) Advantages of biofilm

The presence of biofilm at the solid-liquid interface in man-made systems can be beneficial to man. For example, water quality in treatment plants can be improved due to degradation of the pollutants by the inhabitants of the biofilms (Lappinscott & Costerton, 1989). Biofilms in treatment plants and ponds have also been used to remove heavy metals from solutions because the exopolysaccharide components of their glycocalyx matrices have a very high affinity for metallic cations (Blenkinsopp & Costerton, 1991). It is possible to use biofilms for the selective removal of target metals from mining and refining effluents (Blenkinsopp & Costerton, 1991).

The presence of a thick bacterial biofilm which is a normal inhabitant of the tissue surface constitutes one of the many defenses of animal tissue. This biofilm protects the animal against colonization by pathogenic bacteria. This autochthonous population is highly competitive. Hence, the pathogenic bacteria are prevented

from attaching to the tissue surface (Costerton *et al.*, 1988).

The epithelial tissue of the rumen is a very special environment because very large amounts of urea diffuse through the rumen wall and must be transformed to ammonia to avoid toxic effects. Bacteria that colonize the rumen wall includes several species that produce large amounts of urease. Thus, the adherent autochthonous bacterial population makes an important physiological contribution to the health of the animal (Costerton *et al.*, 1988).

(b) Disadvantages of biofilms

Shipping vessels and marine structures

Biofilms can cause significant financial losses. Biofilm in marine waters is a costly problem for ship operators and owners of offshore structures, including oilrigs and offshore platform (Callow, 1990). Fouling on ship hulls increase the frictional drag on the vessel thereby increasing fuel consumption. This necessitates expensive dry-docking to remove the marine fouling and also the application of antifouling paints (Blenkinsopp & Costerton, 1991; Cooksey & Wiggles Worth-Cooksey, 1992).

Navigation buoys

Moored structures such as buoys are even more prone to microbial fouling than ships, since they remain permanently in sea waters. The tidal currents to which such structures are often exposed also appear to favour the growth of the microbial fouling organisms.

Underwater sound equipment

Naval and commercial ships are commonly equipped with sonic devices. These devices are permanently installed under water for purposes of coastal defense. Such equipments are not usually protected with antifouling paints nor are they constructed of metals which resist fouling. Their functioning as well as life may be seriously impaired due to microbial fouling on their surfaces.

Heat exchangers and cooling towers

Industrial cooling towers and heat exchangers are also adversely affected by biofilms (Characklis & Marshall, 1990; Melo *et al.*, 1992). The cost to control fouling on these surfaces is enormous. The output of power station heat exchangers is affected by biofilms that form on the surface in contact with the river, estuarine or seawater used for cooling (Bott, 1992; Nair & Venugopalan, 1996). Microbial biofilms in industrial systems reduce heat transfer efficiency. Transfer of heat is reduced because the thick surface growth physically prevents an efficient heat exchange between the liquid phase and the cooling surface (Lappin-Scott & Costerton, 1989).

Water distribution system

Tap water distribution system is also influenced by biofilms (Le Chevallier *et al.*, 1987). Biofilm formation on surfaces within water systems can result in significantly increased disinfection resistance of commonly occurring water borne bacteria (Pyle & Mc Feters, 1990; Block, 1992; Vess *et al.*, 1993).

Dental plaque

The association of bacteria within a film on dental enamel is termed plaque. Bacterial plaque is essential for the pathogenesis of dental caries, is the major aetiological factor in chronic gingivitis and is intimately associated with the advancing lesions of chronic periodontitis (Addy *et al.*, 1992).

Bacterial adhesion to inert medical prostheses

Modern medical practice implants large number of plastic and metal prostheses into patients. Bacteria that colonize the surfaces of these prostheses grow in coherent biofilms and often necessitate the removal of the prostheses (Costerton *et al.*, 1988; Gristina, 1994).

Microbial fouling in food and canning industry

The food and canning industry is obviously not immune from the phenomenon of microbial adhesion, followed in some cases by build-up of biofilm (Notermans *et al.*, 1991; Pontifract, 1991; Holah & Kearney, 1992; Mattila & Wirtamen, 1992). Biofilm act as a reservoir for potential pathogens. Bacterial detachment from biofilms also poses health risks in the food industry.

Microbially induced corrosion

In recent years, attention is focussed on another costly and persistent problem associated with the microfouling of metal surfaces i.e. microbiologically induced corrosion (MIC). It was actually in

nineteenth century that the scientists first became aware of the corrosion of metals by microbial activity (Garrett, 1891) and since then MIC has emerged as an important economic problem.

MIC was reported by Garrett (1891), while studying the action of water on lead. According to him bacterial metabolites could have been responsible for the corrosion of lead covered cables. Gains (1910) produced evidence to indicate that iron and sulphur bacteria were responsible for the corrosion of ferrous metals buried in soil. Formation of deposits in water pipes by iron bacteria was also reported after a few years.

Anaerobic corrosion of ferrous metals buried in soils by sulphate reducing bacteria was reported by Von Wolzogen Kuhr and Van der Vlugt (1934). Non-metallic structures like concrete are also subject to microbial degradation (Bock & Sand, 1993). The main organisms involved here are *Thiobacillus* which are sulphur oxidising bacteria (Parker, 1947; Kempner, 1966). Several papers have been published on microbiologically induced corrosion (Booth, 1971; King & Miller, 1971; Iverson, 1972; Miller & King, 1975; Luty, 1980; Deshmukh *et al.*, 1992; Lee *et al.*, 1993; Gouda *et al.*, 1993; Walker *et al.*, 1998).

The corrosion of metals in an aqueous environment is an electrochemical phenomenon involving an anode, cathode and an electron path. The electrons liberated by anodic dissolution are taken up by hydrogen present at the cathode and/or dissolved oxygen. The

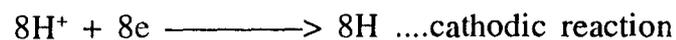
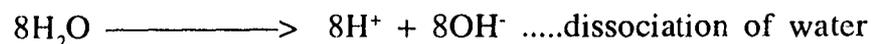
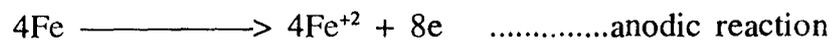
corrosion products formed may also contribute to the overall corrosion reaction. In most cases, the resulting products are loose and bulky and are precipitated. Some corrosion products like Fe_2O_3 , may remain firmly adherent to the metal surface, thereby reducing corrosion. A typical example of this phenomenon is the well preserved archaeological iron objects excavated from a corrosive soil in York (Booth *et al.*, 1962). These objects were covered with a thin, compact and firmly adherent film of ferric and ferrous phosphates.

Microbial corrosion is not fundamentally different from any other type of electrochemical corrosion. It is simply that the chemical or physical conditions creating the aggressive environment are produced by organisms because of their metabolic activities (Fig 1.2 a & b).

Bacteria associated with various types of corrosion processes

1) Bacteria associated with anaerobic corrosion

The most important bacteria associated with anaerobic corrosion processes are sulphate reducers (Fig 1.4). In 1934, Von Wolzogen Kuhr and Van de Vlught put forward the classical cathodic depolarisation theory to explain the role of sulphate reducing bacteria in anaerobic corrosion. The steps of the theory are given below.



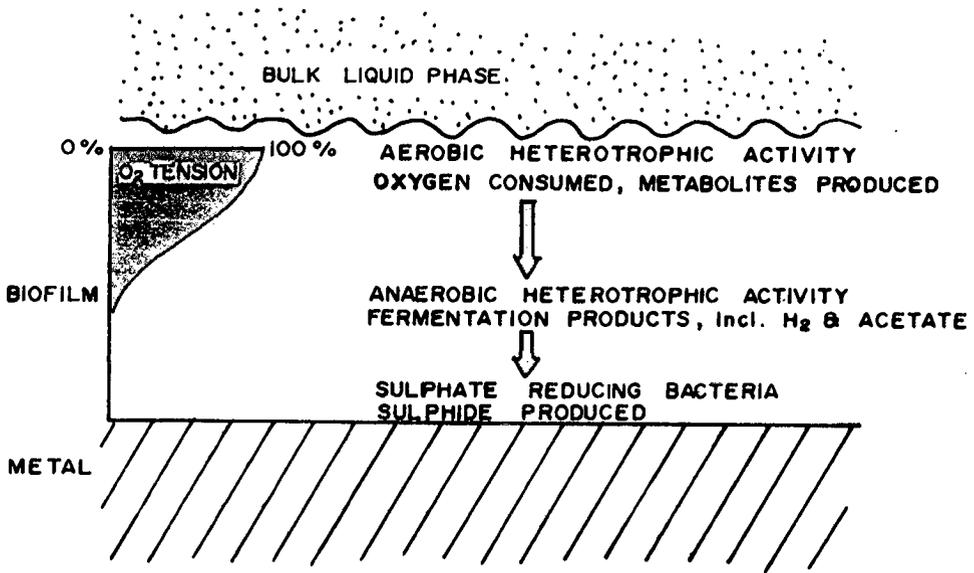


Fig. 1.2 (a) Activities of biofilm microorganisms on a metal surface (from Hamilton & Maxwell, 1986).

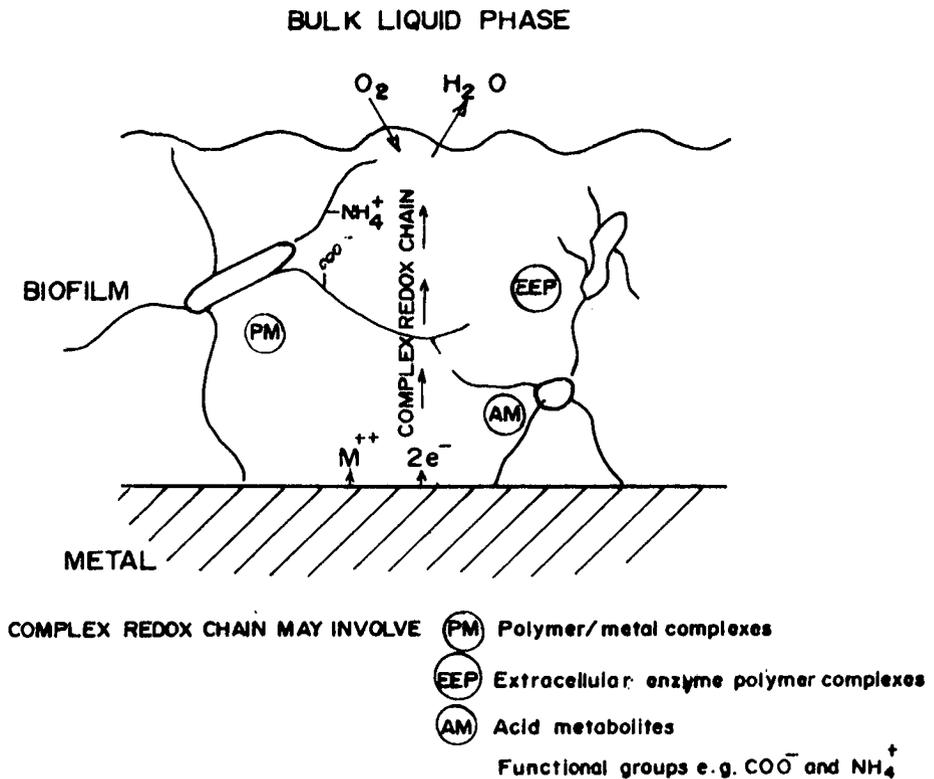


Fig. 1.2 (b) Metabolic activities of the biofilm microorganisms which facilitate the oxidation of metal surface. (from Ford & Mitchell, 1990).

$\text{SO}_4^{-2} + 8\text{H} \longrightarrow \text{S}^{-2} + 4\text{H}_2\text{O}$ cathodic depolarisation by bacteria

$\text{Fe}^{+2} + \text{S}^{-2} \longrightarrow \text{FeS}$ corrosion product

$3\text{Fe}^{+2} + 6\text{OH}^- \longrightarrow 3\text{Fe}(\text{OH})_2$ corrosion product

$4\text{Fe} + \text{SO}_4^{-2} + 4\text{H}_2\text{O} \longrightarrow 3\text{Fe}(\text{OH})_2 + \text{FeS} + 2\text{OH}^-$ overall reaction

Others (King & Miller, 1971; Stott, 1993) however, debated the validity of cathodic depolarization theory. They provided evidence for the occurrence of an alternative mechanism involving the metabolite sulphide and that the presence of iron sulphide films may be more important in MIC. King & Miller (1971) ascribed a role to both hydrogenase and iron/iron sulphide galvanic cells. Thus, even before 1980, anaerobic corrosion of iron and steel by SRB could be explained in electrochemical terms.

Costello (1975) modified the theory developed by Miller and King and replaced H^+ by H_2S . Some studies (Schaschl, 1980) also indicate that the elemental sulphur itself may be corrosive to the metal. The elemental sulphur appears to promote corrosion by a concentration cell mechanism similar to differential aeration cell. An anodic area may develop underneath any material which shields the metal from dissolved sulphur. The corresponding cathodic area is the adjacent region where dissolved sulphur is easily available as a cathodic reactant. The bacterial colonies may provide shielding action

needed for this concentration cell.

While working with *Desulfovibrio*, Iverson (1968) found that the corrosion product was iron phosphide (Fe_2P). Further results indicated that the corrosive agent was a volatile phosphorus compound produced by SRB (Iverson & Olson, 1983). Iverson *et al.* (1986) proposed that the break down of iron sulphide film allows corrosive phosphorus compounds to come into contact with iron surface and initiate corrosion.

2) Bacteria associated with corrosion due to differential aeration cells

A wide range of bacterial species like *Pseudomonas*, *Flavobacterium*, *Aerobacter*, *Gallionella*, *Vibrio* and *Sphaerotilus* have been associated with corrosion due to differential aeration cell. Fungal species of *Trichoderma*, *Monilia* and *Penicillium* are also capable of causing differential aeration cells due to the patchiness of biofilm. The uptake of oxygen by the microbial colony results in the depletion of oxygen under these colonies. An area of oxygen depletion becomes anodic to more oxygenated areas. As a result the metal ions go into the solution at the anode while electrons combine with water and oxygen at the cathode (Fig 1.3). Fouling of industrial cooling water systems and heat exchangers may be subjected to this type of corrosion. Structures immersed in rivers, estuaries or other waters may also be prone to this corrosion.

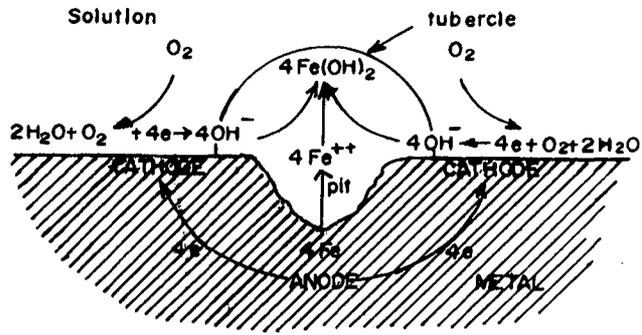


Fig. 1.3. Corrosion due to differential aeration cell.
(from Iverson, 1972).

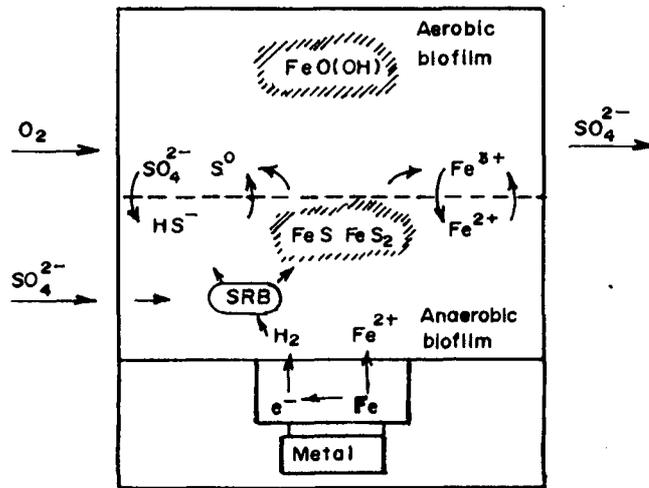


Fig. 1.4. Hypothetical model of corrosion due to microbial activity within deposits
(from Nielsen et al., 1993).

3) Bacteria associated with tubercle formation

This problem is usually found in portable water distribution pipe lines. Tubercles are formed due to microbiological deposition of iron and manganese oxides. The bacteria isolated from tubercles are iron and manganese oxidising bacteria like *Gallionella*, *Sphaerotilus*, *Leptothrix*, *Crenothrix*, *Arthrobacter*, *Caulobacter* or *Hyphomicrobium*. These organisms obtain their energy by the oxidation of ferrous ions to ferric ions. Ferric hydroxide is accumulated on the internal surfaces of the pipe line. Tubercle consists of thin shells of hard magnetite (Fe_3O_4) beneath which is a mixture of hydrated ferrous oxide and iron sulphides. The surface of the tubercle in contact with water is covered with a thin layer of goettite ($\text{FeO}(\text{OH})$) (Fig 1.4).

4) Bacteria causing acidic environments

Inorganic and organic acids produced by microorganisms are known to induce corrosion of metals (Iverson, 1974). For example, bacteria of the genus *Thiobacillus* oxidise sulphur to strong sulphuric acid which play an important role in the degradation of and corrosion of building material. The most well known examples are the corrosion of archaeological structures like Parthenon in Greece and temples of Cambodia (Pochon & Jaton, 1968). A number of fungal species are also capable of producing organic acids. An example is *Hormoconis resinae* (previously known as *Cladosporium resinae*) which brings about the corrosion of the aluminium fuel tanks of aircraft.

Biofouling control

A number of methods can be adopted to prevent microbial fouling. These measures fall into four categories i.e. chemical, physical, protective coatings and biological.

Physical control measures include different techniques like manual cleaning, hydraulic compressed air, water jets, sand blasting and high velocity ice particles (Fisher *et al.*, 1984). Manual cleaning by hand tools is very useful for cleaning fixed offshore structures. Hand tools include diver's knife, scrapers and chipping hammers. Hydrolically powered equipments like rotary brushes and rotary chippers do not need any physical effort from the operator and provide good cleaning standards.

Compressed air needle gun can be used only for shallow waters i.e. upto 30 m. Water jet is well suited for under water cleaning. However, hard calcareous shells are difficult to remove with water jets. Addition of abrasive to water jets may be more effective, but it causes damage to the surface of the structure beneath the fouling growth. Use of high pressure water jets gives fairly good results.

Dry sand blasting can be used to clean ship hull in dry docking. This is a fast method and cleans the surface well. But it generates dust which creates bad working conditions and is dangerous to ship and dock machinery. Wet sand blasting technique can overcome this problem, but the efficiency of this method may

be lesser than dry sand blasting. Bombarding high velocity ice particles has an advantage over sand blasting in that it does not cause surface pitting.

Regular mechanical scraping (pigging) is quite effective in oil industry. Pigging disturbs the highly structured bacterial corrosion consortia and renders them inactive (Blenkinsopp & Costerton, 1991). Drainage of pipes combined with flushing with hot air which results in death through dessication can also be used. A technique of alternating cycles of slow freezing and thawing can be effectively used to prevent fouling of industrial cooling towers and heat exchangers (Blenkinsopp & Costerton, 1991). Slow freezing produces large ice crystals within the biofilm and the thawing of these crystals then leads to the removal of biofilm. Other methods of biofilm control include the use of acoustics, ultraviolet light and gama radiation (Callow, 1993).

Various other methods use electric current to prevent fouling. In this method, metal bars are placed close to the surface of the structure to be protected from fouling. When the current is impressed, large amount of hydrogen or hypochlorite is generated, which prevents settlement of fouling organisms.

Chemical control is used in water bodies where complete biofouling control is essential eg. swimming pools. Chlorination is the most widely used preventive measures and chlorine is the most effectively used biocide (Jenner & Janssen-Mommen, 1993). Chlorine solution is very effective in controlling fouling inside pipes and

conduits, which supplies sea-water on board the ship or for cooling purposes in condensers of coastal installations. The dose required depends on temperature, pH and the chlorine demand of the water (Jenner & Janssen-Mommen, 1993). Residual chlorine concentration of 0.25 ppm is reported to be effective in preventing fouling growth (Anon, 1952). Other compounds used are sodium hypochlorite, chlorine dioxide, ozone, hydrogen peroxide, cyameric acid and copper salts (Callow, 1993). Biocide concentration that is sufficient to kill planktonic bacteria may not be able to kill biofilm bacteria. Hence research is currently directed towards studying biocide penetration into the biofilm.

Application of antifouling paints have only a limited success. Antifouling paints are mostly based on copper, mercury, arsenic and tributyltin compounds. These compounds are toxic to the bacteria which form on the surface submerged in the sea. However, the use of such chemical toxins in marine environment pose threat to marine biota. Hence, research is focussed on the use of environment freindly natural compounds, which are easily biodegradable and cause no harm to marine life (Clare, 1998). Many marine organisms and terrestrial plants have been screened for antifouling properties (Sawant *et al.*, 1995; Sawant, 1996, Prabha Devi *et al.*, 1997).

The biological method of control of fouling includes release of marine organisms which feed on the organisms attached to the surface which is to be cleaned. Callow (1993) has suggested that biological control using viruses or parasitoids may find application for specific fouling organisms. The use of compounds which inhibit

CHAPTER 2A

*Isolation & characterization
of marine fouling bacteria from
aluminium panels immersed
in Mandovi estuary.*

1. INTRODUCTION

Soon after immersion in an aquatic environment solid surfaces adsorb dissolved organic matter, thereby conditioning the surfaces (Loeb & Neihof, 1977; Characklis & Escher, 1988; Zutic & Tomaic, 1988; Callow, 1993). Spontaneous adsorption of these macromolecules onto the surfaces immersed in an aquatic environment results in the concentration of useful nutrients at the surfaces in an otherwise nutrient deficient environment (Marshall, 1980). Such a situation favours colonization of solid-liquid interfaces by small rod shaped copiotrophic bacteria (Marshall *et al.*, 1971; Fletcher & Marshall, 1982). These early colonizing copiotrophic bacteria are predominantly small gram-negative rods, comprising species of *Pseudomonas* (50 to 90%), *Flavobacterium* (10 to 49%) and *Achromobacter* (Marshall *et al.*, 1971).

Adhesion and growth of copiotrophs in the early stages of colonization may lead to rapid utilization of nutrients accumulated at the solid - liquid interface. This creates nutrient deficient condition on the surface which allows oligotrophs a competitive advantage to colonize the surface. It is also likely that the primary colonizers provide specialised nutrients for the secondary colonizers (i.e. oligotrophs) due to secretions, cell leakage and/or cell lysis. Biomass, numbers and diversity of attached microorganisms increase as the succession proceeds over the period of immersion (Jordan & Staley, 1976; Gerchakov *et al.*, 1977). Bacterial colonization of

surface is generally followed by attachment and growth of other micro-organisms including diatoms, fungi and protozoa. Such attachment, growth and succession of micro-organisms on a surface is known as biofilm or microfouling.

Development of microfouling on solid surfaces placed in temperate marine waters has been studied by many researchers (Corpe, 1973; Baier, 1984; Little, 1984). However, most of these studies utilized short immersion period ranging from hours to few days. Conversely, very little is known about the development of microfouling on solid surfaces deployed in tropical marine waters. In view of this, the aim of the work presented in this chapter was to study the development of bacterial fouling on metallic surfaces immersed in sea water over a period of one month. Aluminium, a commonly used engineering material was employed as a model substratum for the assessment of bacterial fouling studies presented here.

2. MATERIALS AND METHODS

2.1 Growth media

Nutrient broth (Table 2A.1) prepared in Basal Salt Solution (BSS) (Table 2A.2) was used as a growth medium for the bacterial cultures throughout the course of the study. Zobell Marine Agar (ZMA) (Table 2A.3) was used for the isolation and purification of bacteria using spread plate method.

Table 2A.1

Nutrient broth composition

Chemicals	Weight (gms) l⁻¹
Peptone	5
Yeast extract	1
Glucose	2
Basal Salt Solution	1000 ml
pH	7.5

Table 2A.2**Basal salt solution (BSS) composition**

Chemicals	Weight (g.l⁻¹)
NaCl	30.00
KCl	0.75
MgSO ₄	7.00
NH ₄ Cl	1.00
K ₂ HPO ₄ (10%)	7.00
KH ₂ PO ₄ (10%)	3.00
Trace metal solution	100.0 ml
Distilled water	1000.0 ml
pH	7.5

Sterilization 121° C for 15 mins

Trace metal solution composition

Chemicals	Weight (mg.l⁻¹)	Final metal Conc(ppm)
H ₃ BO ₃	2850	0.5
MnCl ₂ .4H ₂ O	1800	0.5
FeSO ₄ .7H ₂ O	2490	0.5
Na Tartarate	1770	-
CuCl ₂	26.9	0.01
ZnCl ₂	20.8	0.01
COCl ₂	40.4	0.01
Na ₂ MoO ₄ .2H ₂ O	25.2	0.01

Table 2A.3**Zobell Marine Agar Composition**

Chemicals	g.l⁻¹
Peptone	5.0
Yeast extract	1.0
Ferric Citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.0
Final pH at 25°C	7.6±0.2

2.2 Sterilization

All the media were sterilized at 121°C for 15 min. Glassware was washed with chromic acid and then with sodium hydroxide. Thereafter, the glassware was rinsed with distilled water and dried in an oven.

2.3 Preparation of test panels

Aluminium panels (15 x 10 cm) were cleaned with 20% (V/V) hydrochloric acid (Camerson & Robinson, 1968; Bhosle *et al.*, 1989, 1990), repeatedly washed with tap water followed by distilled water, dried in an oven and kept covered until used.

2.4 Deployment of panels

Panels were immersed in the surface waters (~1m) at a site in the Mandovi estuary (15° 30'N, 73° 52'E). Before immersion they were fixed onto a PVC frame using PVC nuts and bolts. The assembly was maintained at 1 m in surface waters with the help of a float.

2.5 Retrieval of panels

Panels were retrieved in triplicate after 24 h and thereafter at weekly intervals over a four week period. Panels were transported to the laboratory in an ice box containing water from the collection site.

2.6 Estimation of microfouling biomass

After retrieval panels were scraped in a laminar flow chamber using a nylon brush and filter-sterilized ($0.22\mu\text{m}$) estuarine water (White & Benson, 1984; Morikawa, 1988; Bhosle *et al.*, 1989, 1990; Sharma *et al.*, 1990). The volume of the scraped material was adjusted to 100 ml and aliquots (10 ml) were filtered through preignited (450°C , 3h) and preweighed GF/C glass filters (47 mm). Duplicate filters were then analysed for dry weight (DW) and organic carbon (OC) to estimate microfouling biomass. The OC content of the microfouling material was analysed following the method of Parsons *et al.*, (1984). The DW was determined after drying the filters in an oven at 50°C for 24h (Bhosle *et al.*, 1989). The filters were then cooled in a desiccator and weighed on a microbalance (Mettler M3). The coefficient of variation of the analytical methods used for DW and OC based on 6 replicate analysis was 6.50 and 2.08%, respectively.

2.7 Estimation of viable count of bacteria

The microfouling material (1ml) obtained as above was serially diluted and 0.1 ml aliquot was plated in triplicate on Petri dishes containing Zobell Marine Agar (ZMA) by the surface spread plate method (Buck & Cleverdon, 1960). The Petri dishes were then incubated at room temperature ($28\pm 2^\circ\text{C}$) for 24h after which the total viable count and colony morphology were noted. During each sampling twenty colonies (24% of total) were randomly selected from the greatest dilution showing growth, therefore, the

colonies in this study represent the numerically most dominant aerobic strain from the test panels. Colonies were purified by repeatedly streak plating on ZMA plates, transferred on ZMA slants and routinely subcultured at monthly intervals. The cultures were maintained on ZMA slants at 4°C.

2.8 Isolation and characterization of bacterial cultures

One hundred colonies were isolated during the period of study and these were studied for morphological, physiological and biochemical characteristics (Cruickshank *et al.*, 1975) and identified (Gibson *et al.*, 1978; Mitruka, 1979; Krieg & Holt, 1984; Robertward *et al.*, 1986). A taxonomic scheme for the identification of marine bacteria developed by Oliver (1982) was used for the characterization of these isolates. Four cultures were selected for further study depending on their occurrence during the immersion period and their ability for extracellular polysaccharide production (Chapter 6). These four cultures were identified upto species level.

2.9 Diversity

Data on the relative abundance of bacterial genera were utilized to estimate diversity using the Shannon-Weaver equation

$$H = \sum_{i=1}^s P_i \log P_i$$

where P_i is the proportion of the community belonging to the i th species and S is the number of species.

2.10 Growth curve

All the four isolates were grown in nutrient broth (Table 2A.1) prepared in basal salt solution (BSS) (Table 2A.2) for 18hrs. This was then used as a source of inoculum (1%) for another flask (500 ml) containing 100 ml nutrient broth. Flasks were incubated on a rotary shaker (150 rpm) at room temperature. This procedure was employed throughout for the inoculation and growth of isolates for various experiments described in this thesis. At regular intervals, a 2ml aliquot was removed. Turbidity was measured at 400nm using a Beckman DU 64 Spectrophotometer. Observation on bacterial growth was performed until the culture attained stationary phase.

3. RESULTS

3.1 Microfouling biomass

Microfouling biomass measured as dry weight (DW) and organic carbon (OC) ranged from 51.1 to 162.5 mg.dm⁻² and 190.2 to 578.04 μ gC.dm⁻², respectively, and generally increased over the period of study (Fig 2A.1).

3.2 Isolation and characterization

Total viable counts of bacteria on the aluminium panels showed a consistent increase over the period of study and varied from 0.41 to 495 x 10⁶ CFU cm⁻² (Table 2A.4).

Bacterial colonies also showed differences in colony morphology. Colonies isolated after immersion period of 1d were

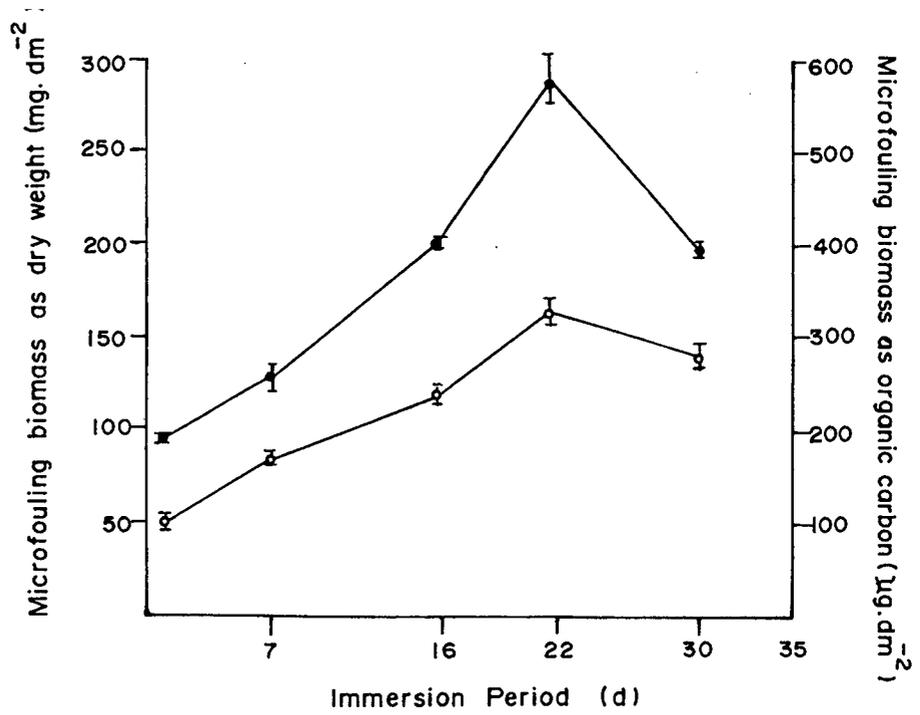


Fig. 2A.1. Development of microfouling biomass as dry weight (○) and organic carbon (●) on aluminium panels immersed in the surface waters of Mandovi estuary for a period of 30 d.

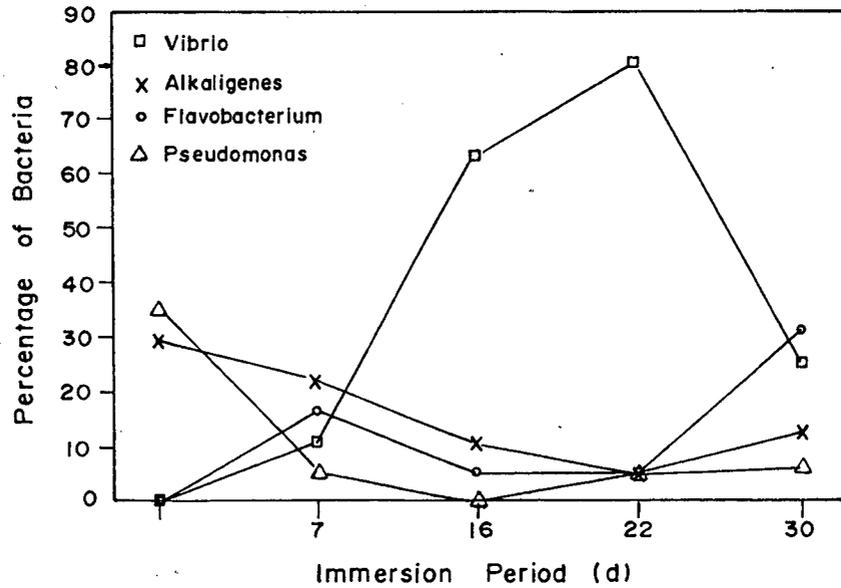


Fig. 2A.2. Variation in the abundance of some bacterial groups (as a % of total isolates) on aluminium panels immersed in the surface waters of Mandovi estuary for a period of 30 d.

Table 2A.4

Total viable count of bacteria on aluminium panels immersed in the surface waters of Mandovi estuary during 30d immersion period

Period of immersion (d)	Viable count CFU cm⁻² x 10⁵
1	0.41 ± 0.012
7	5.16 ± 0.796
16	5.60 ± 0.610
22	10.50 ± 1.200
30	495.00 ± 35.600

CFU = Colony Forming Units

all colourless, regular, smooth and mucoidal; whereas 7d after immersion, many coloured colonies were found. Spreading, spreading and fluorescent and small microcolonies of bacteria were observed at 16, 22 and 30d after the immersion, respectively. Pigments of the isolates varied from lemon yellow to reddish brown during the period of immersion.

Of the hundred colonies isolated, 10 could not be maintained. The remaining 90 colonies were identified using standard, morphological and biochemical characteristics of the isolates (Table 2A.5). Most were gram negative, oxidase positive, facultative anaerobes and oxidative and fermentative in nature. Many were motile, fluorescent and reduced nitrate to nitrite. The isolates in general produced catalase and utilised some sugars including glucose, sucrose, maltose, mannitol and lactose. On the basis of these characteristics the cultures were tentatively identified up to the generic level (Table 2A.5). Of these, 4 cultures produced exopolysaccharides and so selected for further studies. These 4 cultures were identified upto species level (Table 2A.6).

The generic composition of the bacterial biofilm developed on aluminium panels appears to vary over the period of the immersion (Fig 2A.2, Table 2A.7). For example, *Pseudomonas* (35%), *Alkaligenes* (29%) and *Chromobacterium* (23%) were the most abundant biofilm bacteria on aluminium panels during the initial 24h immersion period. Subsequently, as the immersion period

Table 2A.5

Morphology and biochemical characteristics of bacteria on aluminium panels immersed in the surface waters of Mandovi estuary for a period of 30 d															
Organisms	Gm stain & morph	MOT	O	C	G	M	L	S	MA	I	NO ₃ Red	H ₂ S Prod	Hugh - Oxid	Leifson Fermt	Pigment
<i>Vibrio</i> (34)	Gm-ve short rods	+	+	+	+	+	+	+	+	(18)	+	+	+	+	-
<i>Alkaligenes</i> (14)	Gm-ve rods	+	+	+	+	-	-	-	-	-	(3)	-	+	-	-
<i>Flavobacterium</i> (10)	Gm-ve rods	(7)	+	+	+	(2)	+	+	(4)	-	+	-	(1)	-	+
<i>Pseudomonas</i> (9)	Gm-ve rods	+	(3)	+	+	+	-	+	(3)	(1)	(4)	-	(2)	-	-
<i>Chromobacterium</i> (7)	Gm-ve rods	+	+	+	+	(2)	-	+	(2)	-	+	-	+	+	-
<i>Enterobacteriaceae</i> (6)	Gm-ve rods	(2)	-	+	+	(2)	-	+	(2)	-	+	-	+	+	(5)
<i>Corynebacteriaceae</i> (1)	Gm-ve pleiomorphic rods tapered at the ends making sharp angles with one another	+	-	+	+	-	-	+	-	-	-	-	+	-	-
<i>Moraxella</i> (1)	Gm-ve cocci, some in pairs, some in chains	+	-	+	+	-	-	+	-	-	-	-	+	-	-
<i>Staphylococci</i> (1)	Gm+ve cocci in clusters	+	-	+	+	+	+	+	+	-	-	-	+	+	+
<i>Agrobacterium</i> (1)	Gm-ve rods	-	+	+	-	-	-	-	-	-	+	+	-	-	-
<i>Arthrobacter</i> (1)	Gm-ve pleiomorphic rods and cocci (with purple dots inside)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Gm stain = Gram stain & morphology, MOT = Motility, O = Oxidase, C = Catalase, G = Glucose, M = Maltose, L = Lactose, S = Sucrose, MA = Mannitol, I = Indole, + = All isolates giving positive results, - = All isolates giving negative results, (n) = Number of isolates giving positive results

Table 2A.6

Biochemical characteristics of the four bacterial isolates for the identification to species level

Bacteria	Utilization of							Growth at				Production of			
	Glucose	Fructose	Maltose	Sucrose	Mannitol	Lactose	Citrate	4°C	30°C	35°C	40°C	Amylase	Gelatinase	Lipase	Indole
<i>Pseudomonas stutzeri</i>	+	+	+	+	-	-	+	-	+	+	+	+	-	+	-
<i>Vibrio pelagius</i> (<i>S</i> ₁) biovar II	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
<i>Flavobacterium multivorum</i>	+	+	+	+	-	+	+	-	+	+	ND	+	ND	+	-
<i>Vibrio pelagius</i> (<i>S</i> ₂) biovar II	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-

+ All isolates giving positive results.

- All isolates giving negative results.

ND Not done.

Table 2A.7

Abundance of various bacteria (as a % of isolates) on aluminium panels as a function of immersion period

Bacteria	Immersion period (d)				
	1	7	16	22	30
<i>Agrobacterium</i>	-	-	-	-	6.25
<i>Alkaligenes</i>	29.41	22.22	10.52	5.00	12.50
<i>Arthrobacter</i>	-	-	-	-	6.25
<i>Chromobacterium</i>	23.52	11.11	-	-	6.25
<i>Corynebacterium</i>	5.88	-	-	-	-
<i>Enterobacteriaceae</i>	5.88	27.77	-	-	-
<i>Flavobacterium</i>	-	16.66	5.26	5.00	31.25
<i>Moraxella</i>	-	-	5.26	-	-
<i>Pseudomonas</i>	35.29	5.55	-	5.00	6.25
<i>Staphylococcus</i>	-	-	5.26	-	-
<i>Vibrio</i>	-	11.11	63.15	80.00	25.00
Not identified	-	5.55	10.52	5.00	6.25

- = Negative results

increased, *Vibrio* increased in abundance and on day 22 *Vibrio* was the single most abundant group (80%). Thereafter, the group composition of bacteria changed again with *Flavobacterium* (31.25%) and *Vibrio* (25%) being the most abundant groups on day 30.

When expressed as the percentage of the total isolates, collected over the period of study, *Vibrio* was the most abundant biofilm bacterium, comprising about 37% of the total isolates (Table 2A.8). This was followed by the *Alkaligenes* (15%), *Flavobacterium* (11%) and *Pseudomonas* (10%). The relative abundance of *Enterobacteriaceae* and *Chromobacterium* did not vary much over the period of study. The remaining bacteria and the unidentified isolates each accounted for about 5% of the total.

The selected two *Vibrio* cultures utilized different sugars, grew at different temperatures and required NaCl for growth. Furthermore, these cultures hydrolysed starch, liquified gelatin and showed the activity of the enzyme alginase. Based on these characteristics these two cultures were tentatively identified as *Vibrio pelagius* (Table 2A.6) and designated as S₁ & S₂. *Pseudomonas* sp. was gelatin negative, positive for starch hydrolysis and lipase activity. It was a non-pigmented isolate and grew at 40°C. The culture used sodium citrate as a sole source of carbon. This culture also utilized different sugars. Due to these characteristics, this culture was tentatively identified as *Pseudomonas stutzeri*.

Flavobacterium sp was indole negative. Two species of *Flavobacterium* which are indole negative are *F. multivorum* and *F. spiritivorum*. The culture used in this investigation did not utilize mannitol sugar and so it was tentatively identified as *F. multivorum*.

3.3 Diversity

The bacterial generic composition data was analysed using the Shannon-Weaver equation in order to assess the diversity of bacterial population. The indices tended to increase over the first 7d period of immersion (Table 2A.9), decreasing thereafter as the period of immersion increased. The evenness of the bacterial community was apparent for the first 7d, but a consistent decrease was recorded on days 16 and 22 after immersion followed by an increase.

3.4 Growth curve

The growth curve experiment for *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were carried out to define the growth phases of these cultures. All the isolates showed a very short lag phase. This was followed by rapid growth during the exponential phase. The isolates attained stationary phase within 16 h of incubation and showed typical sigmoidal growth curves (Fig 2A.3).

Table 2A.9

Shannon Diversity Index for the bacterial cultures on aluminium panels immersed in estuarine waters for a period of 30 d.

Immersion Period (d)	No of species	Total sum of species	H	E
1	5	17	2.02	0.21
7	6	17	2.42	0.21
16	5	17	1.44	0.14
22	4	19	0.88	0.10
30	7	15	2.47	0.18

H = Shannon Diversity Index; E = Evenness of the community

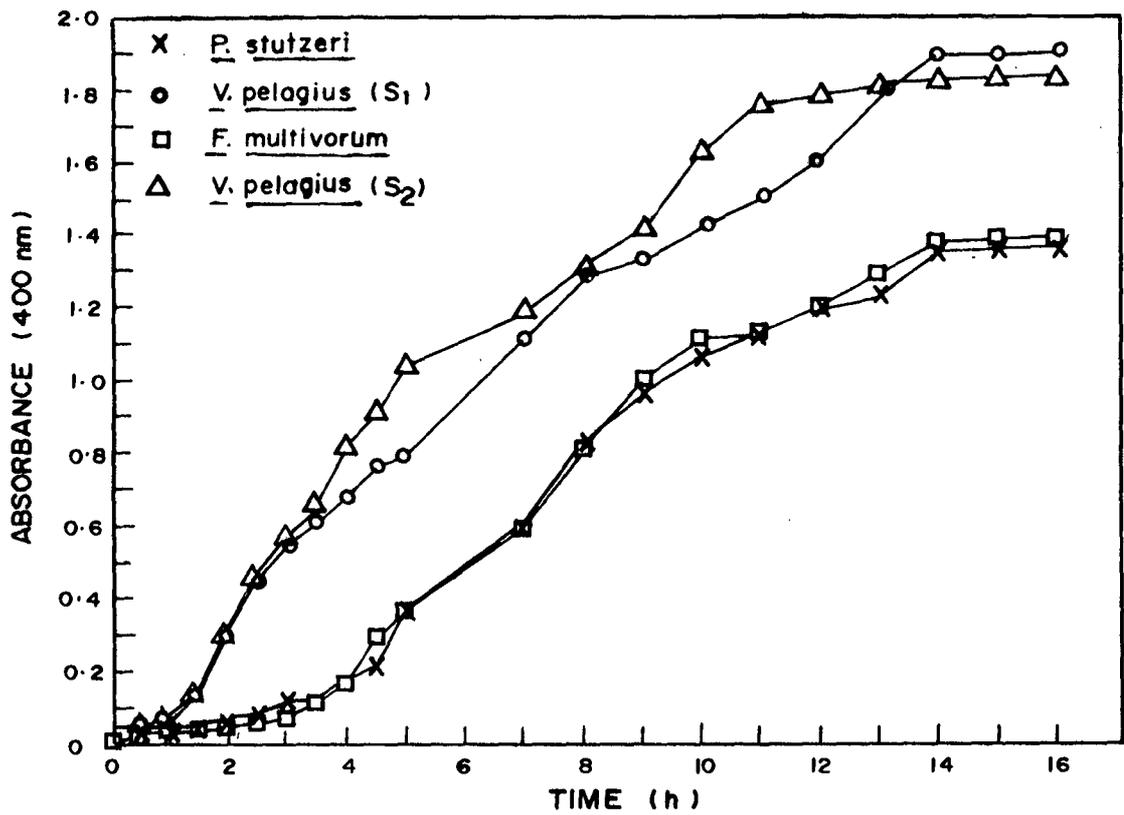


Fig. 2 A·3. Growth curve of the bacterial isolates .
P. stutzeri (x), *V. pelagius* (S₁) (-o-)
F. multivorum (-□-) and *V. pelagius* (S₂) (-△-) in nutrient broth.

4. DISCUSSION

Several parameters such as DW, carbon, nitrogen, ATP and carbonate content of fouling material have been employed to quantify the extent of biofouling (Aftring & Taylor, 1979; Mayback *et al.*, 1984; Bhosle *et al.*, 1989, 1990). In the present study, DW and OC were used because these are good indicators of microfouling biomass (Aftring & Taylor, 1979; Mayback *et al.*, 1984; Bhosle *et al.*, 1989, 1990). Microfouling biomass on aluminium generally increased over the period of immersion in the surface seawater of study area. This agrees well with earlier studies suggesting a non-linear increase in microfouling biomass as a function of the immersion period (Martinez *et al.*, 1984; White & Benson, 1984; Yanshun *et al.*, 1984; Chamberlain & Garner, 1988).

As observed for DW and OC, viable cell counts of the biofilm bacteria also showed an increasing trend over the period of immersion. Therefore, the observed increase in microfouling biomass and in the viable cell count suggests enhanced settlement and/or growth on the aluminium panels (Jordan & Staley, 1976; Gerchakov *et al.*, 1977). Nevertheless, a small decrease was recorded in microfouling biomass on aluminium at day 30 following immersion. The observed decrease was perhaps due to shearing and/or sloughing of the biofilm material.

One hundred bacterial colonies were isolated from the microfouling material developed on the aluminium panels over the

period of immersion. These were randomly selected from the greatest dilution showing growth, therefore, the colonies represent the numerically most dominant aerobic strains from the test panels. Most of the isolates were Gram negative rods. This observation is in agreement with several other studies. For example, Corpe (1973) isolated several strains of bacteria developed on glass which were generally Gram negative. Marshall *et al.* (1971) also found that the early colonising bacteria were gram negative rods.

Pseudomonas (35.29%), *Alkaligenes* (29.41%) and *Chromobacterium* (23.52%) were the most abundant biofilm bacteria at day 1 following the immersion. These have been observed to be the early colonizers on various surfaces immersed in marine waters (Corpe, 1973). As the period of immersion increased, the bacterial community also changed. For example, *Vibrio* showed a consistent increase and was the single most abundant group over the 22d period of immersion (Fig 2A.2). The apparent changes in the bacterial community structure were further assessed by statistical treatment of the data.

A number of indices of species diversity have been developed (Hurlbert, 1971; Peet, 1974; Pielou, 1975). The Shannon Diversity Index is probably the most widely used (Shannon & Weaver, 1949). The indices tended to increase over the 7d immersion period following successional changes within the communities. This agrees well with the theoretically predicted increase from low diversity of pioneer populations to the higher,

the stable diversity of the climax community. Such an increase was reported by earlier workers (Jordan & Staley, 1976; Walch, 1986). Subsequently, as the immersion period increased, the Shannon diversity index decreased. The relatively low diversity indices observed over the 16d immersion period probably resulted from domination by a particular group of bacteria. The observed changes in the diversity indices are suggested to be due to changes in the biofilm community structure.

The observed apparent succession of bacterial types colonizing aluminium surface suggests an alteration of the surface by those bacteria that are sorbed initially (Marshall *et al.*, 1971; Walch, 1986). Any modification of a surface by the initial colonizing bacteria may render it more amenable to colonization by other groups of microorganisms as was observed in this study. Such successional changes in bacterial types may be due to utilization of nutrients accumulated on surfaces, release of the specific nutrients, surface active agents, polymeric compounds and other ligands by the bacteria. Thus, secondary colonizers attaching to a surface after the initial colonizers may be presented with a very different environment than that encountered by the pioneer species. It is possible that some of these factors may have influenced the bacterial succession observed on aluminium panels immersed in marine waters of the study area (Walch, 1986).

CHAPTER 2B

*A simple spectrophotometric
method to assess bacterial
attachment to surfaces*

1. INTRODUCTION

Surfaces immersed in aqueous environments are colonized by microorganisms. Within few hours to few days bacteria attach irreversibly to the surface. In marine and estuarine environments, irreversible attachment and the growth of bacterial on the surfaces may enhance frictional resistance to ship hulls, influence heat transfer efficiency and induce corrosion of metals and alloys. Therefore, it is necessary to assess bacterial attachment in order to better understand their role in material deterioration.

There are several techniques to estimate bacterial biomass including adenosine triphosphate (ATP), lipid phosphate, muramic acid and nucleic acid content and viable and total cell counts (Holm-Hansen, 1969; Hobbie *et al.*, 1977; Paul *et al.*, 1985; Balkwill *et al.*, 1988). Some of these techniques have been employed to assess bacterial adhesion to surfaces (Daves & White, 1980; Jefferey & Paul, 1986; Balkwill *et al.*, 1988). Although these methods are sensitive, they are time consuming and uneconomic. Further, these methods need expensive chemicals and instruments. Such costly equipments are not available to many researchers. Crystal violet adhesion assay has been utilised to evaluate bacterial adhesion to non-metal surfaces (Mc Eldowney & Fletcher, 1986; Shea & Williamson, 1990). This method was modified to assess bacterial adhesion to metal surfaces, during the course of this study.

2. MATERIALS AND METHODS

2.1 Reagents

1. Crystal Violet (0.5%)

Crystal violet (500mg) was dissolved in 10ml ethanol and made upto 100ml using double distilled water.

2. Sodium Deoxycholate (2%)

Sodium Deoxycholate (2g) was dissolved in 100ml of double distilled water.

2.2 Test panels

Aluminium, Copper, Stainless steel and Polystyrene panels (3.5 x 1.5 cm) were used to assess bacterial adhesion to the surfaces. The metal panels were cleaned and sterilized as described in chapter 2A. Polystyrene panels were cleaned following the procedure described for metal panels, however, these panels were sterilized using UV radiation.

2.3 Bacterial Cultures

The four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were used for the calibration of the crystal violet adhesion assay.

2.4 Growth Condition

Cells of *V. pelagius* (S_2) culture were grown at room

temperature ($28\pm 2^{\circ}\text{C}$) for 18 h in nutrient broth (Table 2A.1) prepared in BSS (Table 2A.2). This was then used as a source of inoculum (1%) for another flask (500 ml) containing fresh nutrient broth (100 ml). After inoculation, the flask was incubated on a rotary shaker (150 rpm) at room temperature for 18h. The cells in the stationary phase were harvested by centrifugation at 4000 rpm, washed thrice with 0.01M phosphate buffer and suspended in the same buffer. Cell suspension was adjusted to 0.2 O.D. at 400 nm, using phosphate buffer which corresponds to about 2×10^9 colony forming units/ml (CFU.ml⁻¹).

2.5 Cell Attachment

A known volume of aliquots (100 ml) of *V. pelagius* (S_2) cell suspension prepared as above was dispensed into twelve 250 ml Erlenmeyer flasks. Replicate (3) aluminium panels were suspended in each flask using nylon thread and the flasks were incubated at room temperature. Panels (6) with attached cells were retrieved at an interval of 30 min over a period of 3h. After retrieval panels were rinsed with the BSS to remove any unattached cells. Three panels were used for the crystal violet staining and remaining three were used for the viable cell count of the bacterial cells attached to aluminium panels using ZMA plates.

2.6 Staining of the Attached Cells

The panels with attached cells of *V. pelagius* (S_2) were stained with 0.5 % of crystal violet for 2 min, after which they

were removed, washed five times with the BSS to remove excess stain. The crystal violet stain from the attached cells was eluted with 2ml of 2% deoxycholate. Subsequently, this was diluted with 3ml of distilled water. Absorbance was measured at 570nm using a Beckman DU-64 spectrophotometer. Flasks containing aluminium panels in phosphate buffer without bacteria were treated similarly and were used as controls.

2.7 Estimation of Viable Cell Count

Remaining two panels were used to assess viable cell count of the attached *V. pelagius* (S_2) cells. Cells of *V. pelagius* (S_2) attached to the panels were removed with a nylon brush using sterilized BSS solution. A suitable aliquot (0.1ml) of the scraped material was used for serial dilution. Appropriate dilutions were spread plated on Petri dishes prepared with ZMA. Petri dishes were incubated at room temperature for 24h after which viable cell count was noted.

2.8 Calibration of the assay for P. stutzeri, F. multivorum and V. pelagius (S₁).

In order to calibrate the assay for other three cultures, i.e. *P. stutzeri*, *F. multivorum* and *V. pelagius* (S_1), these cultures were grown in nutrient broth, harvested in the stationary phase, washed and suspended in buffer as described above for *V. pelagius* (S_2) culture. The attachment of the cells to the aluminium panels was also carried out as described for

V. pelagius (S_2) culture. The individual aluminium panels with attached cells of *P. stutzeri*, *F. multivorum* and *V. pelagius* (S_1) were then subjected to both crystal violet staining method and the total viable cell count as described for *V. pelagius* (S_2) cells.

2.9 Accuracy of the crystal violet adhesion assay

In another set of experiment, the accuracy of the results obtained using the crystal violet adhesion assay was tested using the two cultures, *P. stutzeri* and *F. multivorum*. Cells of *P. stutzeri* and *F. multivorum* were grown in nutrient broth, harvested, washed and suspended in buffer as described earlier. Replicate panels (4) of copper, polystyrene and stainless steel were suspended in the flasks containing cell suspensions of *P. stutzeri* and *F. multivorum*. Cells were allowed to attach to these panels for 60 min. The attached cells of *P. stutzeri* and *F. multivorum* were then assessed by both the crystal violet adhesion assay as well as by the total viable cell count using ZMA plates. The cell count was noted after incubating ZMA plates at room temperature ($28^{\circ} \pm 2^{\circ}\text{C}$) for 24 hours.

2.10 Statistical Analysis

Relationship between the crystal violet stained attached cells and viable cell count of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cells attached to aluminium panels was assessed by the simple linear regression analysis using 123 Lotus programme (Sokal & Rohlf, 1981).

The significance of the variations in the attachment of the bacterial cells to the different metals by both the methods i.e. crystal violet adhesion assay and the total viable cell count was assessed using Mann-Whitney U test (Sokal & Rohlf, 1981).

3. RESULTS

3.1 Calibration of the Crystal violet adhesion assay

Few bacterial cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were attached to the aluminium panels during the initial 30 min. of the incubation. Thereafter, the number of attached cells of the four bacterial cultures increased over the incubation period (Fig. 2B.1). A similar trend was observed for crystal violet stained attached cells of these cultures. As observed for the viable cell count, the absorbance of crystal violet stained attached cells of these cultures was also low during the first 30 min. following incubation. A consistent increase in absorbance of crystal violet stained attached cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) was noted with time (Fig. 2B.2).

The data showed non-linear increase in viable cell counts of attached cells as well as in the absorbance of crystal violet stained attached cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2). This apparent relationship between the viable cell count of the attached bacterial cells and the absorbance of the

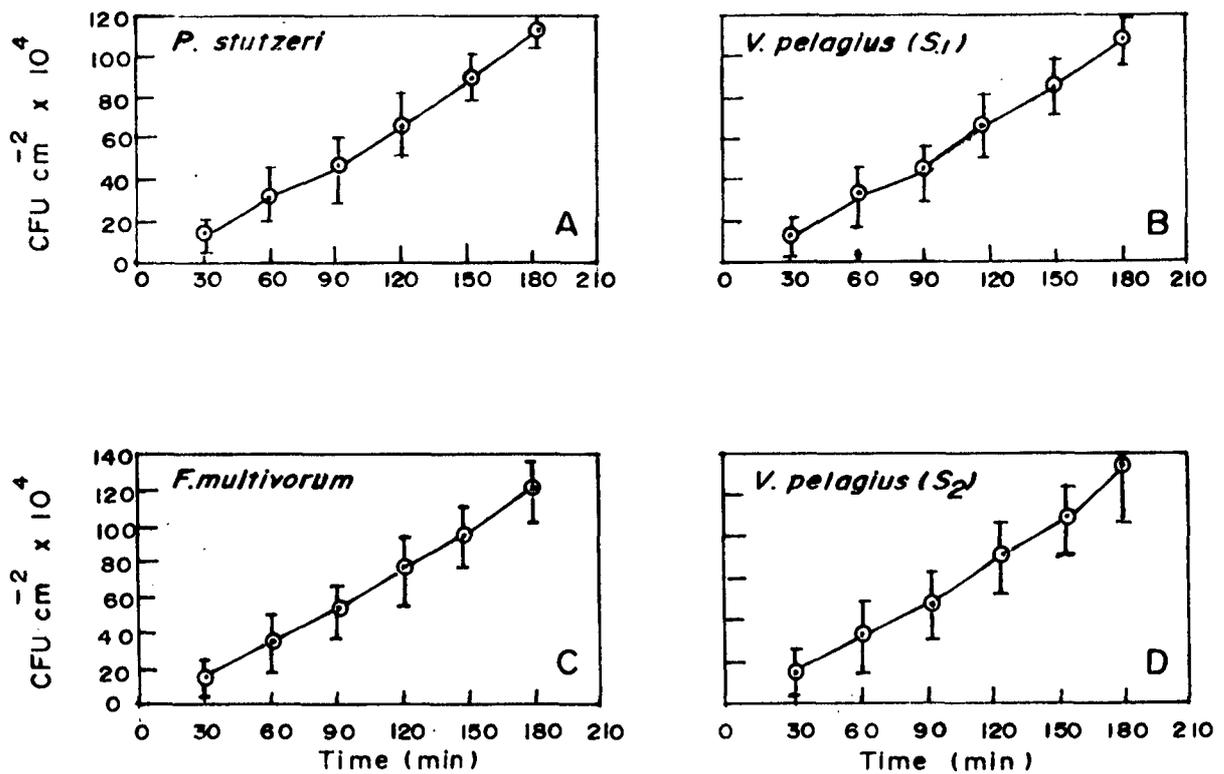


Fig. 2B.1. Changes in the viable cell count of A, *P. stutzeri*; B, *V. pelagius (S₁)*; C, *F. multivorum* and D, *V. pelagius (S₂)* attached to aluminium panels over a 3h incubation period. Points are the means of 3 determinations. Bars = 2 SD.

crystal violet stained cells attached to aluminium panels was assessed by using the simple linear regression analysis between these two parameters. There was a highly significant positive relationship ($r = 0.9994$; $p < 0.001$; $n = 6$) (Fig.2B.3) between the viable cell count of the attached cells and the absorbance of the crystal violet stained *V. pelagius* (S_2) cells attached to aluminium panels. Similar relationship was found between the viable cell count of the attached cells and the absorbance of the crystal violet stained *P. stutzeri* ($r = 0.9997$; $p < 0.001$; $n = 6$), *F. multivorum* ($r = 0.9984$; $p < 0.001$; $n = 6$) and *V. pelagius* (S_1) ($r = 0.9976$; $p < 0.001$; $n = 6$) cells (Fig 2B.3).

3.2 Accuracy of the crystal violet adhesion assay

The accuracy of the crystal violet adhesion assay was tested by comparing the numbers of the cells of *P. stutzeri* and *F. multivorum* attached to various surfaces, using the standardized crystal violet adhesion assay and the total viable cell count on ZMA plates. The difference in the number of *P. stutzeri* and *F. multivorum* cells attached to copper, stainless steel and polystyrene as estimated by both the methods i.e. crystal violet adhesion assay and the total viable cell count was insignificant. At all instances, p was greater than 0.1 as assessed by Mann-Whitney U test (Table 2B.1).

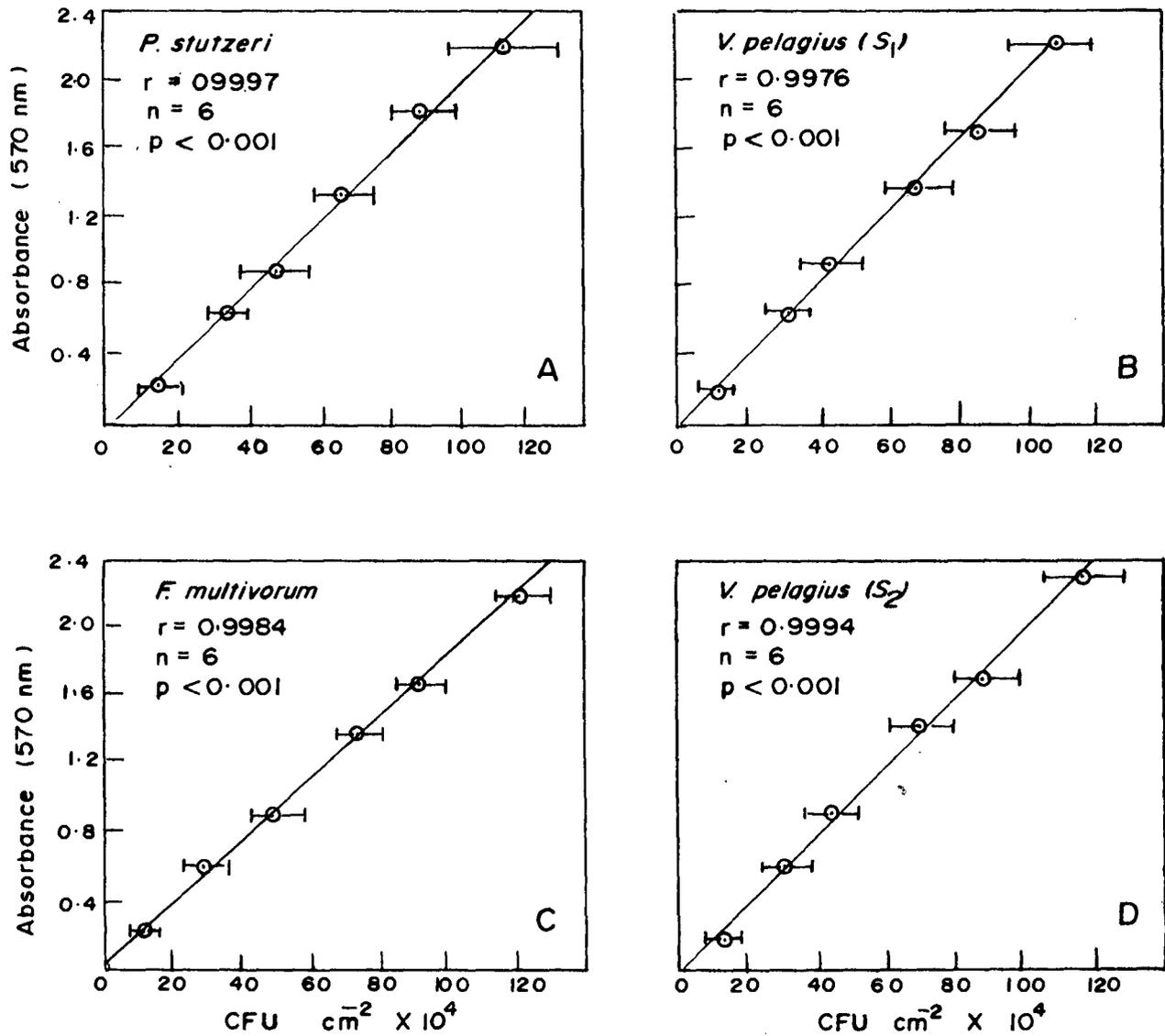


Fig. 2B.3. Relationship between viable cell count and the absorbance of crystal violet stained cells of A, *P. stutzeri*; B, *V. pelagius* (S₁); C, *F. multivorum* and D, *V. pelagius* (S₂) attached to aluminium panels. Points are the means of 3 determinations. Bars = 2SI

Table 2B.1

Number of cells of *P. stutzeri* and *F. multivorum* attached to the various surfaces as estimated by the two methods a) crystal violet adhesion assay and b) total viable cell count by spread plate method. There was no significant difference ($p > 0.1$) in the numbers estimated by the two methods, as assessed by Mann-Whitney U test.

Surface	CFU. cm ² x 10 ⁴				<i>p</i>
	<i>P. stutzeri</i>		<i>F. multivorum</i>		
	a	b	a	b	
Copper	2.96 ± 0.48	2.88 ± 0.44	1.23 ± 0.34	1.21 ± 0.33	> 0.1
Polystyrene	6.60 ± 0.66	6.03 ± 0.66	6.35 ± 0.58	6.26 ± 0.62	> 0.1
Stainless steel	4.92 ± 0.62	4.78 ± 0.56	4.29 ± 0.64	4.23 ± 0.63	> 0.1

p = Level of significance

CFU = Colony Forming Units

4. DISCUSSION

There are numerous methods of estimating microbial biomass and some of these, especially, ATP, viable cell counts and acridine orange direct count (AODC) are utilised for estimating the attachment of bacteria to surfaces (Paul *et al.*, 1985; Pederson, 1990; Vandevivere & Kirchman, 1993). Although these methods are sensitive, they are time consuming and need expensive chemicals and instruments, which are not always available in many laboratories. Moreover, there are practical problems associated with these methods. For example, the ATP content of microorganisms is influenced by the extraction method and by the physiological state of the cells. Similarly, attached bacterial cells may form aggregates, clumps or multilayered biofilm which are difficult to count by the AODC method (Ford *et al.*, 1989).

In view of the foregoing, a simple and sensitive method was required. In order to achieve this, crystal violet microplate adhesion assay has been modified for estimating bacterial attachment to metal and non-metal surfaces. Viable cells counts of the four bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to aluminium panels increased over the period of incubation. A similar trend was also observed for crystal violet stained cells of the four bacterial cultures attached to the same surface. This indicated that the method can be utilised for the qualitative assessment of bacterial attachment to various surfaces.

CHAPTER 3A

*Factors influencing adhesion
of bacteria to surfaces*

1. INTRODUCTION

Bacterial colonization of surfaces is a complex process and is very often influenced by changes in the environmental conditions (Mc Eldowney & Fletcher, 1986). The physico-chemical factors like pH, temperature (Mc Eldowney & Fletcher, 1988 a, b), ionic strength of the medium (Delaquis *et al.*, 1988) and the concentrations of nutrients (Yu *et al.*, 1987) affect adhesion of microorganisms to surfaces. Such changes in physico-chemical factors have pronounced effect on both quality and composition of macromolecular components of bacterial surfaces e.g. lipopolysaccharide, protein and exopolymers which in turn may influence bacterial adhesion (Ellwood & Tempest, 1972). Further, substratum surface texture, topography and chemical nature of the surface also influences adhesion.

In the present chapter, an attempt has been made to assess the effect of various factors like temperature, pH, NaCl, nitrate and phosphate concentrations on the adhesion of four bacterial cultures to hydrophilic and hydrophobic surfaces.

2. MATERIALS AND METHODS

2.1 *Bacterial cultures*

Four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were used to study the effect of various factors on the adhesion of these bacteria to various surfaces.

2.2 Test panels

Test panels (3.5 x 1.5 cms) of four different surfaces such as aluminium, stainless steel, copper and polystyrene were used to assess bacterial adhesion. These test panels were cleaned and sterilized as described in Chapter 2B.

2.3 Growth conditions

Bacterial cultures were grown in nutrient broth (Table 2A.1), prepared in Basal Salt Solution (BSS) (Table 2A.2). The pH was adjusted to 7.5 with 1N NaOH. The medium was sterilized by autoclaving for 20 min at 1.5 lb pressure and 121°C and was then inoculated with 1% (v/v) of an 18h old culture grown in the same medium. The flasks were then incubated at room temperature ($28 \pm 2^\circ\text{C}$) on a rotary shaker at 150 rpm. After 18h of incubation, flasks were removed and the cells in the stationary phase were harvested by centrifugation at 4000 rpm. The cell pellet was washed thrice with 0.01 M phosphate (PO_4) buffer and resuspended in the same buffer. The optical density at 400 nm was adjusted to 0.2 using phosphate buffer. This corresponds to about 1.9×10^9 colony forming units (CFU.ml^{-1}). This cell suspension was then used for various experiments described below.

2.4 Effect of various factors on the adhesion of P. stutzeri, V. pelagius (S₁), F. multivorum and V. pelagius (S₂)

a. Effect of temperature

Aliquots (100 ml) of each of the above bacterial suspensions were dispensed individually in twenty four 250 ml Erlenmeyer flasks. Replicate panels (3) of each test surface were suspended individually in each flask, using nylon thread. Each flask contained three panels of any one test surface. Flasks were incubated at 4, 14, 20, 27, 37 and 50° C over a period of 60 minutes. At the end of this incubation period, panels were removed and cell numbers of the bacteria attached to the different surfaces were estimated.

b. Effect of pH

Cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were suspended in the phosphate buffer of different pH i.e. 5, 6, 7, 8 & 9. Three panels of each test surface were introduced individually into these flasks, with each flask having three panels of any one surface. Test surfaces were kept suspended in the bacterial suspension for 60 minutes, at room temperature ($28 \pm 2^\circ\text{C}$). Panels were removed after the incubation period and cell numbers of the attached bacteria were estimated.

c. Effect of sodium chloride concentration

Bacterial cell suspensions were prepared using BSS (100 ml) containing different NaCl concentrations ($10\text{-}40 \text{ g.l}^{-1}$) and transferred to various Erlenmeyer flasks. Test surfaces were deployed into these flasks as described above. The flasks were incubated over a period

of 60 minutes. After incubation the cell numbers of the bacteria attached to the panels were estimated.

d. Effect of nutrients

The effect of nutrients nitrogen and phosphorus on the adhesion of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) was studied. Cells of these four cultures were suspended in BSS containing various concentrations of NH_4Cl (0 to 0.2%) or K_2HPO_4 (0 to 0.1% K_2HPO_4). Panels of the four surfaces were deployed in the flasks as described for earlier experiments. After 60 minutes, the cell numbers of the bacteria attached to these surfaces were estimated.

2.5 Controls

In each set of experiment, flasks containing BSS without bacterial culture, were incubated with different test surfaces under similar conditions as the experimental set up and were treated as controls.

2.6 Estimation of attached cell numbers

After incubating for 60 min in the bacterial cell suspension, panels were retrieved from the flasks. They were rinsed in BSS to remove any unattached cells. The panels with the attached cells were then stained with crystal violet (0.5%) for two minutes, after which they were removed, washed five times in BSS (to remove any excess stain). The crystal violet stain from the attached cells was

eluted with 2ml of sodium deoxycholate (2%). Subsequently, this was diluted with 3ml of distilled water and the absorbance was measured at 570 nm using a Beckmann DU-64 spectrophotometer. From the absorbance reading corresponding cell numbers were calculated using the calibration curve as described earlier (Chapter 2B). Controls were also treated similarly.

2.7 Statistical analysis

The significance of the observed differences in the number of cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various surfaces under different conditions of temperature, pH, NaCl, nitrogen and phosphate concentrations was assessed using two way analysis of variance (ANOVA) (Sokal & Rohlf, 1981).

3. RESULTS

3.1 Effect of substrata

In the present study, bacterial cell attachment was influenced by various environmental parameters and the nature of the substratum. All the four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) showed highest adhesion to aluminium. Compared to aluminium, relatively lesser number of bacterial cells were found attached to polystyrene followed by stainless steel. While a very few cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the

copper surface. Further, various physico-chemical factors also influenced the adhesion of bacterial cells to these surfaces.

3.2 Effect of various factors on adhesion of different bacteria to various surfaces.

a. Effect of temperature

Adhesion of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) to the four surfaces was influenced by temperature. Statistical treatment of the data also suggests that there was a significant variance ($P < 0.05$) in the bacterial cells attached to the surfaces at different temperatures (Table 3A.1). As the temperature increased from 4° to 27°C, there was an increase in the adhesion of *P. stutzeri* cells to the four surfaces (Fig 3A.1A). With further increase in temperature to 37°C, however, there was a decrease in the number of *P. stutzeri* cells attached to all the four surfaces. At all temperatures, highest number of *P. stutzeri* cells attached to aluminium panels followed by polystyrene panels. As compared to polystyrene, lower number of cells attached to stainless steel. Lowest number of cells attached to the copper panels at all temperatures.

A similar trend was seen for *V. pelagius* (S_1) cells (Fig 3A.1B). However, highest number of cells adhered to surfaces at 37°C. On further increase in temperature to 50°C, a sharp decrease in the attachment of cells was observed. At 50°C, the number of *V. pelagius* (S_1) cells attached to the aluminium was

Table 3A.1

ANOVA analysis to assess the effect of various physicochemical factors i.e. temperature, pH, NaCl concentration, nitrogen & phosphate concentration on the adhesion of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various surfaces.

Surfaces	Physicochemical factors				
	Temperature	pH	NaCl concentration	Nitrogen concentration	Phosphate concentration
Aluminium	$F_1 = 25.8757$ $p_1 = 7.09E-07$	$F_1 = 10.9371$ $p_1 = 0.0001$	$F_1 = 5.9011$ $p_1 = 0.0041$	$F_1 = 14.4221$ $p_1 = 3.59E-05$	$F_1 = 47.8808$ $p_1 = 1.05E-08$
	$F_2 = 4.0704$ $p_2 = 0.0266$	$F_2 = 14.6049$ $p_2 = 3.32E-05$	$F_2 = 140.9911$ $p_2 = 2.95E-12$	$F_2 = 35.8087$ $p_2 = 8.52E-08$	$F_2 = 38.0033$ $p_2 = 5.57E-08$
Polystyrene	$F_1 = 7.9117$ $p_1 = 0.0003$	$F_1 = 6.6091$ $p_1 = 0.0024$	$F_1 = 5.6270$ $p_1 = 0.0050$	$F_1 = 8.7214$ $p_1 = 0.0006$	$F_1 = 19.7298$ $p_1 = 4.99E-06$
	$F_2 = 3.7989$ $p_2 = 0.0186$	$F_2 = 23.5030$ $p_2 = 1.57E-06$	$F_2 = 44.023$ $p_2 = 1.93E-08$	$F_2 = 41.2800$ $p_2 = 3.08E-08$	$F_2 = 1.9439$ $p_2 = 0.1521$
Stainless steel	$F_1 = 8.59110$ $p_1 = 0.0001$	$F_1 = 1.9892$ $p_1 = 0.1447$	$F_1 = 4.9353$ $p_1 = 0.0087$	$F_1 = 4.1816$ $p_1 = 0.0166$	$F_1 = 7.8458$ $p_1 = 0.0010$
	$F_2 = 3.4053$ $p_2 = 0.0280$	$F_2 = 14.4920$ $p_2 = 3.48E-05$	$F_2 = 49.8558$ $p_2 = 7.78E-09$	$F_2 = 16.1596$ $p_2 = 173E-05$	$F_2 = 2.0191$ $p_2 = 0.1401$
Copper	$F_1 = 7.4797$ $p_1 = 0.0004$	$F_1 = 8.4259$ $p_1 = 0.0007$	$F_1 = 3.7170$ $p_1 = 0.0252$	$F_1 = 13.1876$ $p_1 = 6.14E-05$	$F_1 = 4.1258$ $p_1 = 0.074$
	$F_2 = 7.5084$ $p_2 = 0.0007$	$F_2 = 2.6186$ $p_2 = 0.0741$	$F_2 = 1.3990$ $p_2 = 0.2789$	$F_2 = 16.7659$ $p_2 = 1.41E-05$	$F_2 = 6.173$ $p_2 = 0.0033$

F_1 & p_1 are the values for F distribution (Fisher) and probability within the same bacterial culture and F_2 & p_2 are the values for F distribution and probability among the different bacterial cultures.

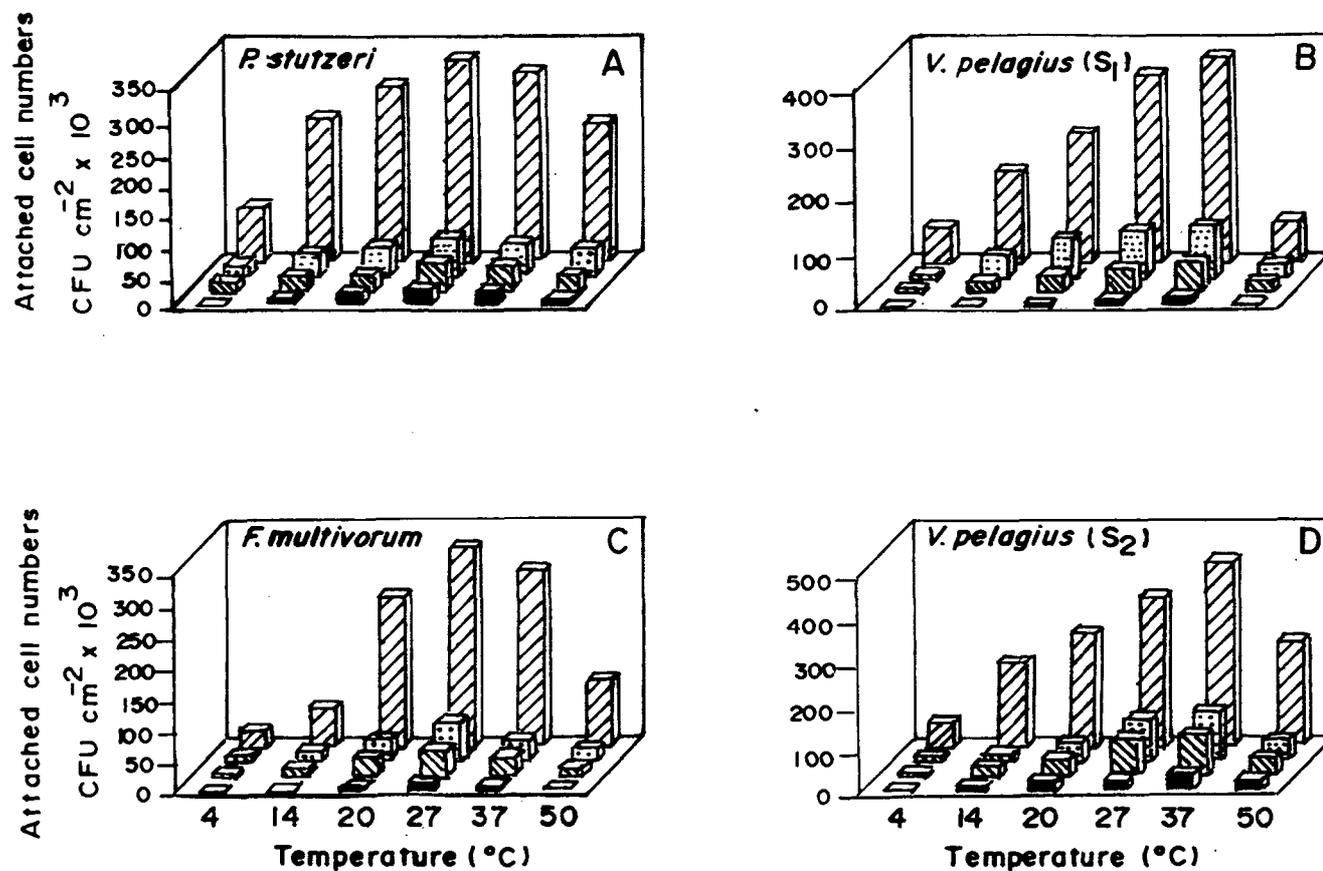


Fig. 3A-1. Effect of temperature on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁) C, *F. multivorum* and D, *V. pelagius* (S₂) to copper ■; stainless steel ▨; polystyrene ▩ and aluminium ▪. Bar values represent the means of 2 determinations.

much lower than that of *P. stutzeri* and *F. multivorum*. Highest adhesion of *F. multivorum* cells was found at 27°C. At 37°C, there was a small decrease in the *F. multivorum* cells attached to different surfaces (Fig 3A.1C). Compared to the other three cultures the adhesion of *F. multivorum* culture was more affected by temperature differences. Very less cells attached to the surfaces at 4°, 14° and 50°C. *V. pelagius* (S_2) cells showed a trend similar to that of *V. pelagius* (S_1) cells (Fig 3A.1D). However, the number of *V. pelagius* (S_2) cells attached to the four surfaces at higher temperatures (37°C, 50°C) was much higher than *V. pelagius* (S_1) cells. In general, cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) showed lowest adhesion to surfaces at 4°C.

b. Effect of pH

The attachment of the bacterial cultures *P. stutzeri* (Fig 3A.2A), *V. pelagius* (S_1) (Fig 3A.2B), *F. multivorum* (Fig 3A.2C) and *V. pelagius* (S_2) (Fig 3A.2D) increased as the pH of the medium increased from 5 to 7. Highest bacterial cells attached to the surfaces at pH 7. As the pH was further increased to 8, the number of bacterial cells attached to all the four surfaces decreased. There was a significant variance ($P < 0.05$) in the number of cells attached to the surfaces at different pH as evident from the statistical treatment of the data (Table 3A.1). However, the attachment of bacterial cells to the stainless steel was not significantly influenced by the variation in the pH of the inoculation

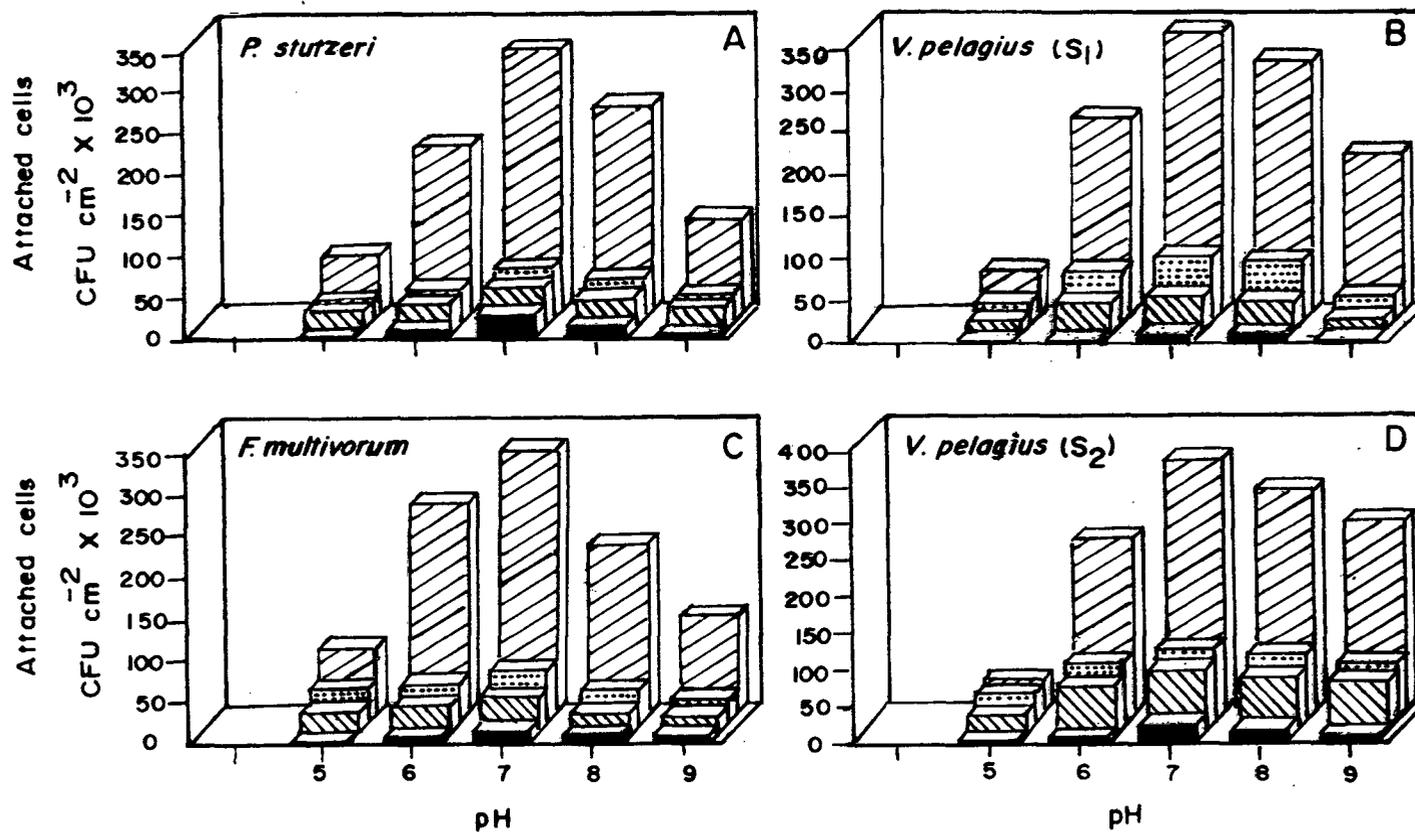


Fig. 3A-2. Effect of pH on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁), C, *F. multivorum* and D, *V. pelagius* (S₂) to copper ■, stainless steel ▨, polystyrene ▩ and aluminium ▪. Bar values represent the means of 2 determinations.

medium. Nevertheless, the decrease in the attached cells was influenced by both the nature of the substratum and the bacterial species. *Vibrio* cultures did not show much decrease in attachment at pH 8. However, when the pH was increased to 9, there was an appreciable decrease in the *Vibrio* cells attached to various surfaces. The decrease in the attached cells to all the surfaces was lowest with *V. pelagius* (S_1) culture. Amongst the four surfaces studied, the effect of pH on bacterial adhesion to aluminium and polystyrene was more noticeable. The number of cells which attached to aluminium surface showed large increase as the pH increased from 5 to 7. For example, *V. pelagius* (S_1) cells attached to aluminium were $52.5 \pm 4.5 \text{ CFU.cm}^{-2} \times 10^3$ at pH 5, which increased to $336.04 \pm 16.4 \text{ CFU.cm}^{-2} \times 10^3$ when the pH was 7.

c. Effect of NaCl concentration

In general with the increase in NaCl concentration from 10 to 30 g.l^{-1} , bacterial adhesion to the surfaces also increased but thereafter, showed a small decrease in their attached cell numbers with further increase in NaCl concentration. The effect of NaCl concentration on the adhesion of *P. stutzeri* cells to the four surfaces was less pronounced than the cells of other three bacterial cultures (Fig 3A.3A). On increasing the concentration of NaCl from 10g.l^{-1} to 30g.l^{-1} , not much appreciable increase in the adhesion of *P. stutzeri* cells to the four surfaces was found. Nevertheless, the number of cells attached to the four surfaces at $40\text{g.l}^{-1}\text{NaCl}$ was considerably

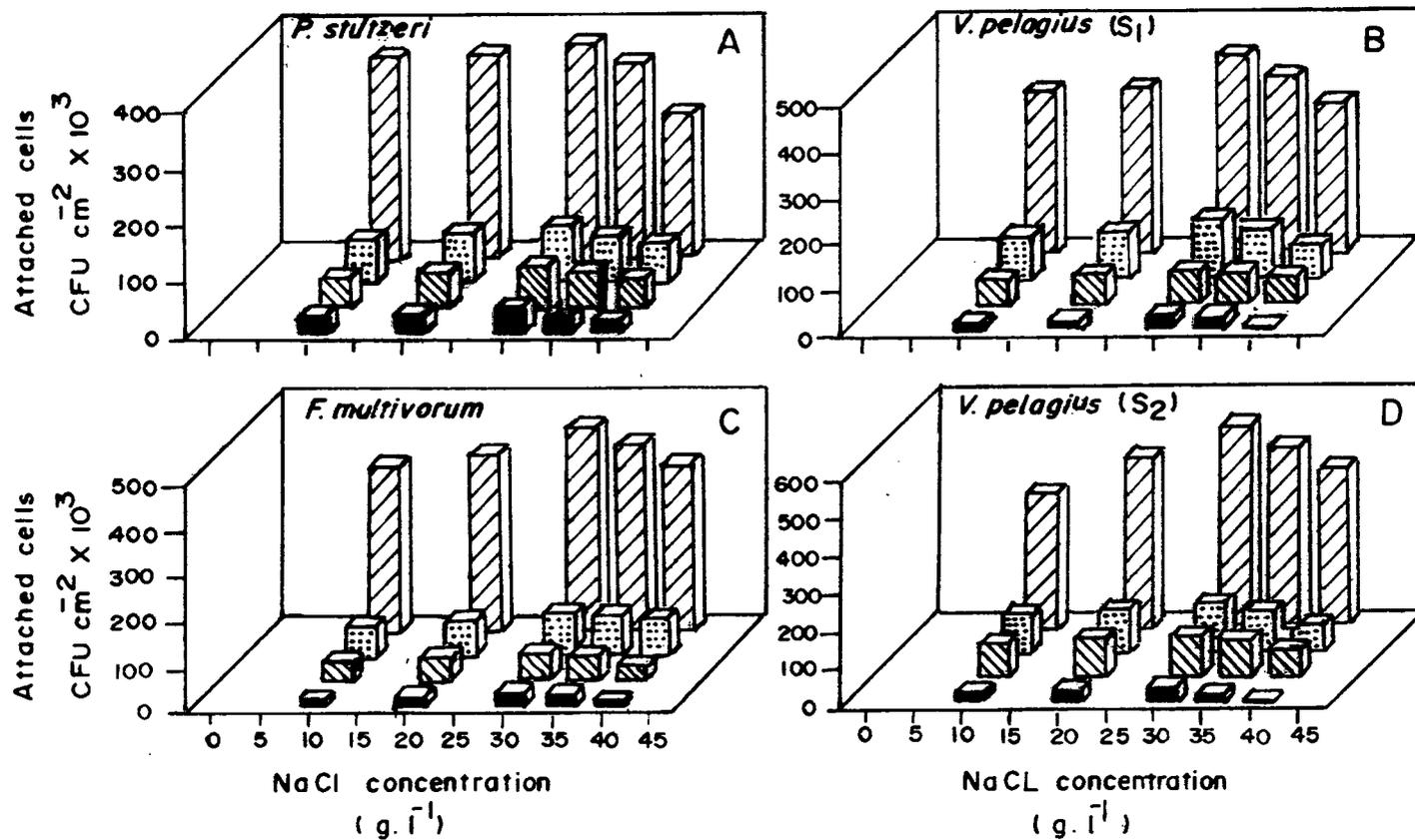


Fig. 3A-3. Effect of NaCL concentration on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁), C, *F. multivorum* and D, *V. pelagius* (S₂) to copper ■, stainless steel ▨, polystyrene ▩ and aluminium ▤. Bar values represent means of 2 determinations.

less than those attached to surfaces when NaCl concentration was 30g l^{-1} and 35g l^{-1} . A similar trend in adhesion of *V. pelagius* (S_1) cells to the surfaces as a function of NaCl concentration was recorded (Fig 3A.3B). The attachment of *F. multivorum* cells to the four surfaces was also affected by the NaCl concentrations (Fig 3A.3C). Least adhesion of *F. multivorum* cells to the four surfaces was noted at 40g NaCl l^{-1} . Except for the attachment of *V. pelagius* (S_2) to the aluminium, the other three cultures showed lower adhesion to the surfaces at 40g NaCl l^{-1} than at 10g NaCl l^{-1} . The number of *V. pelagius* (S_2) cells attached to aluminium surface was lowest at 10g l^{-1} NaCl concentration (Fig 3A.3D). Attachment of *V. pelagius* (S_2) cells to copper surfaces was more affected at 35 & 40g l^{-1} NaCl concentration than the attachment of cells to the other surfaces. The observed differences in bacterial cell adhesion to the surfaces at different NaCl concentrations were significant ($P < 0.05$) (Table 3A.1).

d. Effect of nitrogen concentration

The changes in nitrogen concentration showed differences in the number of bacterial cells attached to various surfaces. In general, as the nitrogen concentration increased from 0 to 0.1% NH_4Cl , the number of cells attached to the surfaces also increased. However, further increase in nitrogen concentration to 0.15% NH_4Cl , did not show any appreciable increase in the attachment of bacterial cells to surfaces (Fig 3A.4ABCD). At all the nitrogen concentrations studied, highest numbers of bacterial cells attached to aluminium. However, greater

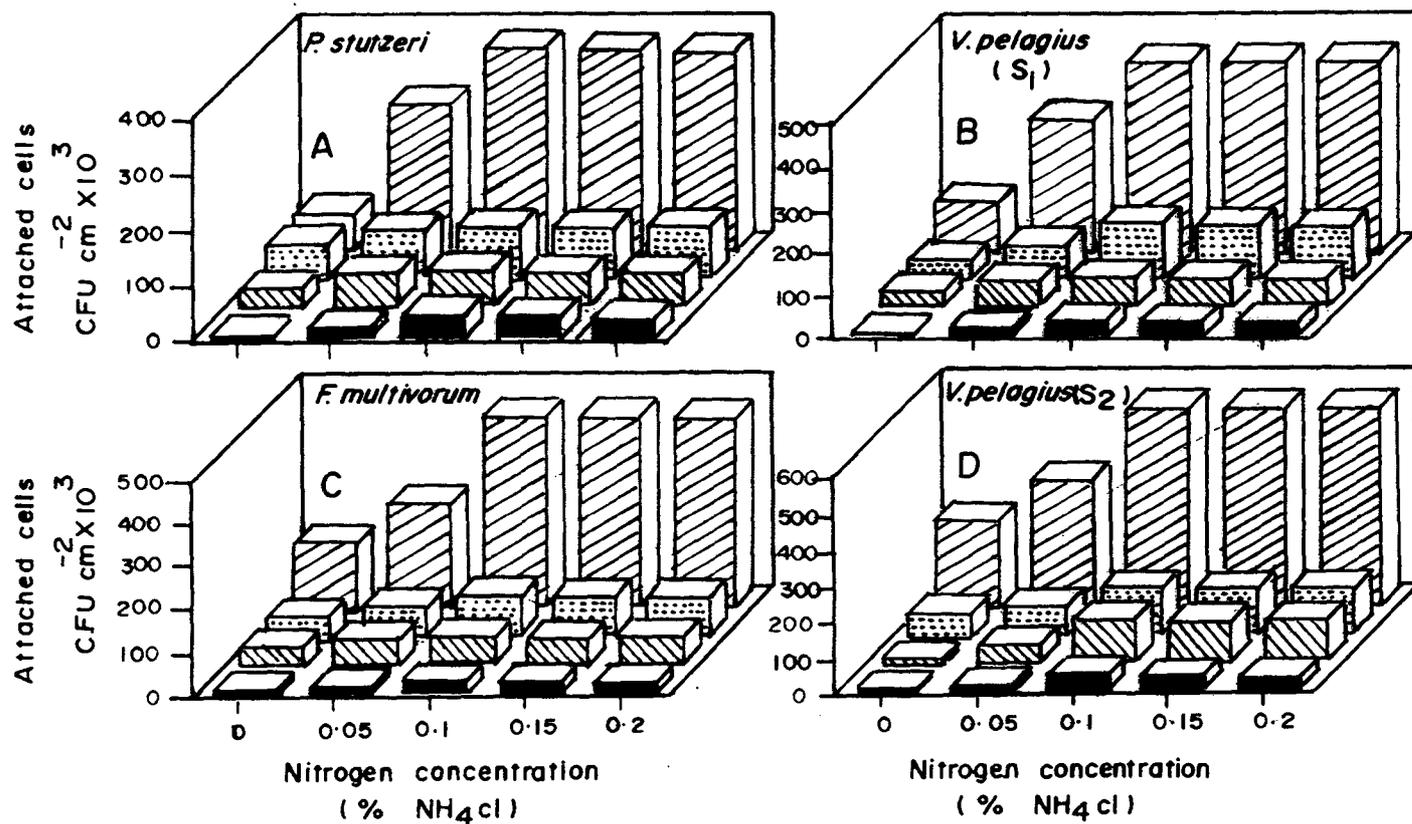


Fig. 3A-4. Effect of nitrogen concentration on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁); C, *F. multivorum* and D, *V. pelagius* (S₂) to copper ■; stainless steel ▨; polystyrene ▩ and aluminium ▪. Bar values represent the means of 2 determinations.

increase in the attached cells with increasing nitrogen concentration was found on copper surfaces, especially with respect to *P. stutzeri* (Fig. 3A.4). Further, statistical treatment of the data showed significant differences ($P < 0.05$) in the cell numbers of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the surfaces at different nitrogen concentrations (Table 3A.1).

e. Effect of phosphate concentration

Phosphate concentrations also affected the attachment of bacterial cells to the surfaces (Fig. 3A.5). This is evident from the statistical treatment of the data (Table 3A.1). The differences in the attachment of cells to the surfaces at different phosphate concentrations were significant ($P < 0.05$) within any bacterial culture. However, the differences in the attachment of cells to polystyrene and stainless steel surfaces among the four bacterial cells were not significant ($P > 0.05$). As the PO_4^{-3} concentration increased from 0 to 0.075 (% K_2HPO_4) the number of *P. stutzeri* cells attached to the surfaces also increased (Fig. 3A.5A). Further increase in PO_4^{-3} concentration to 0.1 (% K_2HPO_4) did not show any increase in *P. stutzeri* cells attached to the surfaces. *V. pelagius* (S_1) culture was also similarly affected by PO_4^{-3} concentration (Fig. 3A.5B). Although attachment to all surfaces was affected by PO_4^{-3} concentration, in case of *V. pelagius* (S_1), this effect of PO_4^{-3} concentration was more pronounced for adhesion to polystyrene.

Adhesion of *F. multivorum* to the surfaces was markedly

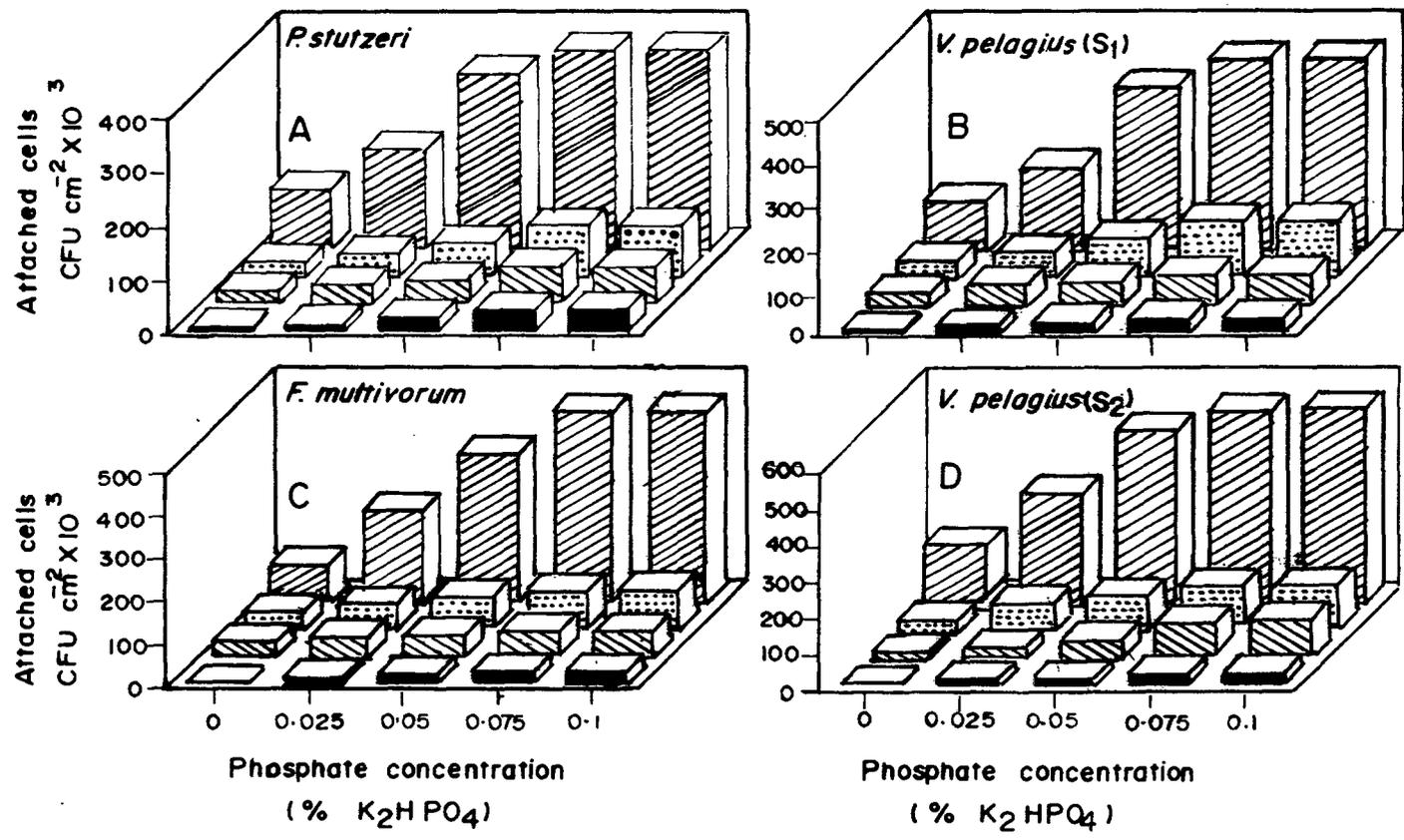


Fig. 3A-5. Effect of phosphate concentration on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁); C, *F. multivorum* and D, *V. pelagius* (S₂) to copper ■, stainless steel ▨; polystyren ▩ and aluminium ▪. Bar values represent the means of 2 determinations.

influenced by the PO_4^{-3} concentration (Fig. 3A.5C). In the absence of PO_4^{-3} very few cells of *F. multivorum* attached to the aluminium and copper, as compared to other three cultures. As the PO_4^{-3} concentration increased from 0 to 0.075 (% K_2HPO_4) there was a sharp rise in the attachment of *F. multivorum* cells. Nevertheless, like other three cultures, further increase in PO_4^{-3} concentration did not increase the adhesion of *F. multivorum* cells to the surfaces (Fig. 3A.5C). *V. pelagius* (S_2) showed a trend similar to that of *V. pelagius* (S_1). However, in the absence of PO_4^{-3} , *V. pelagius* (S_2) cells attached to the stainless steel and polystyrene surfaces were relatively lower than that of *V. pelagius* (S_1). *V. pelagius* (S_2) cells attached to the surfaces at different concentrations of PO_4^{-3} were higher than that of *V. pelagius* (S_1).

4. DISCUSSION

The effect of temperature, pH, NaCl, nitrogen and phosphate on the adhesion of bacteria to the surfaces was assessed in the present study. These physico-chemical factors influenced the adhesion of the bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) to aluminium, polystyrene, copper and stainless steel. The observed differences in the bacterial attachment to these surfaces under different conditions of temperature, pH, NaCl concentrations, nitrogen and phosphate concentrations, were significant ($P < 0.05$) as evident from statistical treatment of the data.

As the temperature increased, the adhesion of bacteria to the test surfaces also increased. Nevertheless, the optimum temperatures for adhesion varied with the bacterial species. *P. stutzeri* and *F. multivorum* showed higher adhesion to all the surfaces at 27°C, whereas, higher attachment of *V. pelagius* (S_1 & S_2) were observed at 37°C. At higher temperatures (> 37°C), adhesion of all the bacteria to the surfaces generally decreased. Temperature may influence adhesion by affecting the physiological properties of the organisms. High temperatures denature and inactivate proteins and damage cell membranes (Stanier *et al.*, 1992) which might be responsible for the decrease in the adhesion of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cells to the surfaces observed at high temperature. Moreover, at low temperatures cell proteins undergo conformational changes, due to the weakening of their hydrophobic bonds (Stanier *et al.*, 1992). Such changes in proteins may contribute to the decreased adhesion of cells at low temperatures. Further, while colonizing the surface, bacteria produce exopolysaccharides. The production and properties of bacterial exopolysaccharides are influenced by the temperature (Knox *et al.* 1986). It has been suggested that at higher temperature (> 37°C) viscosity of the adhesive polymer decreases (Stanley & Rose, 1967; Patc, 1983). Thus, the observed decrease in the bacterial attachment to the test surfaces at higher temperature (> 37°C) was perhaps due to the changes in the viscosity of the adhesive polymers of these bacteria.

pH was also found to influence the adhesion of four bacterial cultures to the surfaces. The number of cells of the bacterial cultures attached to the test surfaces increased as the pH increased from 5 to 7. On further increase in pH, a decrease in the attachment of the bacterial cells to the test surfaces was found. Such a decrease was perhaps due to decrease in the viscosity of the adhesive polymers due to increase in the pH of the medium. It has been reported that the pH can modify the viscosity of bacterial polymers (Sutherland, 1983). For example, the viscosity of an exopolysaccharide produced by a marine isolate decreased as the pH increased (Boyle & Reads, 1983). Similarly, viscosity of the exo-polysaccharide of a fresh water bacterium decreased at pH 3 and 11, while maximum viscosity of the polymers was observed at pH 7 (Sutherland, 1983).

Alternatively, pH could also influence other factors important in permanent adhesion including electrical double layer thickness and the dissociation of charged groups on the solid as well as cell surfaces. Variations in the dissociation of cell surface ionogenic groups due to pH changes would alter the adhesion interaction between cell and the surface, which may either enhance/induce or inhibit bacterial adhesion to surfaces (Mc Eldowney & Fletcher, 1988a). Similar changes in either electrical double layer and/or ionogenic groups of the bacteria due to pH change may be associated with the observed effects of pH on the attachment of the bacteria to the surfaces. Changes in pH may also cause denaturation

of cell surface proteins which result in the decreased adhesion at low or high pH values.

The presence of the nutrients had a marked effect on the bacterial adhesion. Very few bacterial cells attached to the surfaces in the absence of nitrogen and/or phosphate. Similar results are reported by earlier workers (Yu *et al.*, 1987; Delaquis *et al.*, 1989). Brown *et al.*, (1977) demonstrated poor bacterial adhesion from a nitrogen limited culture, despite large extracellular polymer production. Similarly, working with glass surfaces, Delaquis *et al.*, (1989), reported an active mechanism of cell immigration away from the surface into the bulk phase on depletion of carbon and nitrogen source. The observed decrease in the attached cell biomass was suggested to be a nutrient induced form of behaviour. Moreover, changes in nutrients alter the bacterial cell surface hydrophobicity, which can influence the adhesion of the bacterial cells to the surfaces (Delaquis *et al.*, 1989, Chapter 3B).

Furthermore, the presence of nutrients in the liquid phase would have physiological effects. The liquid surface tension changes because of the interaction between water molecules and the nutrients present in the solution, which varies with the nutrients and its concentration. The effect of liquid surface tension on bacterial attachment to surfaces has been described by several workers (Absolm *et al.*, 1983; Fletcher & Pringle, 1985). In the presence of nutrients a conditioning layer may form on the substrata and this

may result in the increased attachment of bacterial cells to the surface. The observed increase in the attachment of bacterial cells of the four cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) to the surfaces with increasing concentration of nitrogen and phosphate may be attributed to the formation of a conditioning layer on the surfaces in the presence of these nutrients.

Concentration of NaCl was also found to influence the adhesion of the four bacterial cultures to the surfaces. Adhesion of bacterial cultures to the test surfaces increased with increase in NaCl concentrations and higher number of cells attached to the surfaces at 30g.l⁻¹ of NaCl. This was followed by a decrease in the adhesion of bacteria to surfaces at NaCl concentrations of 40 and 45g.l⁻¹. Similar results have been reported by other workers (Koga *et al.*, 1986; Delaquis *et al.*, 1986; 1988; Roller, 1991). Delaquis *et al.*, (1986) reported increased growth of attached bacteria when NaCl concentration was used at 10g.l⁻¹ and 20g.l⁻¹. However, with further increase in NaCl concentration (30g. to 40g.l⁻¹) there was a decrease in the bacterial cells attached to the test surfaces. In an experiment carried out by Roller (1991) adhesion of *P. fragi* increased in the presence of NaCl. Roller & Woods (1987) have provided evidences to show the presence of an amorphous, outer layer surrounding the cells when *P. fragi* was grown in presence of 20g.l⁻¹ NaCl. It is, therefore, possible that the increased bacterial adhesion to surfaces with increasing concentrations of NaCl, observed in the present study, was mediated by the presence of an outer layer of polymers.

Further, increased adhesion of bacteria to inert surfaces in response to sub-lethal concentrations of an osmotically active solute in growth medium have been demonstrated previously. Koga *et al.*, (1986) have shown that the presence of 10g.l^{-1} sucrose caused an increase in the attachment of *S. mutans* strains to glass surfaces. Further, Zita & Hermansson, (1994) observed that at increasing concentrations of electrolytes (< 0.1), the stability of the flocs increased. In some other studies, increasing the ionic strength of the aqueous solution has been shown to increase the extent of bacterial sorption to a variety of surfaces (Marshall *et al.*, 1971; Gordon & Millerno, 1984; Sharma *et al.*, 1985; Yates & Yates, 1988; Van loosdrecht *et al.*, 1989; Mills *et al.*, 1994; Zita & Hermansson, 1994).

This increased adhesion of bacterial cells to the surfaces in the presence of increasing concentration of electrolytes as found in the present study can be explained in terms of DLVO theory. According to DLVO theory, surfaces with similar charges can attract one another at high electrolyte concentrations when the electrical double layer is compressed due to the increased concentration of electrolytes (Gannon *et al.*, 1991; Harvey, 1991; Mills *et al.*, 1994). The mechanistic basis of the observed ionic strength effect is related to the shrinking of the double layer as the ionic strength increases. Further, as the concentration of counter ions increases, the secondary free energy minimum i.e. the location at which reversible sorption occurs as a result of balanced attractive and repulsive forces

between particles is forced closer to the actual surface (Marshall *et al.*, 1971; Mills *et al.*, 1994). The potential energy barrier to the secondary minimum in the DLVO theory shrinks, and the bacterial cells more readily occupy reversible adsorption sites with increasing ionic strength of the aqueous solution (Van Loosdrecht *et al.*, 1989; Harvey, 1991). There is a limit, however, to the compression of the double layer. Beyond a certain level, continued addition of electrolyte does not influence the compression of the double layer (Sharma *et al.*, 1985; Mills *et al.*, 1994). In the present study, adhesion of the bacteria to the surfaces increased with NaCl concentrations from 0 to 30g l⁻¹. This is perhaps due to the compression of the double layer as further increase in NaCl concentration (> 30g NaCl l⁻¹) did not enhance bacterial adhesion to surfaces.

Nature of the substrata also influenced the adhesion of *P. stutzeri*, *V. pelagius* (*S*₁), *F. multivorum* and *V. pelagius* (*S*₂). For example, higher bacterial cells attached to aluminium as compared to those attached to polystyrene and stainless steel, while a few cells attached to copper. Similar results have been reported by others (Fera *et al.*, 1989; Ford *et al.*, 1989). Fluid shear stress and the concentration of suspended cells can influence cell attachment (Fletcher, 1977; Bott, 1993). However, these parameters were almost constant in the present experiments. Thus, any variation in cell density is probably due to differences in substratum characteristics. The substratum surface texture and/or topography can influence

biofilm development. (Ista *et al.*, 1996; Wiencek & Fletcher, 1997; Hunt & Parry, 1998). It is reported that the chemical nature of the inert surface also influences bacterial attachment in the early phases of biofilm development (Wiencek & Fletcher, 1995).

In the present study, polystyrene was the second most favoured surface for colonization by bacteria. Of the four surfaces used in this study, polystyrene was hydrophobic. It is well documented that different numbers of bacteria initially attach to hydrophilic and hydrophobic surfaces (Pringle & Fletcher, 1983; Pedersen, 1990). Substratum wettability is an important factor in bacterial adhesion (Bidle *et al.*, 1993; Meinders *et al.*, 1994). For example, a direct relationship between hydrophobicity measured as water contact angle and percent adhesion was established by Doss *et al.*, (1993) using conidia of *Botrytis cinerea* and the substrata with different hydrophobicities. The results suggest that the intermediate adhesion of conidia of *B. cinerea* depends, at least in part, on hydrophobic interactions between the conidia and substratum. Similarly, Husmark & Ronner (1993) found a linear relationship between the relative surface hydrophobicity of the materials tested and the number of adhered cells. In the present study, high number of cells attached to the polystyrene surfaces. As the polystyrene surface is hydrophobic, higher attachment of bacterial cells to this surface can be related to the hydrophobicity of the substratum.

It is suggested that in case of a hydrophobic surface, the water layer from the surrounding medium which separates the bacterium and substratum can be readily expelled due to the repulsive forces existing between the water layer and the substratum thus bringing the bacterial cell in close proximity to the surface (Rittle *et al.*, 1990), whereas, in case of a hydrophilic surface, there are attractive forces between the water layer and the substratum surface and for the adhesion of the bacterial cell to take place to the surface, additional work has to be done against this water layer. Because of this, relatively more bacterial cells attach to the hydrophobic surface, which is energetically more favourable, than the hydrophilic surface, as observed in the present study.

However, the number of bacterial cells attached to aluminium panels was markedly greater than those observed on polystyrene panels. This was probably because of the roughness of the aluminium panels. Surface roughness appears to control bacterial adhesion to surfaces (Sjollema *et al.*, 1990; Shreve *et al.*, 1991). It is generally observed that rough surfaces are more conducive to bacterial attachment than smooth surfaces. For example, it has been reported that electropolished stainless steel, and fluorinated ethylene propylene and glass tubes were less amenable to biofilm growth than untreated 316 stainless steel (Bott, 1993). Similarly, Pedersen (1990), also reported that more bacterial cells attached to rough steel surface as compared to the electropolished steel. Such higher bacterial

attachment to rough steel surface was due to decreased detachment due to shear forces since cells can be shielded from the bulk flow on a rough surface. Alternatively rough surface may have more surface area for the biofilm attachment and growth (Pedersen, 1990).

As compared to aluminium and polystyrene, adhesion of bacteria to stainless steel was relatively lower. Stainless steel is known to be more resistant to microbial attack (Prabha Devi, 1995). This is perhaps due to the iron containing crystalline protective structures normally present on the steel coupons (Westlake & Obuekwe, 1981). This layer serves as a protective coating and prevents further bacterial settlement. Furthermore, nickel and chromium present in the stainless steel are found to be toxic to bacteria. Hence relatively low numbers of bacteria were found on this surface.

All the four bacteria *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were isolated from an area not known for copper pollution. Therefore, it was not surprising that relatively few cells attached to copper panels, probably due to the toxic effect of copper ions. It is suggested that copper tolerance is an inducible characteristic dependent on a past history of copper tolerance (Ford et al., 1989). Toxic effects of copper have been reported by other workers. For example, Roger *et al.*, (1994), in a study of influence of temperature and plumbing material on biofilm

formation and growth of *Legionella pneumophila* in a water system, observed that copper surfaces were inhibitory to total biofouling and induced only low numbers of *L. pneumophila* organisms. Copper was the least colonized surface of the various test panels. Lowest numbers of bacterial cells attached to the copper surfaces in the present study, could be a response to environmental stress, caused perhaps by toxicity of copper corrosion products or frequent disturbance due to the instability of corrosion product films. *Efird* (1976) reported that the resistance of copper alloys to fouling in seawater is due to the formation of the adherent cuprous oxide layer, which is toxic to marine organisms. An overlayer of cuprous hydroxy chloride, which may form is not as toxic and may be subjected to fouling, but it is easily removed and organisms, therefore, cannot attach themselves very firmly to the copper surfaces.

1. INTRODUCTION

Microbial cell surface hydrophobicity plays an important role in the adhesion of microbial cells to various surfaces (Doyle & Rosenberg, 1990; Savage, 1992). Hydrophobic bonding is one of the several short range attractive forces involved in the adhesion of bacteria to surfaces (Marshall *et al.*, 1971). The hydrophobicity of both the solid surface and the cell surface determines the extent of the hydrophobic interaction and thus influences cell adhesion (Husmark & Ronner, 1992).

Several factors including growth conditions, growth rate, growth phase, carbon source and starvation may influence the cell surface hydrophobicity of bacteria (Rosenberg, 1984; Walch, 1986). Such changes in the hydrophobicity of a cell can also influence its ability to attach to a substratum (Walch, 1986). Therefore, the objectives of the present chapter were to check (i) the effect of various factors like hexadecane concentration, temperature, growth phase, carbon source and concentration, nitrogen concentration on hydrophobicity and the (ii) effect of hydrophobicity on adhesion of bacteria.

2. MATERIALS AND METHODS

2.1 Bacterial cultures

Bacterial cultures (90) which were isolated from aluminium surface immersed in Mandovi estuary during different immersion

periods (Chapter 2A) were studied to see if their cell surface hydrophobicity varied during the period of immersion. Four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) which were selected for further studies described in this, were also used to evaluate the effect of various factors on the hydrophobicity of bacteria. Further, effect of hydrophobicity on adhesion was also studied using twelve more bacterial cultures which varied in their cell surface hydrophobicities.

2.2 Hydrophobicity of the bacterial cells isolated over different immersion periods.

Bacterial cultures (90) isolated from aluminium surface at different periods of immersion (1-30d) were grown to the stationary phase (24h) in nutrient broth (Table 2A.1) prepared in BSS (Table 2A.2). Cell surface hydrophobicity was measured by the method of Rosenberg *et al.*, (1980) following the modifications suggested by Walch (1986). Aliquots (3ml) of the broth were transferred to glass test tubes and a known volume (50 μ l) of hexadecane was added to each tube. All the tubes were vortexed for 2 min and the phases were allowed to separate for 15 min. The absorbance of the aqueous phase was measured spectrophotometrically at 400nm using a Beckman DU-64 Spectrophotometer. The absorbance of the bacterial suspension without addition of hexadecane was used as a control. Hydrophobicity as the percentage of hexadecane bound cells was subsequently calculated using the following formula

$$\text{Hydrophobicity (\% Hexadecane)} = (1-A/A_0) \times 100$$

Where A_0 is the absorbance of the bacterial suspension without hexadecane at 400 nm, A is the absorbance after addition of hexadecane, and % hexadecane is the percentage of bacteria in the hexadecane phase.

2.3 Effect of cell hydrophobicity on adhesion

From the hundred bacterial cultures isolated from aluminium panels immersed in Mandovi estuary, (Chapter 2A) twelve cultures were randomly selected to assess the effect of hydrophobicity on the adhesion of bacteria to aluminium. The cultures were grown in nutrient broth prepared in BSS for 18 h. The cells were harvested in the stationary phase, washed three times in BSS and resuspended in BSS and the O.D. was adjusted to 0.2. Hydrophobicity was measured as described earlier.

The adhesion of these bacterial cultures to aluminium panels was also assessed simultaneously. The panels were washed and cleaned as described earlier (Chapter 2A). The panels were then suspended in duplicate in flasks containing different bacterial cultures (OD = 0.2) for one hour. At the end of one hour, the panels were removed, washed and scraped in sterile seawater using a nylon brush. Serial dilutions were prepared in sterile seawater and appropriate dilutions were plated on Zobell Marine Agar (ZMA) plates to quantify the attached bacterial cells. The bacterial colonies were counted after incubating the ZMA plates at room temperature ($28 \pm 2^\circ\text{C}$) for 24 h.

2.4 *Effect of various factors on the hydrophobicity of P. stutzeri, V. pelagius (S₁), F. multivorum and V. pelagius (S₂)*

Further detailed work on hydrophobicity was carried out using four bacterial isolates viz. *P. stutzeri*, *V. pelagius (S₁)*, *F. multivorum* and *V. pelagius (S₂)*. All the experiments were done in duplicates.

a. **Effect of hexadecane concentration**

Bacterial cultures *P. stutzeri*, *V. pelagius (S₁)*, *F. multivorum* and *V. pelagius (S₂)* were grown in nutrient broth prepared in BSS for 18 hrs. and the stationary phase cells were harvested, washed thrice in BSS and suspended in BSS. Aliquots (3 ml) were transferred to glass test tubes and different amounts (25, 50, 75 μ l) of hexadecane were added to each tube. Hydrophobicity was measured as described above.

b. **Effect of temperature**

All four bacterial isolates were grown in nutrient broth prepared in BSS and harvested in the stationary phase. The cell pellets were washed thrice in BSS and then suspended in BSS. Aliquots (3 ml) were transferred to glass test tubes. These were incubated at different temperatures (27, 60 & 100°C) for 30 mins, after which they were assessed for hydrophobicity.

c. **Effect of carbon source**

Bacterial isolates *P. stutzeri*, *V. pelagius (S₁)*, *F. multivorum*

and *V. pelagius* (S_2) were grown in BSS using either glucose or citrate as carbon source (0.3%). Cells were harvested in the stationary phase and processed as above for measuring hydrophobicity. One culture *V. pelagius* (S_1) was further studied to assess the effect of various other carbon sources viz. galactose, fructose, sucrose, maltose and melibiose, on the hydrophobicity.

d. Effect of growth phase

Bacterial isolates were grown in nutrient broth to mid-exponential phase (7 h) and stationary phase (24 h) after which they were harvested, washed thrice in BSS, resuspended in BSS and their hydrophobicity was measured, as described earlier.

e. Effect of starvation

Bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were grown in nutrient broth prepared in BSS. The cultures in stationary phase were harvested, centrifuged and washed thrice in BSS so that the cells are free of the nutrients. The cells were suspended in BSS. Aliquots (3ml) were then used to measure hydrophobicity. The bacterial cells suspended in BSS were then incubated to starve at room temperature ($28 \pm 2^\circ\text{C}$). At various time intervals i.e. 6, 12, & 24 h, aliquots (3 ml) of these starved bacterial cell suspensions were removed and assessed for hydrophobicity. The hydrophobicity of the bacterial cells after starvation for 6, 12, and 24 h was compared with the hydrophobicity of the cells measured immediately after harvesting. A

much longer starvation period (192 h) was used for *V. pelagius* (S_1). Starved *V. pelagius* (S_1) cells were removed at 48, 72, 96, 144, 168 and 192 h and hydrophobicity was measured as above.

f. Effect of carbon concentration

Effect of carbon concentration on the hydrophobicity was assessed using *V. pelagius* (S_1). The isolate was grown in BSS with different concentrations of glucose (0.5 to 10%). After 72 hrs of growth, the cells were harvested and processed as described above and the hydrophobicity was estimated.

g. Effect of nitrogen concentration

Bacterial culture *V. pelagius* (S_1) was used to study the effect of nitrogen concentration on hydrophobicity. The cells were grown in BSS with different concentrations of nitrogen (0.05 to 2.0% NH_4Cl). Glucose (0.5%) was used as a carbon source. The cells were harvested after 72h and processed to measure hydrophobicity.

2.5 Statistical analysis

The relationship between the hydrophobicity and the adhesion of bacteria to aluminium was assessed using a simple linear regression analysis employing 123 Lotus programme (Sokal & Rohlf, 1981). The relationship between the carbon concentration and hydrophobicity as well as nitrogen concentration and hydrophobicity of the bacteria was assessed using the same programme.

3 RESULTS

3.1 *Effect of immersion period*

The isolates were divided into five groups based on their cell surface hydrophobicity i.e. the percentage of bacteria in the hexadecane phase (Table 3B.1). Group A isolates showed very low hydrophobicity and their abundance generally decreased over the 22d immersion period, whilst group B (10 - 20% Hex); Group C (21 - 30% Hex), Group D (31 - 40% Hex) and Group E (> 40% Hex) isolates did not show any particular trend. In general, most of the isolates were hydrophilic. However, the cell surface hydrophobicity of the isolates increased over a 22d immersion period with some exceptions.

3.2 *Effect of hydrophobicity on bacterial adhesion*

Cell surface hydrophobicity of the bacteria appear to influence its adhesion to metal surface. Twelve bacterial cultures were selected to study the effect of hydrophobicity on adhesion. The cell numbers of bacteria attached to the aluminium increased with increase in cell hydrophobicity (Fig 3B.1). There was a highly significant positive relationship ($r = 0.9425$, $p < 0.001$, $n = 12$) between the cell hydrophobicity and bacterial cell adhesion to aluminium (Fig. 3B.2).

3.3 *Effect of various factors on hydrophobicity*

a. **Effect of hexadecane concentration**

The four isolates *P. stutzeri*, *V. pelagius* (*S*₁), *F. multivorum*

Table 3B.1

Changes in cell surface hydrophobicity of bacterial isolates as a function of the immersion period and as a % of the total isolates obtained during each sampling period

Group	% Hex	Immersion period (d)				
		1	7	16	22	30
A	<10	61.11	50.00	17.64	13.33	64.28
B	10 - 20	16.66	28.57	23.52	46.66	35.71
C	21 - 30	-	7.14	35.29	40.00	-
D	31 - 40	-	7.14	11.76	-	-
E	> 40	22.22	7.14	11.76	-	-

Figures indicate percentage of isolates giving positive results

- = negative results

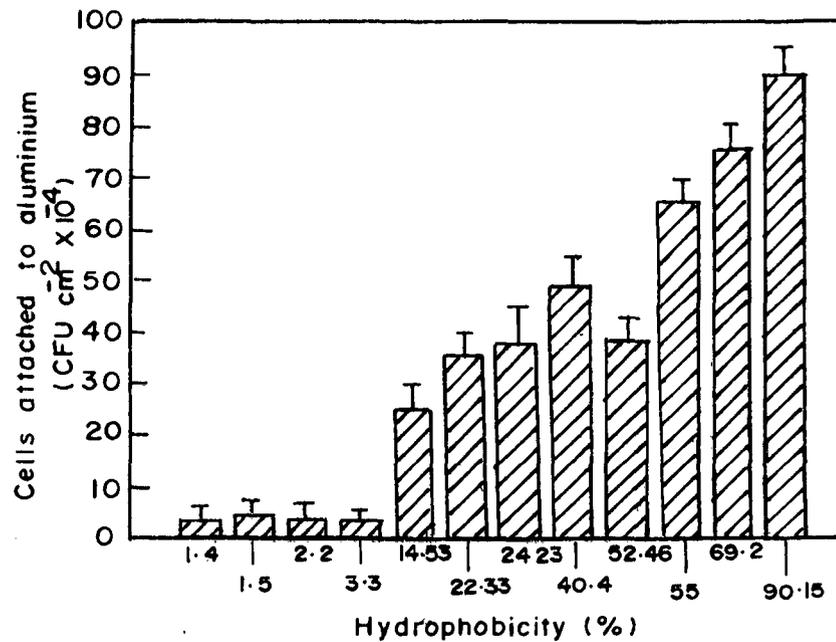


Fig. 3B-1. Effect of cell surface hydrophobicity on the adhesion of bacterial cells to the aluminium surface. Bar values are the means of 2 determinations. Error bars indicate standard deviation.

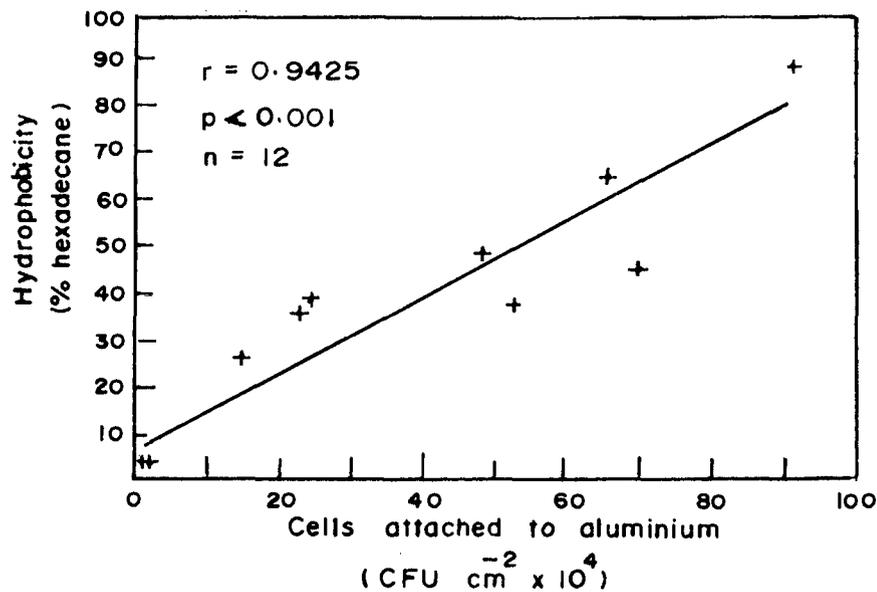


Fig. 3B-2. Relationship between hydrophobicity and attached cell numbers (CFU cm⁻² x 10⁴) of twelve bacterial cultures isolated from aluminium panels immersed in Mandovi estuary.

and *V. pelagius* (S_2) showed greater hydrophobicity with 50 μ l of hexadecane than 25 μ l. However, when the amount of hexadecane was further increased to 75 μ l, there was no significant change in the hydrophobicity of of the four isolates. *V. pelagius* (S_1) showed a relatively higher hydrophobicity in all the concentrations of hexadecane (Fig. 3B.3). This was followed by *V. pelagius* (S_2) and *F. multivorum*. *P. stutzeri* exhibited the lowest hydrophobicity regardless of the concentration of hexadecane.

b. Effect of temperature

The hydrophobicity of all the isolates decreased with increase in temperature (Fig 3B.4). The effect was more noticeable at 100°C, particularly with *V. pelagius* (S_2) cells as its hydrophobicity decreased from 24.23 at room temperature to 8.9 at 100°C. However, *P. stutzeri* showed a different trend. Hydrophobicity of *P. stutzeri* decreased when the cells were heated at 60°C, and increased again when heated at 100°C.

c. Effect of carbon source

The carbon source on which the bacteria were grown appeared to influence their hydrophobicity. All cultures showed higher hydrophobicity when grown on glucose as compared to the citrate grown cells (Fig 3B.5). Effect of additional carbon sources on hydrophobicity was assessed using *V. pelagius* (S_1). Hydrophobicity of *V. pelagius* (S_1) cells showed increasing trend when grown on sucrose, melibiose, galactose, maltose, fructose and glucose in that order. (Table 3B.2).

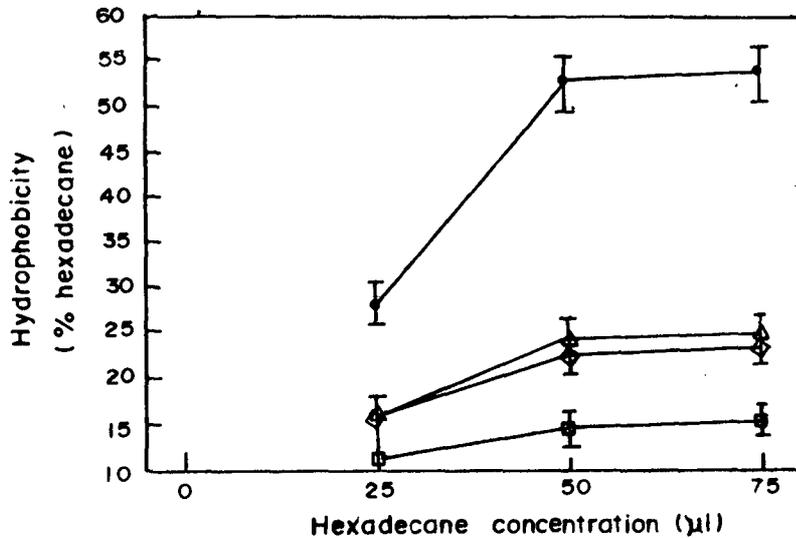


Fig. 3B-3 Effect of amount of hexadecane on the hydrophobicity of *P. stutzeri* —□—, *V. pelagius* (S₁) —●—, *F. multivorum* —◇— and *V. pelagius* (S₂) —△—. Points are the means of measurements done in duplicate. Bars indicate standard deviation.

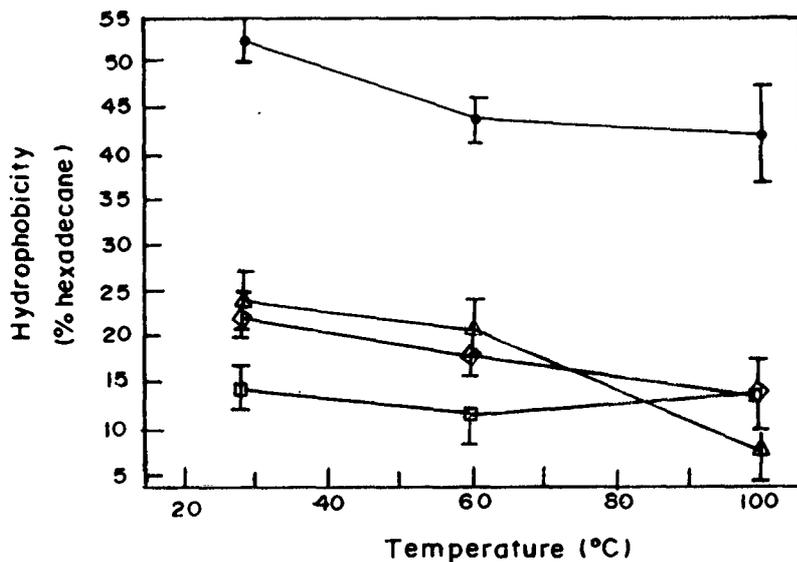


Fig. 3B-4. Effect of temperature on the hydrophobicity of *P. stutzeri* —□—, *V. pelagius* (S₁) —●—, *F. multivorum* —◇— and *V. pelagius* (S₂) —△—. Points are the means of measurements done in duplicate. Bars indicate standard deviation.

Table 3B.2

Cell surface hydrophobicity of *V. pelagius* (S_1) grown on different carbon sources.

Carbon source	% hydrophobicity
Sucrose	39.55 \pm 0.45
Melibiose	40.25 \pm 0.95
Galactose	43.50 \pm 0.90
Maltose	46.95 \pm 1.05
Fructose	54.05 \pm 0.25
Glucose	56.00 \pm 0.70

d. Effect of growth phase

Growth phases of bacteria also seemed to have an effect on the hydrophobicity of the bacterial cells. For all the four cultures, stationary phase bacteria were more hydrophobic than the mid-exponential phase bacteria (Fig 3B.6). For example, the hydrophobicity of *V. pelagius* (S_1) increased from 35.6 ± 4.2 in the exponential phase to 53.1 ± 4.8 in the stationary phase. Similarly, hydrophobicity of *P. stutzeri* increased from 10.8 ± 0.8 in the exponential phase to 15.2 ± 0.7 in the stationary phase.

e. Effect of starvation

The effect of starvation on the hydrophobicity of four bacterial isolates is presented in Fig 3B.7. The data indicate that starved bacteria became more hydrophobic than the bacteria which were not starved. There was a sharp rise in the hydrophobicity when bacteria were starved for 12h. However, thereafter there was not much appreciable increase in cell surface hydrophobicity of bacteria. *V. pelagius* (S_1) which was starved for 190 h showed increase in the hydrophobicity for the first 24h of starvation. However, further increase in starvation period did not increase the cell hydrophobicity of *V. pelagius* (S_1) (Fig 3B.8).

f. Effect of carbon and nitrogen concentration

Increasing concentration of the carbon source in the growth medium had a negative effect on the hydrophobicity of

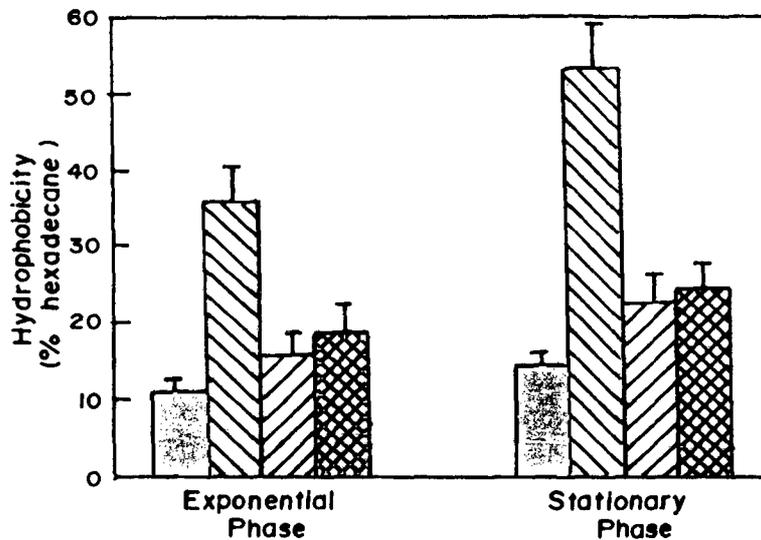


Fig. 3B-6. Effect of growth phases on the hydrophobicity of *P. stutzeri* □, *V. pelagius* (S₁) ▨; *F. multivorum* ▩ and *V. pelagius* (S₂) ▤. Bar values are the means of 2 determinations. Error bars indicate standard deviation.

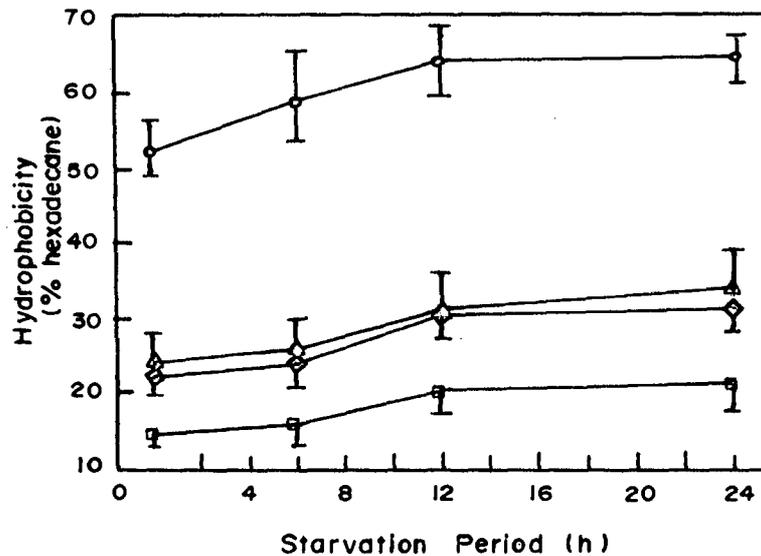


Fig. 3B-7. Effect of starvation on the hydrophobicity of *P. stutzeri* —□—, *V. pelagius* (S₁) —○—, *F. multivorum* —◇— and *V. pelagius* (S₂) —△—. Points are the means of measurements done in duplicate. Bars indicate standard deviation.

V. pelagius (S_1) cells. As the carbon concentration increased, the hydrophobicity of *V. pelagius* (S_1) cells decreased (Fig 3B.9). In contrast to this, nitrogen concentration had a positive effect on cell hydrophobicity. The hydrophobicity of *V. pelagius* (S_1) appeared to increase with the increase in nitrogen concentration of the growth medium (Fig 3B.10). Further, there was a negative relationship between the concentration of carbon source and the cell hydrophobicity ($r = - 0.6823$, $p > 0.05$, $n = 5$) (Fig 3B.11) whereas the relationship between nitrogen concentration and the cell hydrophobicity was highly significant and positive ($r = 0.9196$, $p < 0.01$, $n = 5$), (Fig. 3B.12).

4. DISCUSSION

Hydrophobic bonding is one of the several short range attractive forces in the adhesion of bacteria to surfaces (Marshall *et al.*, 1971). Cellular hydrophobicity is also known to be associated with the bacterial adhesion to various surfaces (Doyle & Rosenberg, 1990; Rosenberg, 1991; Parment *et al.*, 1992). Moreover, it is suggested that, during short exposure times, hydrophobic interactions play a major role in the attachment (Rittle *et al.*, 1990). Effect of hydrophobicity on the bacterial adhesion to surfaces and various factors influencing cell surface hydrophobicity of the bacteria were studied in this chapter. Results of the present study suggest that the hydrophobicity plays an important role in bacterial adhesion to surfaces and is influenced by several factors.

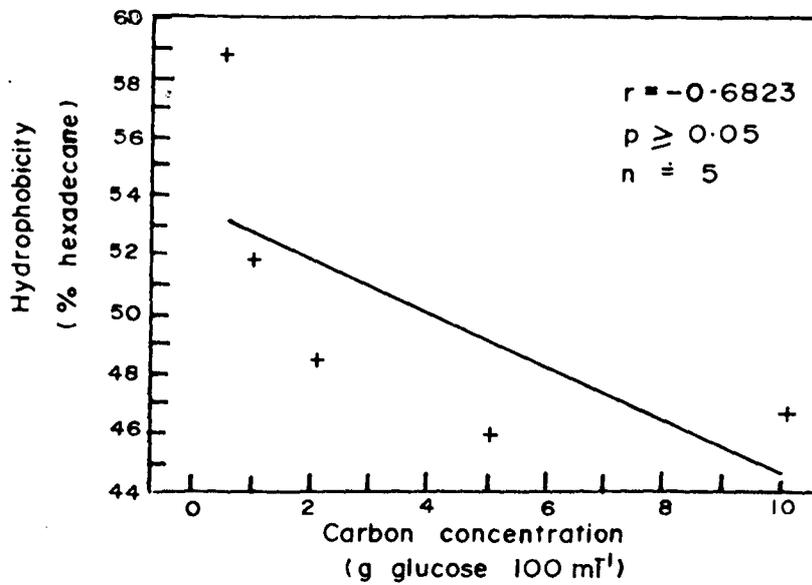


Fig. 3B-11. Relationship between carbon concentration and hydrophobicity of *V. pelagius* (S_1).

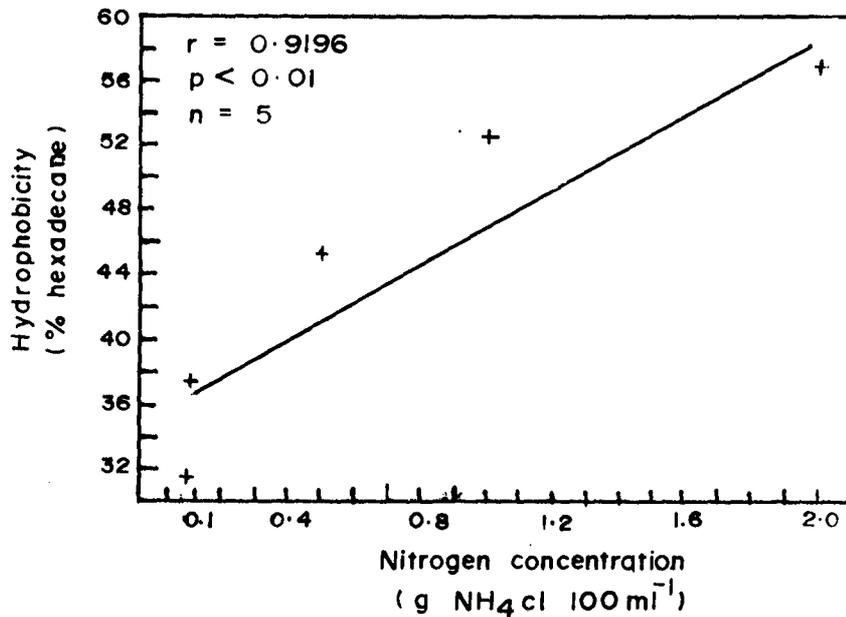


Fig. 3B-12. Relationship between nitrogen concentration and hydrophobicity of *V. pelagius* (S_1).

Bacterial colonies (90) which were isolated from aluminium panels immersed in estuarine waters over a period of 30d were screened for hydrophobicity. Most of the cultures were hydrophilic with less than 40% of absorbance in hexadecane. This observation corroborated with the earlier observation. Walch, (1986) isolated several bacterial isolates from various metal surfaces immersed in seawater over a period of 8 days which were also hydrophilic. This implies that hydrophilic bacteria are more abundant on hydrophilic surface. Although bacteria were hydrophilic, there were apparent changes in the cell surface hydrophobicity of bacteria as a function of the immersion period. Bacteria appeared to become relatively more hydrophobic especially over 22d period of immersion. This is in contrast with the observation of Walch (1986), who did not observe any change in the cell surface hydrophobicity of the bacterial isolates collected from several surfaces over a 8d period of immersion in marine waters.

There was a highly significant positive correlation ($r = 0.9425$, $p < 0.001$, $n = 12$) between the cell surface hydrophobicity and bacterial adhesion to aluminium panels. This implies that the hydrophobic interactions play a major role in the adhesion of bacterial cells to the surfaces. Hydrophobic interactions played an important role in the adhesion of *Bacillus cereus* spores to solid surfaces (Husmark & Ronner, 1990). The relative hydrophobicities of the spore and the solid surfaces, and the polarity of the bulk phase appear to determine the extent of the hydrophobic

interactions (Husmark & Ronner, 1990; 1993). Similarly, hydrophobic nature of the outer surface of bacteria has been shown to be involved in the attachment of bacteria to mammalian cells (Smyth *et al.*, 1978; Rosenberg *et al.*, 1981; Magusson, 1982; Rosenberg *et al.*, 1983). Moreover, Shinjo *et al.*, (1987) showed that hydrophobic strains of *Fusobacterium necrophorum* were more adherent than the non-hydrophobic strains. In another study, Grotenhius *et al.*, (1992) studied the hydrophobicity of the isolates from methanogenic anaerobic sludge and reported that the hydrophobic bacteria were selected by the granular sludge. Hydrophobicity was also shown to be associated with capacities of the lactobacilli strains to adhere to porcine intestinal epithelial cells (Wadstrom *et al.*, 1987). Similarly, adhesion to urinary epithelial cells was correlated with bacterial hydrophobicity (Reid *et al.*, 1992). Van Loosdrecht *et al.*, (1987) have shown that hydrophobic cells adhered to a greater extent than hydrophilic cells.

Several factors including growth conditions, growth phase, carbon and nitrogen source, temperature, starvation may influence the cell surface hydrophobicity of bacteria. Effect of these factors on the hydrophobicity of bacteria was studied using four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2). The hydrophobicity of these bacterial cultures was influenced by the concentrations of hexadecane used for the experiment. It was found that 50 μ l of hexadecane gave greater hydrophobicity, and hence was used to study the effect of various factors on the cell surface hydrophobicity of bacteria.

Increase in the temperature lowered the cell surface hydrophobicity of the bacterial cultures. An increase in temperature may lead to changes in the non-polar moieties present on the cell surface (Rosenberg, 1991). The observed decrease in the cell surface hydrophobicity of the four bacterial cultures with increase in temperature may be due to the changes in the non-polar moieties leading to the changes in the hydrophobic interactions.

Growth phase of bacteria also controlled the cell surface hydrophobicity (Spenceley *et al.*, 1992). Cell surface hydrophobicity of the bacterial cultures was lower in the exponential phase and was relatively higher in the stationary phase. Our results agreed well with the results of Walch (1986), who also observed increase in hydrophobicity of the stationary phase cells of *Deleya marina*. Similar results were reported for *Acinetobacter calcoaceticus* RAG (Rosenberg *et al.*, 1980) and *Streptococcus pyogenus* (Kjelleberg & Hermansson, 1984).

Conversely, increased cell surface hydrophobicity is reported during exponential phase by other workers. For example, cyanobacteria became more hydrophobic during the exponential growth phase (Fattom & Shilo, 1984). Van Loosdrecht *et al.*, (1987) observed increase in cell surface hydrophobicity of *Pseudomonas fluorescens* during the exponential growth phase, followed by a rapid decrease with the onset of the stationary phase. *Lactobacillus* strains were less hydrophobic when in the stationary phase than in the

exponential phase (Savage, 1992). These contradictory reports could be due to the difference in the composition of exopolysaccharides produced by the bacteria in the different growth phases. It was suggested that capsular exopolysaccharides of the mid-exponential cells interfered with adherence to hexadecane (Kjellberg & Hermanson, 1984; Rosenberg *et al.*, 1983). In some other studies, Christensen *et al.*, (1985) and Wrangstadh *et al.*, (1990) showed that the composition of the EPS of a marine *Pseudomonas* sp varied according to the growth phase which influenced the cell surface hydrophobicity. Further, Bonet *et al.*, (1993) also found that the production of a capsular exopolysaccharides (CPS) decreased the cell surface hydrophobicity of *Aeromonas salmonicida*. They suggested that the production of CPS may mask a surface protein array (A - layer), which is associated with cell surface hydrophobicity, thereby effectively blocking the hydrophobic interactions of the protein layer. Therefore, difference in the composition of exopolysaccharides of bacteria may influence the hydrophobicity of the bacteria. All the cultures employed in this study produced exopolysaccharides (see Chapter 6), therefore it is possible that the exopolysaccharides produced by the bacteria might have influenced cell surface hydrophobicity of the bacteria employed in this study.

Starvation also influenced cell surface hydrophobicity of bacteria. There was a sharp rise in the cell surface hydrophobicity of the bacteria starved for 12h duration. No further appreciable increase

was found after 24h of starvation. This agreed well with the studies reported by other workers. After starving several strains of marine bacteria, Kjelleberg & Hermansson (1984) observed an increase in cell surface hydrophobicity which was accompanied by cell division with growth and continuous size reduction of the cells. Similarly, Dawson *et al.*, (1981) also found a marine *Vibrio* sp. to become more hydrophobic during starvation. Changes in the cell surface proteins during starvation of *Flexibacter* sp. were involved in increased cell surface hydrophobicity (Sorongon *et al.*, 1991). Therefore, the observed increase in hydrophobicity of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) following starvation may be associated with the changes in the cell surface proteins occurring during starvation. Many copiotrophic marine bacteria develop an increasingly hydrophobic surface when starved (Kjelleberg, 1984). Such copiotrophic bacteria are the first to colonize the surfaces after immersion. Starving bacteria with a higher cell surface hydrophobicity as observed in the present study may have better chance to adhere to surfaces and use the nutrients adsorbed on the surfaces, thereby enhancing their chance of survival in aquatic environment (Kjelleberg *et al.*, 1983; Sorongon *et al.*, 1991).

Carbon source utilized for the cultivation of bacteria had a profound effect on the cell surface hydrophobicity. Amongst the different carbon sources employed, glucose grown bacteria were relatively more hydrophobic than that grown on citrate. Walch (1986) has reported that the cells of *Deleya marina* grown on glucose were

more hydrophobic as compared to the citrate grown cells. This observation was particularly evident in our study with respect to *V. pelagius* (S_1) cells which showed increasing hydrophobicity when grown with either sucrose, melibiose, galactose, maltose, fructose, and glucose. It is not known why cells grown on glucose are more hydrophobic. Moreover, cell surface hydrophobicity of the bacteria varied with the carbon concentration. As the concentration of carbon source (i.e. glucose) in the growth medium increased, the hydrophobicity of all four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) decreased. It is well known that exopolysaccharides production by bacteria increases with increase in carbon concentration. Such increased exopolysaccharide production might be responsible for the observed reduction in the cell surface hydrophobicity of these bacteria. This was further evident from the significant inverse relationship observed between the carbon concentration and the cell surface hydrophobicity. Conversely, Delaquis *et al.*, (1989) have reported an increase in cell surface hydrophobicity of *Pseudomonas fluorescens* as a function of the carbon source concentration.

In contrast to this, cell surface hydrophobicity of the bacteria increased with the increase in nitrogen concentration of the growth medium. There was a highly significant positive relationship between the cell surface hydrophobicity and the nitrogen content ($r = 0.9196$, $p < 0.01$, $n = 5$). High nitrogen containing medium reduces the exopolysaccharide production in many, if not all, bacteria. Reduced

production of exopolysaccharides with increasing nitrogen concentration was perhaps associated with the enhanced hydrophobicity of these bacteria (Chapter 6).

It is reported that greater hydrophobicity of the cells and the substratum results in greater attractive forces and higher levels of adhesion (Rijnaarts *et al.*, 1993). The cells that peel off from the biofilms are far more hydrophilic than the cells remaining in the biofilm (Allison *et al.*, 1990; Gilbert *et al.*, 1991). Vanhaecke *et al.*, (1990) used fifteen different isolates of *Pseudomonas aeruginosa* to study the kinetics of adhesion to 304 and 316-L stainless steel and reported that bacterial cell surface hydrophobicity as determined by bacterial adherence to hydrocarbons test and the contact angle measurement was the major parameter influencing the adhesion rate constant at least for the first 30 min of adhesion.

Further, there are numerous studies suggesting that bacterial adhesion to surfaces is dependent on cell surface hydrophobicity. For example, detachment of *Cyanobacteria* from surfaces was due to production of hydrophilic rather than hydrophobic surface structures and layers (Breznak *et al.*, 1984). Bacteria obtained from the human tooth surface exhibited very high cell surface hydrophobicity (Rosenberg *et al.*, 1983). It is also reported that in many biological systems, hydrophobic interactions adjacent to ionic or hydrogen bonds can stabilize an otherwise energetically weak binding complex

(Doyle *et al.*, 1982). The effect of both cell surface and the substratum hydrophobicity can be explained in terms of surface free energy. If both surfaces (substratum and bacterial) are hydrophilic, there is a net increase in free energy of the displaced water molecules. This is because the local ordered structure near the surfaces has to be broken down. This leads to the short range repulsion force which may be sufficient to prevent the cell particles coming close to the substratum surface. On the other hand, when both the surfaces involved are hydrophobic, the short range interactions is a net attraction. In effect this leads to a deepening of the primary minimum. This arises because the water molecules displaced into bulk solution now decrease the free energy, since in bulk solutions there is a net increase in their H-bonding. The decrease in free energy leads to the attraction between the cell surface and the substratum and this in turn leads to the increased adhesion of bacteria to the surfaces.

CHAPTER 4A

*ETS and respiratory activities
of free and attached bacteria*

1. INTRODUCTION

In aqueous environments, a large population of bacteria is found attached to surfaces which may even outnumber the planktonic bacterial (free living) population (Blenkinsopp & Costerton, 1991). Attached bacteria play an important role in various biological and chemical processes in aquatic habitats. Biofilm bacteria are generally more active and differ radically from their free-living counterparts. (Zobell, 1943; Marshall, 1976; Goulder, 1977; Harvey & Young, 1980; Kirchman & Mitchell, 1982; Pearl & Merkel, 1982; Jefferey & Paul, 1986). Conversely, some workers have reported that the attachment of bacteria to surfaces results in decreased activity (Estermann & McLaren, 1959; Hattori & Hattori, 1981).

Different methods including bacterial production utilizing ^3H thymidine incorporation, uptake of radiolabelled compounds, ATP (adenosine triphosphate), DNA (deoxyribonucleic acid) content and respiration measurements are utilized to assess the activity of attached and free living bacteria (Bright and Fletcher, 1983; Jefferey and Paul, 1986; Blenkinsopp and Lock, 1990; Bhosle *et al.*, 1994). Of these, the direct measurement of respiration is difficult, especially for oceanic samples. Hence, several indirect methods have been employed. One of the widely used methods is the measurement of the activity of Electron Transport System (ETS) (Blenkinsopp & Lock, 1990; Bhosle *et al.*, 1994), which is based on the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) to

INT-Formazan. Measured ETS activity can then be transformed to actual oxygen consumption rates using appropriate conversion factors. Such conversion factors can be obtained from the relationship between the ETS activity and the respiration rates.

There are a few studies comparing the activity of attached bacteria with their free cells (Bright & Fletcher, 1983; Jefferey & Paul, 1986). In these studies, various hydrophobic and glass surfaces were utilized to compare the activities of attached and free bacteria. However, very little is known about the effect of adhesion to metal surface on the activity of bacteria. In order to address this aspect, during the present work the effect of bacterial adhesion to metal and non-metal surfaces on the activity of attached bacteria was assessed. Measurements of ETS and respiration were utilized as tools to evaluate the activity of the attached and free bacteria. Further, although there are numerous reports about the relationship between ETS and respiration in marine populations, such information about R:ETS ratio in fouling bacterial community is lacking.

Therefore, the aims of this chapter were to measure the ETS activity and the respiration of attached and free bacteria; to identify the differences in the ETS activity, respiration rate and the R:ETS ratio of free living and attached bacteria and to assess the effect of growth phases on ETS activity, respiration rate and R:ETS ratio of the free living and attached bacteria. Our data suggest that the bacteria attached to metal and non-metal surfaces are relatively more active than their free cells.

2. MATERIALS AND METHODS

2.1 Test panels

Aluminium, copper, stainless steel and polystyrene panels (3.5 x 1.5cms) were cleaned and sterilized as described earlier in chapter 2.

2.2 Bacterial cultures

Four bacterial cultures *Pseudomonas stutzeri*, *Vibrio pelagius* (S_1), *Flavobacterium multivorum*, and *Vibrio pelagius* (S_2) were used for the present study.

2.3 Growth conditions

Cells of *V. pelagius* (S_2) were grown at room temperature in nutrient broth (100 ml) (Table 2A.1) prepared in BSS (Table 2A.2). After 18h of incubation at room temperature, these cells were used to inoculate (1% inoculum) several conical flasks (500 ml) containing 100 ml of fresh nutrient broth and were incubated at room temperature ($28\pm 2^\circ\text{C}$) on a rotary shaker at 150 rpm. After 7, 12, 18 and 24 h of incubation the cells of *V. pelagius* (S_2) in exponential, early stationary, stationary and late stationary phase, respectively, were harvested by centrifugation. The cell pellets were washed thrice with sterile BSS and resuspended in the same solution. The optical density of *V. pelagius* (S_2) cell suspensions at 400 nm was adjusted to 0.2 using BSS. This corresponds to 1.86×10^9 cells.ml⁻¹ of *V. pelagius* (S_2).

2.4 Preparation of attached cells

Test panels of stainless steel were suspended in the bacterial cell suspensions, representing various growth phases of *V. pelagius* (S_2) prepared as above, for 2 h to allow attachment of bacterial cells to the stainless steel panels. At the end of 2 h, the panels were retrieved and rinsed with sterile BSS to remove any unattached cells. Cells attached to the stainless steel panels were utilized to assess the effect of various factors on the ETS activity. Attached cells prepared from the cells collected after 7, 12 and 24 h of following incubation were employed to assess the effect of growth phases on the ETS activity of *V. pelagius* (S_2). Attached cells prepared from *V. pelagius* (S_2) cells grown for 18 h were used to assess the effect of INT concentration, incubation period on ETS activity. Whereas, cells in the exponential phase (7 h) were used to study the effect of adhesion period on the ETS activity.

In order to assess the effect of attachment period (contact time) on the ETS activity, *V. pelagius* (S_2) cell (exponential phase) suspension (100 ml) was transferred to 15 conical flasks. Three stainless steel panels were suspended in each flask. Three flasks were removed at 30 minutes interval over a period of 150 min. Three panels were used as experimental and three were used to prepare control panels. Remaining three panels were used for the estimation of attached cells.

2.5 Preparation of control cells

The stainless steel panels with the attached cells of *V. pelagius* (S_2) prepared as above were suspended in 2% formaldehyde solution for a period of 60 minutes. These panels with the killed attached cells of *V. pelagius* (S_2) were used as controls. The control panels were treated the same way as that of experimental panels as above. Since INT is light sensitive, all the experiments were carried out in dark.

2.6 Estimation of attached cell numbers

To estimate the cell numbers of the attached cells, stainless steel panels with attached cells of *V. pelagius* (S_2) were stained with crystal violet for 2 minutes (For details see chapter 2B). The panels were then removed, washed five times with BSS to remove any excess stain. The crystal violet stain from the attached cells was eluted with 2 ml of sodium deoxycholate. Subsequently, this was diluted with 3 ml of distilled water and the absorbance was measured at 570 nm using a Beckmann DU-64 spectrophotometer. Crystal violet absorbance was then converted to cell numbers using the calibration factor as described in chapter 2B.

2.7 ETS Measurements of Attached *V. pelagius* (S_2) cells

I. Effect of INT concentration

The stainless steel panels with attached cells of *V. pelagius* (S_2) (in triplicate) were placed in test tubes containing

20 ml of sterile seawater having various concentrations (0.005 to 0.4%) of INT. After an incubation period of 2 h at room temperature, the panels were removed. Each panel was rinsed with sterile BSS to remove any unbound INT-formazan and immersed in tubes containing 2 ml of chilled methanol to extract INT-formazan from the attached cells of *V. pelagius* (S_2) at 4°C for 1h. The methanol solution containing INT-formazan was measured spectrophotometrically at 480 nm against a solvent blank (methanol) (Blenkinsopp and Lock, 1990). The concentration of INT-formazan in methanol was calculated using a calibration curve prepared from various concentrations of standard INT-formazan dissolved in methanol.

II. Effect of incubation period

Experimental and control stainless steel panels with the attached *V. pelagius* (S_2) cells were suspended in test tubes containing 20 ml of INT solution (0.1%) and were incubated at room temperature. The 3 experimental and 3 control panels were removed at every 30 minute interval over a period of 150 minutes in order to optimize the incubation period for the measurement of ETS activity of the attached cells of *V. pelagius* (S_2). After removal of panels, ETS activity of the experimental and the control cells attached to the stainless steel panels was measured as described above.

III. Effect of growth phase

Stainless steel panels with attached cells of *V. pelagius* (S_2) were prepared from *V. pelagius* (S_2) cells harvested during different growth phases i.e. exponential (7 h), early stationary (12 h) and late stationary (24 h) and were employed to assess the effect of growth phase on the ETS activity.

Test (3) and control (3) panels representing different growth phases of *V. pelagius* (S_2) were immersed individually into the test tubes containing 0.1% INT solution (20 ml) and incubated for 90 minutes at room temperature. After this the panels were removed and analysed for ETS activity following the procedure described earlier. Cell number of the attached cells was estimated as above.

IV. Effect of adhesion (contact) period

Stainless steel panels were suspended in the *V. pelagius* (S_2) cell (exponential phase) suspension for different intervals of time (30-150 min) and were used to assess the effect of adhesion period i.e. the time allowed for the attachment of *V. pelagius* (S_2) cells to the panels on the ETS activity. Control and experimental panels with the attached cells (in triplicate) were transferred to different test tubes containing INT solution and incubated for 90 minutes. ETS activity of the control and experimental cells attached to stainless steel panels was measured as above.

V. Effect of substrata on the ETS activity of different bacteria

Effect of adhesion of *V. pelagius* (S_2) and other three bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), and *F. multivorum* to various surfaces like aluminium, polystyrene, stainless steel and copper on the ETS activity of these cultures was evaluated. Growth conditions for *P. stutzeri*, *V. pelagius* (S_1) and *F. multivorum* were similar to that described for *V. pelagius* (S_2). Cells of each bacterium were grown for 18 h using nutrient broth and BSS. The flasks were incubated at room temperature on a rotary shaker at 150 rpm for 18 h. At the end of incubation period, the cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were harvested, washed thrice in BSS and resuspended in BSS individually. The optical density of each of the individual bacterial cell suspension was adjusted to 0.2 at 400 nm using BSS. This corresponds to 2×10^9 cells ml^{-1} .

Cell suspensions (200 ml) of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were transferred to a number of flasks. Twelve flasks were employed for each culture. Nine panels each of aluminium, stainless steel, polystyrene and copper were introduced into the individual cell suspension prepared as above. Three panels of each surface were introduced into one flask. The cells were allowed to attach to the panels for 2 h. At the end of 2 h, the panels were retrieved and rinsed with BSS to remove

any unattached cells. Three panels were used as test and three were used to prepare the controls as described earlier. Remaining 3 panels were used for the estimation of cell count.

Test panels of aluminium, copper, stainless steel and polystyrene with attached cells of either *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were utilized to measure the ETS activity of the attached cells following the procedure described earlier.

2.8 ETS measurement of free cells of bacteria

Free cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) obtained for various experiments described above were utilised to measure ETS activity of free cells. Free cell suspensions of the *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were prepared following the procedure described earlier.

Aliquots (0.5 ml) of the individual bacterial suspension were used for the estimation of ETS activity. Aliquots (in triplicate) were introduced into the test tubes containing INT solution (0.1%). The test tubes were incubated for 90 minutes at room temperature. At the end of the incubation period, 1 ml was removed, transferred to another test tube containing 2 ml of chilled methanol and was incubated at 4°C for 1 h. The INT-formazan extracted in methanol was filtered through 0.2 μ m filter (Millipore, US) to remove bacterial cells and the filtrate was measured spectrophotometrically at

480 nm against a solvent blank (methanol). The concentration of the INT-formazan formed was calculated as described earlier.

The total viable cell count of the free cells was estimated by plating appropriate dilutions of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cell suspensions onto Zobell Marine Agar (ZMA), using the spread plate method. Plates were incubated at room temperature for 24 h and colonies were counted.

2.9 Calculation of ETS activity

ETS activity or equivalent oxygen utilization is calculated from INT-formazan absorption at 480 nm (Kenner and Ahmed, 1975). In the cell membrane two electrons and two protons are used to convert $1/2$ O_2 to H_2O . Two electrons are also used in the reduction of INT to INT-formazan. Thus two μ moles of INT-formazan are equivalent to one μ mole of O_2 and since 1 μ mole of O_2 is equivalent to $22.4 \mu l O_2$ and 2 μ mole INT-formazan ml^{-1} is equivalent to $31.8A_{480nm}$, there are $1.42A_{480nm} \mu l^{-1} O_2$. ETS activity is thus expressed as $\mu l O_2 cell^{-1} h^{-1}$.

2.10 Measurement of Respiration of attached *V. pelagius* (S_2) cells.

I. Effect of growth phase

V. pelagius (S_2) cells harvested during different growth phases i.e. exponential (7 h), early stationary (12 h) and late stationary (24 h) and attached to stainless steel panels were used to assess the effect of growth phases on respiration. Experimental (3) and the

control (3) panels with attached cells representing different growth phases were introduced into clean BOD bottles with sterile seawater (120 ml.). These bottles were stoppered immediately and kept in dark for 60 min. At the end of incubation period, the panels were removed from the BOD bottles. Respiration or oxygen consumption by the attached cells was measured by analysing dissolved oxygen content of the BOD bottles following the winkler method as described by Parsons *et al.* (1984).

Measurement of dissolved oxygen concentration

After the incubation period, to each BOD bottle 1 ml of manganous chloride (3M) and 1 ml of sodium iodide (3M) in NaOH (8N) reagents were added using a syringe pipette. The bottle was stoppered again and the precipitate allowed to settle. The precipitate was then dissolved in 1 ml of sulphuric acid (10N) and an aliquot (50 ml) of this sample was titrated against 0.01N thiosulphate using starch as indicator to estimate the dissolved oxygen content in the bottle. Calibration of thiosulphate was done using 0.01N standard iodate.

Final oxygen consumption values were calculated by subtracting the dissolved oxygen values of the experimental bottles from the values of dissolved oxygen of the control bottles. Dissolved oxygen consumption was expressed as $\mu\text{l O}_2 \text{ cell}^{-1} \text{ hr}^{-1}$.

II. Effect of adhesion period

V. pelagius (S_2) cells were grown in nutrient both prepared in BSS for 7 h. Cells were harvested by centrifugation and the cell suspension was prepared as earlier. Stainless steel panels were deployed in the cell suspension of *V. pelagius* (S_2) and the cells were allowed to attach to these panels for different time intervals as described for ETS activity.

Stainless steel panels with attached *V. pelagius* (S_2) cells were suspended into clean BOD bottles (120 ml) containing sterile seawater. Bottles with the attached cells were incubated at room temperature. Respiration or oxygen consumption was estimated following the procedure described earlier. Respiration values were expressed as $\mu \text{IO}_2 \text{ cell}^{-1} \text{ h}^{-1}$.

The number of cells attached to the stainless steel panels at various intervals of incubation period was estimated using crystal violet adhesion procedure as described earlier.

2.11 Respiration rates of free living cells

Free cells of *V. pelagius* (S_2) prepared during experiments described above were used to measure the respiration of free cells.

Aliquots (0.5 ml) of the *V. pelagius* (S_2) cell suspension were introduced into the BOD bottles (120 ml) containing sterile seawater and incubated for 60 minutes. Oxygen utilised during the period of incubation was measured as above.

2.12 Statistical analysis

The significance of the observed differences in the activity of the free living cells and the cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various surfaces was assessed using one way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Similarly, the differences in the respiratory and ETS activity during various growth phases between the free living cells and cells attached to surfaces were also assessed using the same programme.

Furthermore, relationship between the respiration and ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels was assessed by simple linear regression analysis between these two parameters using 123 Lotus programme (Sokal and Rohlf, 1981).

3. RESULTS

3.1 ETS activity of the Attached cells of *V. pelagius* (S_2)

I. Effect of INT concentration

ETS activity of the cells of *V. pelagius* (S_2) attached to stainless steel increased as the INT concentration increased from 0.005 to 0.1% (Fig. 4A.1) This was evident from the observed increase in the INT-formazan formed. Maximum INT-formazan was formed when INT concentration was 0.1%. With further increase in INT concentration, there was a decrease in the INT-formazan production. Hence, INT concentration of 0.1% was chosen for all further experiments.

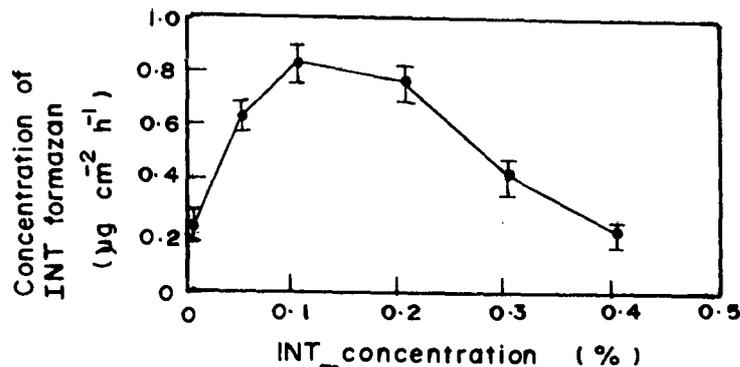


Fig. 4A-1. Effect of INT [2-p (idophenyl)-3- (p-nitrophenyl) -5- phenyl tetrazolium chloride] concentration on the ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels. Points are the means of measurements done in triplicate. Bars = 2 SD

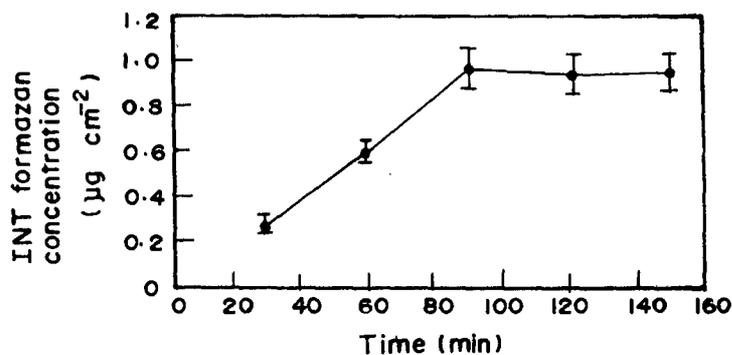


Fig. 4A-2. Effect of time on ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels. Points are the means of measurements done in triplicate. Bars = 2 SD

II. Effect of incubation period

There was a non-linear increase in the production of INT-formazan during the period of incubation of stainless steel attached cells of *V. pelagius* (S_2) in 0.1% INT solution (Fig. 4A.2). Further increase in the incubation period did not enhance the production of INT-formazan by the attached *V. pelagius* (S_2) cells. This implies that 90 min incubation period was optimal for the estimation of ETS activity of the stainless steel attached cells of *V. pelagius* (S_2).

III. Effect of growth phase

ETS activity of free and attached cells of *V. pelagius* (S_2) was influenced by growth phases (Fig 4A.3). ETS activity of both free and attached cells of the *V. pelagius* (S_2) was highest during the exponential phase. As the cells reached stationary phase, ETS activity decreased. A further decrease in ETS activity of both free and attached cells was observed in late stationary phase (Fig 4A.3). Cells of *V. pelagius* (S_2) attached to stainless steel showed relatively higher ETS activity than that observed for free *V. pelagius* (S_2) cells. The observed difference in ETS activity of attached and free cells of *V. pelagius* (S_2) during various growth phases was highly significant (Table 4A.1).

IV. Effect of adhesion period

Attached cells of *V. pelagius* (S_2) harvested during

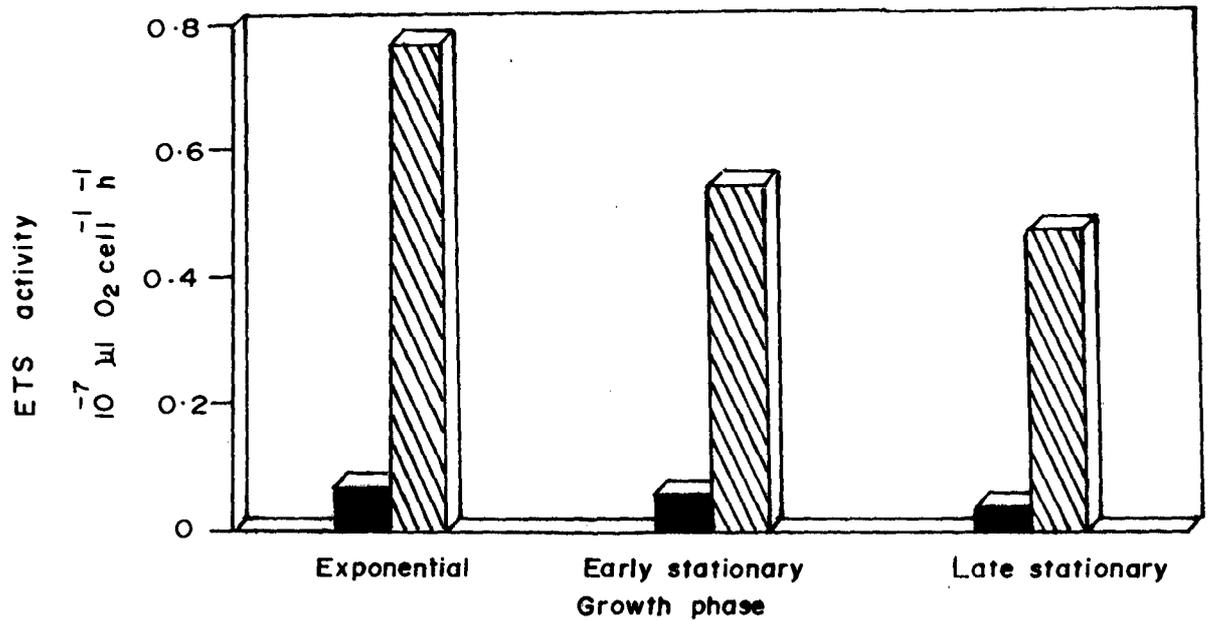


Fig. 4A.3. Effect of growth phase on ETS activity of free living and cells of *V. pelagius* (S_2) attached to stainless steel . Bar values represent the means of 3 determinations. In all cases, the standard deviation is less than 10% value of the plotted mean bar.

Table 4A.1a

ANOVA analysis to assess the effect of different growth phases on the ETS activity of free and attached cells of *V. pelagius* (S_2).

Cells	Attached		Free living	
	LS	ES	LS	ES
Log	F = 58.979 p = 1.546E-03	F = 33.358 p = 4.462E-03	F = 185.561 p = 1.682E-04	F = 6.844 p = 0.0590
ES	F = 5.585 p = 0.0774		F = 13.488 p = 0.0213	

Table 4A.1b

ANOVA analysis to assess the effect of same growth phase on ETS activity of attached and free living cells of *V. pelagius* (S_2)

Free cells	Attached cells		
	Log	ES	LS
Log	F = 544.727 p = 1.998E-05		
ES		F = 652.640 p = 1.394E-05	
LS			F = 579.687 p = 1.765E-05

Log - Logarithmic
 ES - Early Stationary
 LS - Late Stationary

exponential phase were employed to study the effect of contact time on the ETS activity. As the time allowed for the attachment of *V. pelagius* (S_2) cells to the stainless steel panels increased, ETS per cell decreased to a small extent (Fig 4A.4). ETS activity was $11.3 \pm 0.92 \mu\text{l O}_2 \text{ cell}^{-1} \text{ hr}^{-1} \times 10^{-6}$ after 30 mins. which decreased to $7.23 \pm 0.46 \mu\text{l O}_2 \text{ cell}^{-1} \text{ hr}^{-1} \times 10^{-6}$ after 150 mins.

V. Effect of adhesion to various surfaces on ETS activity

The cells of the four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to either aluminium, copper, stainless steel and polystyrene panels showed higher ETS activity than their free living counterparts (Fig 4A.5). Cells of all the four bacterial cultures attached to polystyrene surfaces showed highest ETS activity. This was followed by cells attached to stainless steel surface. Cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to aluminium surfaces showed lower ETS activity compared to the cells attached to polystyrene or stainless steel. Lowest ETS activity was shown by the cells attached to copper surfaces (Fig 4A.5). Statistical treatment of the data showed significant differences between the ETS activity of cells attached to various surfaces and the free suspended cells (Table 4A.2).

Amongst the four bacterial cultures studied, highest ETS activity was recorded for *P. stutzeri* cells attached to polystyrene panels. *P. stutzeri* cells attached to the stainless steel panels also

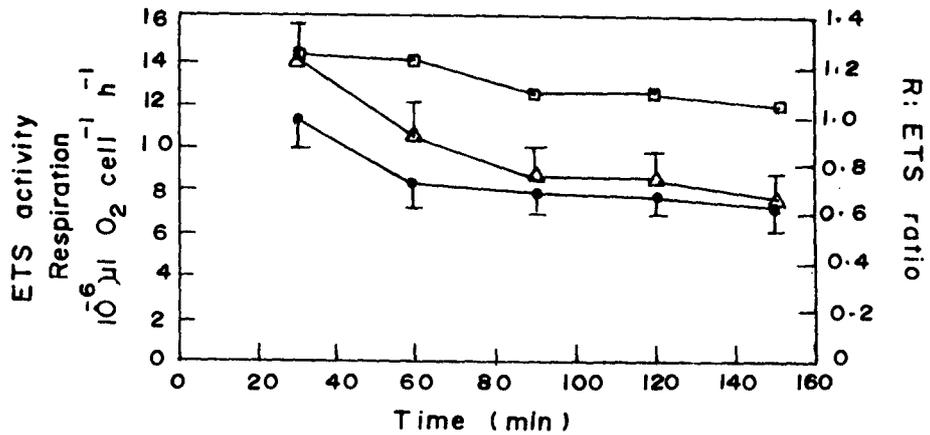


Fig. 4A·4. Effect of period of attachment on ETS activity (\bullet), respiration (\triangle) and R:ETS ratio (\square) of *V. pelagius*(S₂) cells attached to stainless steel panels points are the means of measurements done in triplicate. Bars represent standard deviation.

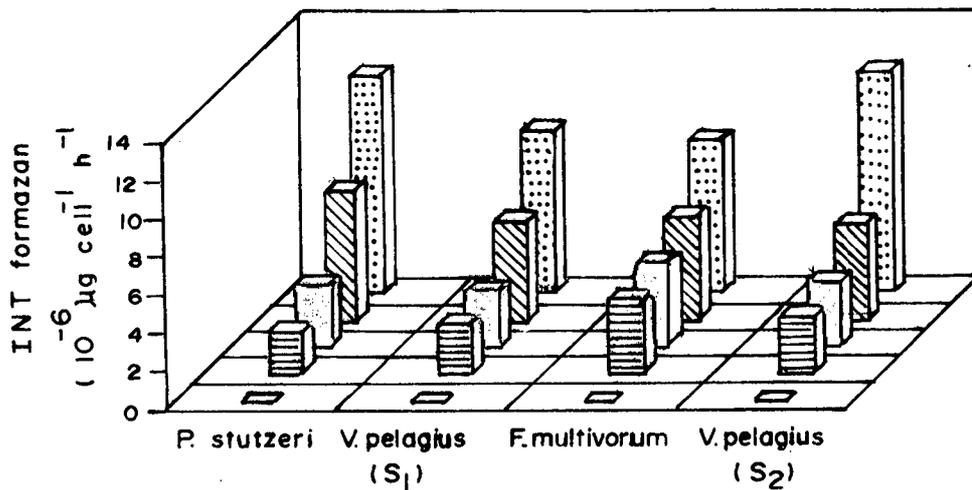


Fig. 4A-5. Effect of adhesion of *P. stutzeri*, *V. pelagius* (S₁), *F. multivorum* and *V. pelagius* (S₂) to Copper , Aluminium , Stainless Steel  and Polystyrene  on the ETS activity. ETS activity of free living cells  is also shown. Bar values represent the means of 3 determinations. In all cases, the standard deviation is less than 10% value of the plotted mean bars.

Table 4A.2

ANOVA between ETS activity of free cells and cells attached to various surfaces of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cultures.

<u>Bacteria</u>	<u>Surfaces</u>			
	<u>AL</u>	<u>PS</u>	<u>Cu</u>	<u>SS</u>
<i>P. stutzeri</i>	F=4152.859 p=3.473E-07	F=6298.969 p=1.511E-07	F=255.286 p=8.971E-05	F=8180.104 p=8.959E-08
<i>V. pelagius</i> (S_1)	F=1.6869E+09 p=2.000E-14	F=10211.031 p=5.751E-08	F=165.835 p=2.097E-04	F=6102.547 p=1.609E-07
<i>F. multivorum</i>	F=3005.217 p=6.629E-07	F=11034.026 p=4.925E-08	F=566.491 p=1.848E-05	F=14301.305 p=2.932E-08
<i>V. pelagius</i> (S_2)	F=1414.608 p=2.984E-06	F=539.764 p=2.034E-05	F=14.470 p=0.0190	F=499.712 p=2.371E-05

AL = Aluminium, PS = Polystyrene, Cu = Copper, SS = Stainless steel

showed higher activity than the cells of the *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the stainless steel panels. *V. pelagius* (S_2) cells attached to polystyrene showed higher ETS activity than that observed for *F. multivorum* and *V. pelagius* (S_1) cells. However, higher ETS activity was exhibited by *F. multivorum* followed by *V. pelagius* (S_2) cells attached to aluminium and copper. *P. stutzeri* cells showed lower activity on attachment to aluminium and copper surfaces compared to the other bacterial cultures (Fig 4A.5). There were significant differences in the ETS activity of the bacterial cells of the four cultures attached to the four different surfaces as evident from the statistical treatment of the data (Table 4A.3).

3.2 Respiration rates of attached *V. pelagius* (S_2) cells

I. Effect of growth phase

Cellular respiration rates of the *V. pelagius* (S_2) cells of different physiological states were significantly different (Table 4A.4). During exponential growth, cell specific respiration rates (R per cell) were higher, for both the free living as well as the attached cells (Fig 4A.6), whereas, respiration rate (R per cell) of the stationary phase free cells and the stationary phase attached cells of *V. pelagius* (S_2) decreased. The observed decrease in the respiration of stationary phase cells was more pronounced for the free living cells of *V. pelagius* (S_2). When the *V. pelagius* (S_2) cells of the late stationary phase were used, there was a further decrease in the respiratory activity of both the free and the attached cells of *V. pelagius* (S_2).

Table 4A.3

Differences in the ETS activity of the cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various surfaces as assessed by ANOVA.

Bacteria	<i>P. stutzeri</i>		
Surfaces	SS	Cu	PS
AL	F = 1505.991 p = 2.634E-06	F = 42.262 p = 2.888E-03	F = 2817.231 p = 7.542E-07
PS	F = 782.546 p = 9.715E-06	F = 1991.786 p = 1.507E-06	
Cu	F = 777.261 p = 9.847E-06		

<i>V. pelagius</i> (S_1)			Bacteria
PS	Cu	SS	Surfaces
F = 4287.706 p = 3.2593E-07	F = 0.128 p = 0.7382	F = 1131.820 p = 4.656E-06	AL
	F = 458.042 p = 2.819E-05	F = 907.601 p = 7.231E-06	PS
		F = 76.923 p = 9.318E-04	Cu

Bacteria	<i>F. multivorum</i>		
Surfaces	SS	Cu	PS
Al	F = 104.976 p = 5.115E-04	F = 9.109 p = 0.0392	F = 964.307 p = 6.408E-06
PS	F = 802.389 p = 9.242E-06	F = 489.168 p = 2.47E-05	
Cu	F = 78.311 p = 9.003E-04		

<i>V. pelagius</i> (S_2)			Bacteria
PS	Cu	SS	Surfaces
F = 256.844 p = 8.864E-05	F = 0.241 p = 0.6493	F = 47.266 p = 2.345E-03	AL
	F = 81.066 p = 8.425E-04	F = 136.215 p = 3.081E-04	PS
		F = 6.342 p = 0.0655	Cu

AL = Aluminium, PS = Polystyrene, Cu = Copper, SS = Stainless steel

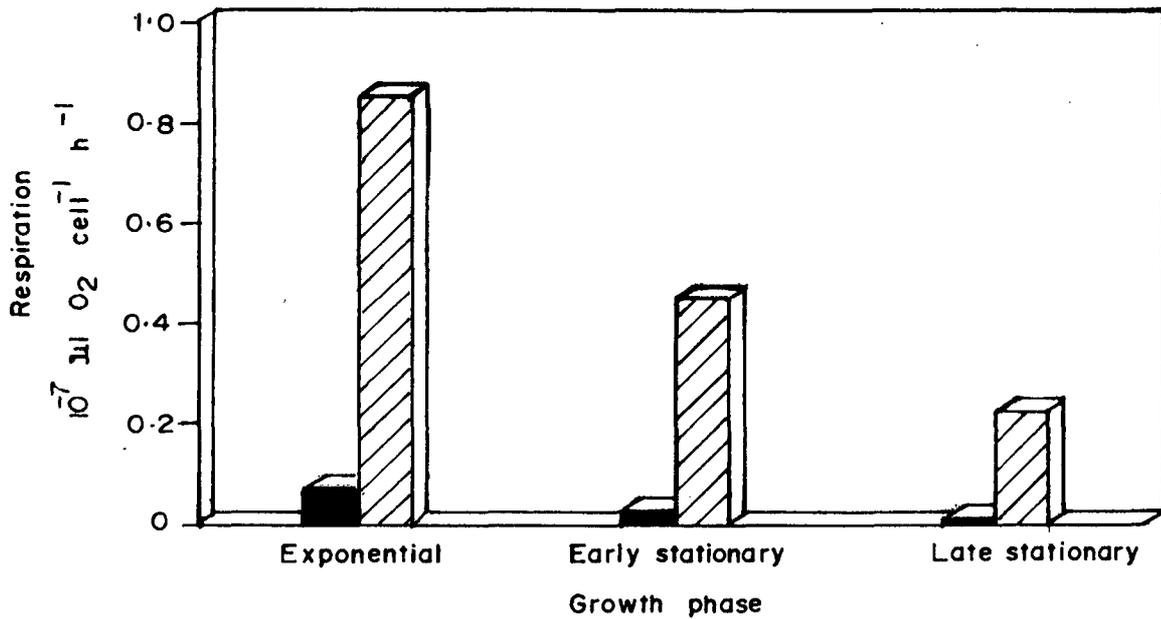


Fig. 4A-6. Effect of growth phase on respiration of free living and attached cells of *V. pelagius* (S_2). Bar values represent the means of 3 determinations. In all cases, the standard deviation is less than 10% value of the plotted mean bars.

II. Effect of adhesion period

Exponential phase cells of *V. pelagius* (S_2) when allowed to attach to stainless steel over a period of 150 min, showed small changes in the cell specific respiration rate (R per cell). Respiration rate decreased from $14.2 \mu\text{l O}_2 \text{ cell}^{-1} \text{ hr}^{-1} \times 10^{-6}$ at 20 mins to $7.53 \mu\text{l O}_2 \text{ cell}^{-1} \text{ hr}^{-1} \times 10^{-6}$ after 150 mins (Fig 4A.4).

3.3 R:ETS ratio of *V. pelagius* (S_2)

The apparent relationship between the respiration and ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels was assessed using simple linear regression analysis. There was a highly significant relationship ($r = 0.9460$, $n = 7$, $p < 0.001$) between the respiration and ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels (Fig 4A.7).

However, the R:ETS ratio varied with the growth phase for both attached and free cells of *V. pelagius* (S_2). During the exponential phase, R:ETS ratio of both attached and free living cells of *V. pelagius* (S_2) was similar. R:ETS ratio of the attached *V. pelagius* (S_2) cells decreased from exponential phase to late stationary phase. The R:ETS ratio of the attached *V. pelagius* (S_2) cells was 1.09 in the exponential phase which decreased to 0.51 during the late stationary phase. However, the decrease in R:ETS ratio was more obvious for the free living cells. R:ETS ratio for free cells of *V. pelagius* (S_2) was 1.03 in the exponential phase. It decreased to 0.31 in the late stationary phase. The consistent

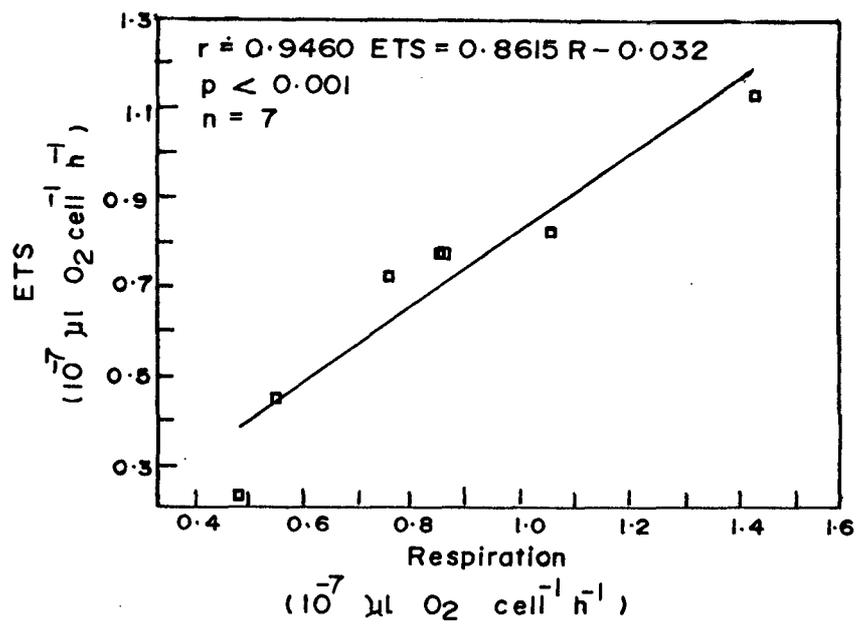


Fig. 4A-7. Relationship between respiration and ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels.

decrease observed for R:ETS ratio of the free and attached cells as a function of growth phase, was due to sharp decline in the respiration rate of these cells from exponential phase to late stationary phase (Fig 4A.8). Exponential phase cells of *V. pelagius* (S_2) when allowed to attach to stainless steel over a period of 150 min, showed small changes in R:ETS ratio (Fig 4A.4).

4. DISCUSSION

A method to measure ETS activity of the attached bacteria was calibrated using *V. pelagius* (S_2) and then employed to compare ETS activity of the attached and free cells of bacteria. Similarly, respiration rates of the attached and free bacteria were also measured. Measurements of the ETS and the respiration help in understanding energy flow and metabolic turnover in marine biofilm. However, the direct measurement of respiration is very difficult, especially in oceanic waters (Williams, 1984). To overcome this problem indirect methods based on biochemical activity rates have been proposed. One of the most widely used of these alternative techniques is the determination of the activity of the enzymes of the respiratory electron transport system (ETS) (Packard, 1971, 1985; Vosian, 1982). This technique depends on homogenization of the bacteria or other microorganisms using a suitable buffer. Such physical disruption methods, however, cannot be employed for biofilm as it influences the measured ETS activity. In order to overcome this problem, we used a method of ETS measurement

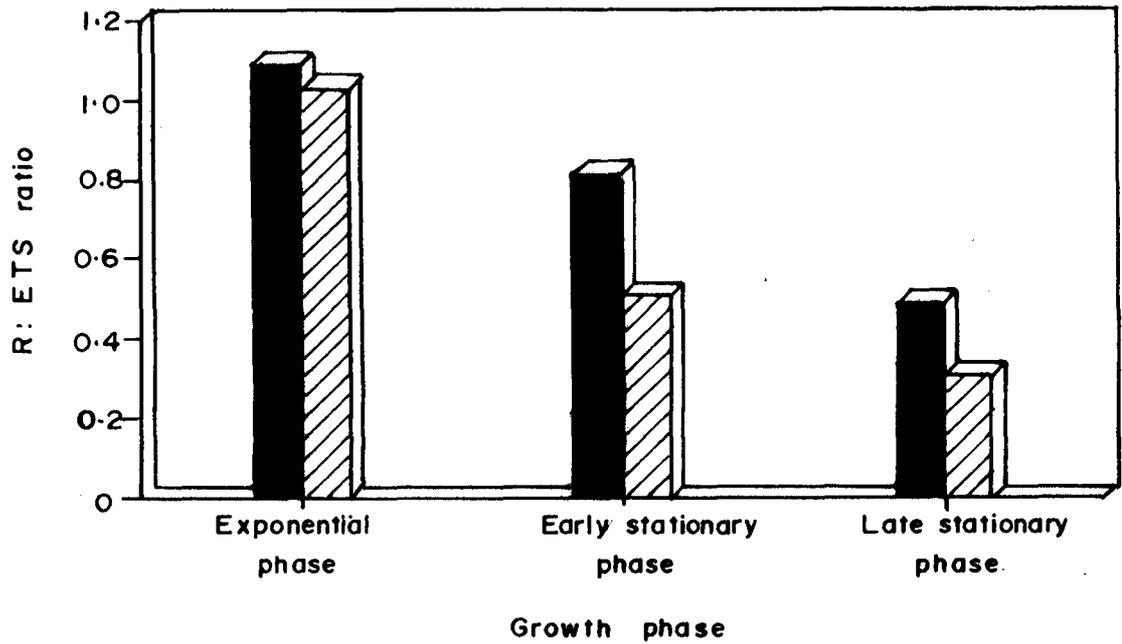


Fig. 4A-8. Effect of growth phase on R:ETS ratio of attached and free living cells of *V. pelagius* (S_2) culture.

which is based on intact biofilm (Blenkinsopp and Lock, 1990). However, some factors including INT concentration and incubation period were evaluated before the method was actually employed for ETS measurements of attached bacteria.

ETS activity of the *V. pelagius* (S_2) cells attached to stainless steel increased with the increase in INT concentrations. When INT concentration was used at 0.1%, highest amount of INT-formazan was produced. Further increase in INT concentration (>0.1%), reduced the amount of INT-formazan produced by the attached *V. pelagius* (S_2). This implies that 0.1% INT was the optimal concentration for the measurement of ETS activity of the attached cells of *V. pelagius* (S_2). It appears that the optimal INT concentration required to measure ETS activity varied with the biofilm. For example, Jeffery & Paul (1986), while working on natural biofilm reported that 0.4% INT was optimal for the ETS activity of the biofilm developed on polystyrene panels. Whereas, 0.02% of INT was found to be optimal to measure ETS activity of the river biofilm (Blenkinsopp & Lock, 1990). Further increase in INT concentration reduced the production of INT-formazan. The decrease in INT-formazan production at higher INT concentration observed in the present study, was perhaps due to toxic effect of INT on the attached bacterial cells. Furthermore, chemical reduction of INT was observed by Packard and Williams (1981). Therefore, killed controls were used to ensure that the INT-formazan production was due to the enzymatic activity of the attached bacteria and not due to chemical INT reduction.

Results of this study suggest that the attached cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were significantly more active than their free cells. This observation compares well with a few earlier studies which indicate that the attached microorganisms are more active than their free cells. For example, Hodson *et al.*, (1981) reported greater dissolved ATP uptake by particle bound bacteria than by unattached cells. Jeffery & Paul (1986) also found that the activity of the attached bacterial cells measured by different methods was greater than that of unattached cells. In another study, the specific glutamate uptake rates by the biofilm bacteria were 26 times greater than those of the planktonic bacteria (Ladd *et al.*, 1979). Conversely, some exceptions to the above observations, have also been reported (Williams, 1970; Hodson *et al.*, 1981; Bright & Fletcher, 1983).

There are two possibilities for the greater activity of the attached bacteria. Either the higher activity is due to the stimulation on attachment of bacteria to the surfaces or the subpopulation of bacteria which becomes attached is inherently different and is more active (Jefferey & Paul, 1986). The latter process is unlikely because the same bacterial strains i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were used to study the activities of free living and attached cells. The observed greater ETS activity and respiration rates of the attached bacteria, therefore, might have been associated with the stimulation on attachment of bacteria to the test surfaces. Further, Brozel *et al.*, (1995) have suggested that

changes in surface free energy of the bacterial cell membrane due to adhesion to surfaces may influence the activity of cell membrane. Thus, it is possible that similar changes in surface free energy of cell membrane of the attached bacteria employed in this study are perhaps responsible for the enhanced ETS activity because ETS is essentially a cell membrane associated process.

Alternatively, it is also possible that the different conditions exist at the solid-liquid interface as compared with the bulk liquid phase which may be responsible for the observed differences in the activities of the attached and free living bacteria (Bright & Fletcher, 1983). It is well documented that surfaces immersed in aquatic environment adsorb dissolved organic matters onto their surfaces thereby accumulating nutrients on the surfaces. Such accumulation of nutrients onto surfaces may be responsible for the differences in the activity of free and attached cells of the bacteria. Moreover, diffusion of the substrate molecules into the bulk liquid may be reduced due to the presence of bacterial exopolymers at the surface or by the substratum adsorption, thus making them easily available for the bacterial uptake. This entrapment of the macromolecules at the surfaces may partially contribute to the differences in the activity of free living and attached bacteria observed in the present study.

There were significant differences in the activities of bacteria attached to the different surfaces. Bright & Fletcher (1983) have also reported large differences in the activity of the bacteria attached to

various surfaces. They ascribed these differences to changes in surface charge, surface free energy and wettability which affect the concentration of ions or charged molecules at the solid surface. Similar changes may explain the observed differences in the activities of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to different surfaces.

ETS activity of the attached bacteria was influenced by the nature of the surface. Of the four surfaces used in the present study, three were hydrophilic and one was hydrophobic. As compared to hydrophilic surfaces, the cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the hydrophobic surface i.e. polystyrene showed higher activity. This indicates that the activity may have been more important in attachment of bacteria to hydrophobic surfaces than that to more hydrophilic ones (Fletcher, 1979; 1980). Similar results are reported by other workers who found an increase in the activity of attached bacteria with an increase in the hydrophobicity of the substrate or bacteria (Bright & Fletcher, 1983).

Bacteria attached to hydrophilic surfaces showed significant differences in the ETS activity. Bacteria attached to stainless steel, a hydrophilic surface, showed higher ETS activity than those attached to aluminium. Aluminium, being a rough surface, large number of cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to it. The decrease in ETS activity of

P. stutzeri, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the aluminium surface could be due to less surface area available for the individual bacterial cell, which may decrease availability of nutrients and other macromolecules.

Cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the copper surface showed lowest ETS activity. Copper surface is known to be inhibitory to bacterial growth (Roger *et al.*, 1994). The lower activity of the bacteria attached to copper surfaces observed in the present study was perhaps due to the toxicity of copper ions.

Further, as the contact time allowed for the adhesion of the bacterial cells to the surfaces increased, both ETS and R per cell of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) decreased. This suggests that the bacterial cells which attach to the surfaces initially are more active than the ones which attach later. It is possible, that the bacterial cells which attach to the surfaces in the initial stages are exposed to a more favourable environment of high nutrient concentration and hence are more active. The cells attached to the surfaces at the later stages may face a nutrient deficient condition thereby showing less activity. Similar behaviour of the attached bacteria was reported by Fletcher & Marshall (1982).

Growth phase of the bacteria also influenced respiration rates and ETS activities. Cell specific respiration rates and the ETS activities were higher in exponential phase and decreased during the

stationary phase for both attached bacteria and their free living counterparts. Similar results are reported by other workers. Christensen *et al.* (1980), while working with five species of marine *Vibrio* found that R:ETS ratio was low during the senescent phase. Observed decrease in R:ETS ratio was attributed to the decrease in the respiration rates following growth termination. Respiratory activity drops in senescing cells, since energy is no longer required for the synthesis of nucleic acids, proteins and other compounds associated with normal cell division and maintenance. As compared to respiration, however, ETS activity does not decrease much, as there is a turnover of respiratory proteins until the cells are dead and disintegrated. This may explain the decrease in R:ETS ratio from the exponential to the stationary phase observed in the present study.

Further, there were also differences in the R:ETS ratio of the free living and attached bacteria. This is because though ETS is a measure of respiratory potential of the community, respiration is not a linear function of ETS and thus the R:ETS ratio does not remain constant under different environmental conditions (del Giorgio, 1992). In the present study, R:ETS ratio was similar for both free living and attached bacteria during the exponential growth phase. Though R:ETS ratio decreased from exponential phase to stationary phase for both attached and free cells the decrease was more pronounced for free cells than the attached cells. This is because respiration rates for the free living cells (R per cell) decreased more

rapidly than the attached cells. The decrease in respiration rates was higher than the decrease in ETS activity. The decrease in respiration rates, ETS and R:ETS ratio from experimental growth phase to the stationary phase was less in case of attached bacteria than the free cells, which may enhance their chances of survival in aquatic environment.

Highly significant relationship ($r = 0.9460$, $n = 7$, $p < 0.001$) was observed between the respiration rates and the ETS activity of *V. pelagius* (S_2) cells attached to stainless steel panels. This relationship between ETS activity (potential respiration) and oxygen consumption (actual respiration) can be employed to determine the respiration rates in biofilm community. The present results suggest that the measurement of ETS activity and consideration of the different physiological states of the attached bacterial population will allow reasonably accurate estimation of the respiratory activity and metabolic rates in bacterial fouling community.

CHAPTER 4B

*Enzyme activities
of free and attached bacteria*

1 INTRODUCTION

Enzymes like alkaline phosphatase, protease, amylase and lipase convert organic matter to inorganic nutrients thereby playing an important role in the biogeochemical cycling of organic matter in ocean environment (Hoppe, 1983). For example, lipase activity provides information on the potential for lipid degradation which is essential for the continuous supply of low molecular substrates in marine water. Furthermore, nitrogen and phosphorus compounds are the most essential elements for biological growth in marine waters. Abundance of these essential nutrients depends on the hydrolysis of naturally occurring proteins and organic phosphates by microbial protease and phosphatase activities, respectively. Proteases can be critical to cell survival (Berges & Falkowski, 1998) and the occurrence of alkaline phosphatase is correlated with phosphorus deficiency in the environment (Li *et al.*, 1998; Thingstad *et al.*, 1998). Distribution of easily utilizable monosaccharides is controlled by amylase activity. Because of the importance of microbial enzymes in nature and fairly high abundance of attached bacteria, these enzymes were employed to measure the activity of surface attached bacteria.

The aims of the research presented in this chapter were to measure the enzyme activities of the bacteria attached to various surfaces, compare the activities of free and surface attached bacteria and to identify the effect of surfaces on the enzyme activity of the bacteria.

2 MATERIALS AND METHODS

2.1a *Enzyme Substrates*

I **p-nitrophenylphosphate** was used as a substrate to estimate the activity of Alkaline phosphatase. p-nitrophenylphosphate (0.002 to 0.01 M) was dissolved in 0.1 M tris buffer (Sigma chemical Co.) and was used as substrate to carry out various experiments on the activity of alkaline phosphatase enzyme.

II. **p-nitrophenyl acetate** was used as a substrate for lipase. Various concentrations (0.002 to 0.01 M) of p-nitrophenylacetate were prepared in tris buffer (0.1 M).

III. **Soble Starch** was used as a substrate to estimate the activity of the amylase enzyme. Starch solution (0.25 to 2%) was prepared in 0.1 M tris buffer.

IV. **Casein** Protease activity was determined using casein as a substrate. Casein solution was prepared by dissolving various amounts of casein (0.25 to 0.2%) in 0.1 M tris buffer.

2.1 b *Reagents*

I. **Tris Buffer** Tris buffer (0.1M) was prepared using Trizma base (Sigma chemicals) The required pH was adjusted with 1N HCl.

II **p-nitrophenol** p-nitrophenol was used to prepare a calibration curve to estimate alkaline phosphatase and lipase activity.

III. DNSA reagent 1g of 3, 5 dinitrosalicylic acid (DNSA) reagent was dissolved in 20 ml of 2N NaOH and 50 ml of H₂O. 30 g of Rochelle salt (sodium potassium tartarate) was added to this and the volume was made to 100 ml with distilled water.

IV. Maltose Amylase activity was calculated using maltose as standard. Various concentrations of maltose (10 μ g - 50 μ g) were used to prepare a standard curve.

V. Buffered reagent for amino acid analysis Buffered reagent was prepared by mixing 1.5 ml of o-phthaldehyde solution (10 mg ml⁻¹ in ethanol) with 90 ml of borate buffer (0.05 M) of pH 9.5. To this 1.5 ml of 2-mercaptoethanol was added.

VI. Tyrosine Tyrosine was used as a standard to calculate the activity of protease enzyme. Various concentrations (10 to 50 μ g) of tyrosine were used to prepare the standard curve.

2.2 *Bacterial cultures*

Bacterial cultures, *P. stutzeri*, *V. pelagius* (*S*₁), *F. multivorum* and *V. pelagius* (*S*₂) were used to study the effect of adhesion of these bacteria to metal and non-metal surfaces on the activity of four enzymes i.e. alkaline phosphatase, lipase, amylase and protease.

2.3 *Growth conditions*

P. stutzeri, *V. pelagius* (*S*₁), *F. multivorum* and

V. pelagius (S_2) were grown at room temperature using nutrient broth and BSS as described in earlier chapters.

2.4 *Preparation of cells attached to surfaces*

Bacterial cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were grown to stationary phase using nutrient broth prepared in BSS as described in earlier chapters. Cells of individual cultures were collected by centrifugation, washed with BSS and resuspended in BSS. Cell suspension of individual cultures was made to 0.2 OD. Cell suspension of each culture was dispensed in conical flasks. Replicate test panels of aluminium, stainless steel, copper and polystyrene were kept suspended in the flasks containing individual cell suspension of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2). Test panels were incubated for 60 min. After the incubation period, test panels were removed and rinsed with sterile BSS to remove unattached cells. Cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various test surfaces were then employed to assess the enzyme activities.

2.5 *Preparation of controls*

Panels of aluminium, stainless steel, polystyrene and copper were suspended in sterile BSS without bacterial cells for 60 min. At the end of the incubation period, panels of the four surfaces were removed, rinsed with sterile BSS and were used as controls. These panels were treated in a manner similar to that of the experimental panels.

2.6 Calibration of enzyme assays

V. pelagius (S_2) cells attached to stainless steel were utilized to optimise the experimental condition to estimate activities of the enzymes. All the experiments described below were run in triplicate.

A. Alkaline phosphatase

The alkaline phosphatase activity (APA) was estimated by measuring the release of p-nitrophenol from p-nitrophenylphosphate (Reichardt *et al.*, 1967). The method used here was the modification of the technique originally described by Morita & Howe (1957) and Tabatabai & Bremer (1969).

I. Effect of substrate concentration Various concentrations of p-nitrophenyl phosphate (0.002 to 0.01M), were used to check the optimum substrate concentration required for APA. The stainless steel panels with attached *V. pelagius* (S_2) cells were suspended individually into the test tubes containing various concentrations (0.002 to 0.001M) of p-nitrophenyl phosphate. The test tubes were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for a period of 5 h. At the end of the incubation period, panels were removed and the absorbance of the product (p-nitrophenol) in the test tubes was measured at 410 nm using Beckman DU-64 UV-Spectrophotometer. Absorbance readings were converted to concentration of p-nitrophenol using a calibration curve prepared by employing p-nitrophenol as a standard. APA was expressed as nmol p-nitrophenol formed per cell per hour i.e. $\text{nmol.p-np. h}^{-1}.\text{cm}^{-2}$.

I Effect of incubation period To assess the optimum incubation period for APA, the test tubes containing 0.006 M p-nitrophenyl phosphate were incubated with *V. pelagius* (S_2) cells attached to stainless steel panels for various time intervals (2 to 6 h). At the end of each incubation period, panels were removed and the APA was measured as described earlier.

III. Effect of pH The substrate i.e. p-nitrophenyl phosphate in tris buffer was adjusted to different pH (7.5 to 9.5) using 1 N HCl or 1N NaOH. *V. pelagius* (S_2) cells attached to stainless steel panels were introduced into different test tubes. The test tubes were incubated at room temperature for 5h and the enzyme assay was carried out as described earlier.

IV. Alkaline phosphatase activity of the bacteria attached to various surfaces Enzyme assay was carried out with the cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2), following their attachment to aluminium, polystyrene, stainless steel and copper surfaces. The panels with attached cells were suspended individually into the test tubes containing p-nitrophenyl phosphate (20 ml, 0.006 M). The test tubes were incubated at room temperature for a period of 5 h. At the end of this incubation period, panels were removed. The concentration of the product (p-nitrophenol) in the test tubes was measured as described earlier.

2.6 B *Lipase*

In order to estimate lipase activity of the bacteria, p-nitrophenyl acetate was used as a substrate.

I. Effect of substrate concentration : *V. pelagius* (S_2) cells attached to stainless steel panels were incubated in test tubes containing p-nitrophenyl acetate of various concentrations (0.002 to 0.01 M). The test tubes were incubated at room temperature for a period of 5h. At the end of 5h, panels were removed. The resulting p-nitrophenol from the test tubes was estimated spectrophotometrically at 410 nm. p-nitrophenol was used to prepare standard curve as described for the APA. Final results were expressed as nmol p-nitrophenol formed $\text{h}^{-1}.\text{cm}^{-2}$.

II. Effect of incubation period : The test tubes containing p-nitrophenyl acetate (0.006 M) and *V. pelagius* (S_2) cells attached to stainless steel panels were incubated for various periods of time (2 to 6h). At the end of the incubation period, lipase assay was carried out as described earlier.

III. Effect of pH : Effect of pH on lipase activity was assessed with p-nitrophenyl acetate (0.006 M) of different pH (6.0 to 8.0) using *V. pelagius* (S_2) cells attached to stainless steel panels. Lipase activity was measured at the end of incubation period (5h) as described above.

IV. Lipase activity of the different bacteria attached to various surfaces Following the attachment of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cells to the aluminium, polystyrene, stainless steel and copper surfaces, these panels with attached cells (in triplicate) were individually suspended in the test tubes containing p-nitrophenyl acetate (20 ml., 0.006 M). The test tubes were incubated at room temperature for a period of 5h. At the end of 5 h, panels were removed. The resulting p-nitrophenol concentration in each test tube was estimated spectrophotometrically at 410 nm. Final results were expressed as nmol-p-nitrophenol formed cell⁻¹ h⁻¹.

2.6 C. *Amylase*

I Effect of substrate concentration : The optimum concentration of substrate required for amylase activity was assessed by incubating *V. pelagius* (S_2) cells attached to stainless steel panels in test tubes containing various concentrations of starch (0.25 to 2.0%). At the end of the incubation period, the panels were removed. Substrate solution from the test tubes was filtered through 0.22 μ m millipore filters to remove any bacterial cells detached from the panels. Aliquots (1ml) of the filtrate were transferred to test tubes. Two ml of the freshly prepared DNSA reagent was added to each test tube and heated for 5 minutes in boiling water bath and then cooled in running tap water. After addition of 20ml of H₂O, the OD of the solution is determined spectrophotometrically at 510 nm using Beckman DU - 64 UV - spectrophotometer. The

amount of reducing sugars released by hydrolysis was quantified by using a calibration curve prepared with maltose as standard. The activity was expressed as $\mu\text{mol maltose h}^{-1}\text{.cm}^{-2}$.

II. Effect of incubation period : *V. pelagius* (S_2) cells attached to stainless steel panels were incubated individually in the test tubes containing 20 ml of starch (1.5%) solution. At different intervals of time (2 to 6h) the individual panels from the test tubes were removed, and the production of maltose in the test tubes was measured as above to estimate the amylase activity.

III. Effect of pH : The starch solution in tris buffer (20 ml., 1.5%) was adjusted to different pH (6.0 to 8.0) and transferred to test tubes. Stainless steel panels with attached cells of *V. pelagius* (S_2) were introduced individually in these test tubes. The test tubes were incubated at room temperature for 4 h. After 4 h, the panels were removed and amylase activity was estimated by analysing the maltose content in the test tubes, as described earlier.

IV. Amylase activity of different bacteria attached to various surfaces : Bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were used to study the effect of adhesion to the four surfaces on amylase activity. Test panels with the attached bacterial cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were transferred individually into test tubes containing 20 ml of starch (1.5%) solution and incubated for 4h at room temperature At the end of the incubation

period, the panels were removed and the amylase activity was estimated by analysing maltose content in the tubes as described earlier. Final results were expressed as $\mu\text{mol maltose cell}^{-1}\text{h}^{-1}$.

2.6 D *Protease*

Proteolytic activity was measured by estimating the degradation of casein at room temperature. The amino acids they produced formed were measured spectrofluorometrically using the method of Roth (1971).

I. Effect of substrate concentration : *V. pelagius* (S_2) cells attached to stainless steel panels were suspended in the test tubes containing various concentrations of casein (0.25 to 2%). At the end of the incubation period, panels were removed. Unused casein was precipitated with 5% trichloroacetic acid and centrifuged. An aliquot (100 μl) of supernatant was mixed with a buffered reagent (3 ml). Samples were measured on a spectrofluorometer set at 340 nm excitation and 455 nm emission. Actual concentration of the amino acids formed was quantified as tyrosine equivalent using the calibration curve prepared with tyrosine. The enzyme activity was expressed as nmol tyrosine released $\text{cm}^{-2}\text{h}^{-1}$.

II. Effect of incubation period : *V. pelagius* (S_2) cells attached to stainless steel panels were introduced in the test tubes containing casein solution (1%) and were incubated for various intervals of time (2 to 6h). At the end of each incubation period, panels were removed and the liberated amino acids due to

protease activity was estimated following the method described above.

III. Effect of pH : Casein solution (1%) was adjusted to various pH (7.5 to 9.5) using 1N NaOH or 1N HCl, and transferred into the test tubes. *V. pelagius* (S_2) cells attached to stainless steel panels were deployed individually in these test tubes and the test tubes were incubated for 4 h. At the end of this incubation period, protease activity was estimated as above.

IV. Protease activity of the cells of different bacteria attached to various surfaces : Bacteria *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were allowed to attach to aluminium, polystyrene, stainless steel and copper panels. Test panels with attached bacterial cells, were suspended individually in the test tubes containing 20 ml of 1% casein solution (pH 8.5) for 4 h. At the end of the incubation period (4 h), protease activity was estimated as described earlier. Final results were expressed as nmol tyrosine cell⁻¹h⁻¹.

2.7 Enzyme activity of free cells :

An aliquot of cell suspension (0.2 ml) of each of the four bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) prepared as above was dispensed individually into the test tubes (in triplicate) containing either p-nitrophenyl phosphate, p-nitrophenyl acetate, soluble starch or casein solution as substrates

for estimating the activities of APA, lipase, amylase and protease, respectively. The activity of the enzymes were estimated following the methods as above.

2.8 Measurement of bacterial cell numbers :

The number of bacterial cells attached to the test panels (in triplicate) was estimated following the crystal violet adhesion procedure described in earlier chapters (2B). The number of free (bacteria) cells in the suspension was estimated using the spread plate method and ZMA plates. The colony forming units (CFU) were counted after incubating the Petri dishes at room temperature ($28 \pm 2^\circ\text{C}$) for 24 h.

2.9 Statistical analysis :

The relationship between various enzyme activities was assessed using simple linear regression analysis with 123 LOTUS programme (Sokal & Rohlf, 1981).

3. RESULTS

Enzyme assays

The effect of substrate concentration, incubation period and pH, was studied to establish the optimum conditions for the measurement of APA, lipase, amylase and protease activities using *V. pelagius* (S_2) cells attached to stainless steel panels.

3.1 Alkaline Phosphatase

I Effect of substrate concentration : Alkaline phosphatase activity of *V. pelagius* (S_2) cells attached to stainless steel panels increased non-linearly as the substrate (p-nitrophenyl phosphate) concentration increased from 0.002 to 0.006 M (Fig. 4B.1A). Highest alkaline phosphatase activity for *V. pelagius* (S_2) cells attached to stainless steel panels was observed when the substrate concentration was 0.006 M. On further increase in substrate concentration, there was no increase in the alkaline phosphatase activity. Hence, 0.006M concentration of p-nitrophenyl phosphate was chosen for all further experiments carried out on this enzyme.

II. Effect of incubation period : As the incubation period increased from 2 to 5h, alkaline phosphatase activity of *V. pelagius* (S_2) cells attached to stainless steel panels also increased (Fig. 4B.2A). However, when the incubation period was increased from 5 to 6 h, alkaline phosphatase activity did not increase any further. Optimum activity of alkaline phosphatase was found at 5h.

III. Effect of pH : The amount of p-nitrophenol formed increased with increase in pH from 7.5 to 8.5. Highest production of p-nitrophenol was found at pH 8.5 (Fig 4B.3A). Alkaline phosphatase activity decreased on further increase in pH from 8.5 to 9 and 9.5.

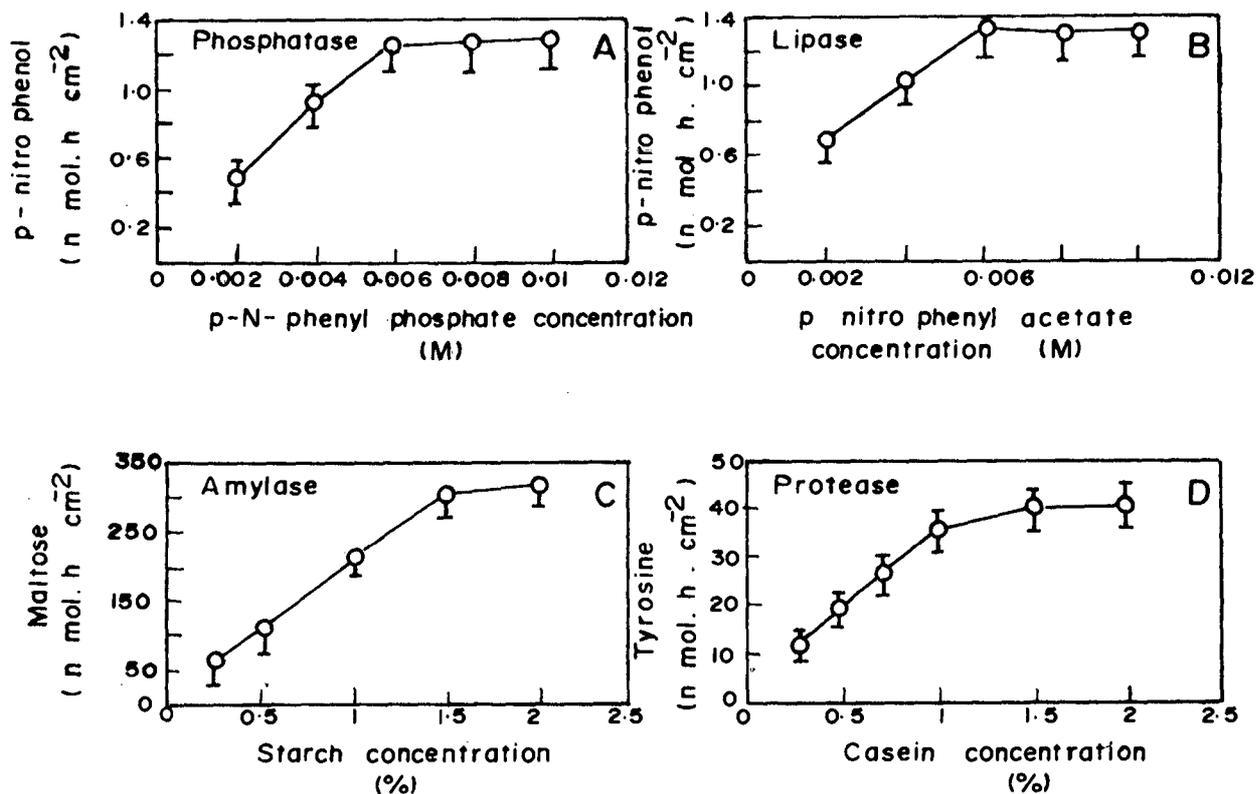
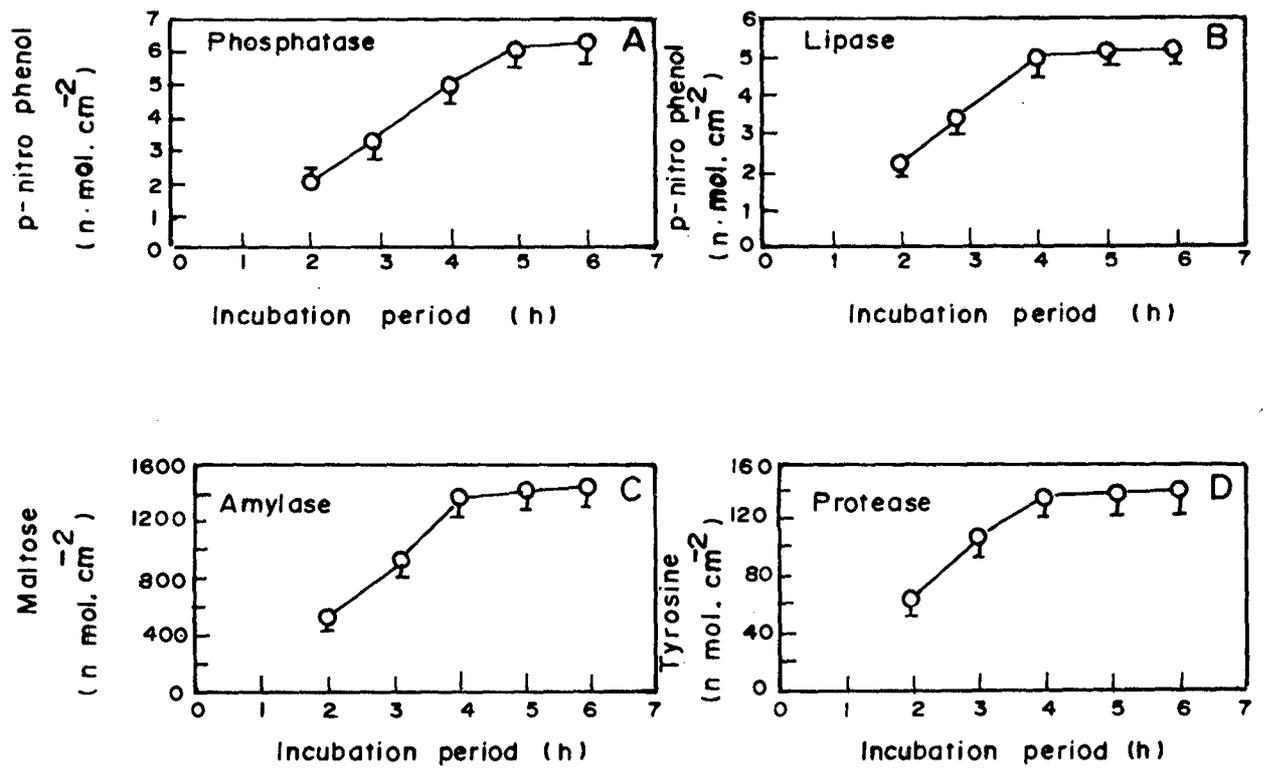


Fig. 4B-1. Effect of substrate concentration on A) Alkaline phosphatase B) Lipase, C) Amylase, D) Protease activity of *V. pelagius* (S_2) cells attached to stainless steel surface. Points are the means of measurements done in triplicate. Error bars represent standard deviation from the mean value.



ig. 4B-2. Effect of incubation period on A) Alkaline phosphatase B) Lipase, C) Amylase and D) Protease activity of *V.pelagius*(S₂) cells attached to stainless steel surface. Points are the means of measurements done in triplicate. Error bars represent standard deviation.

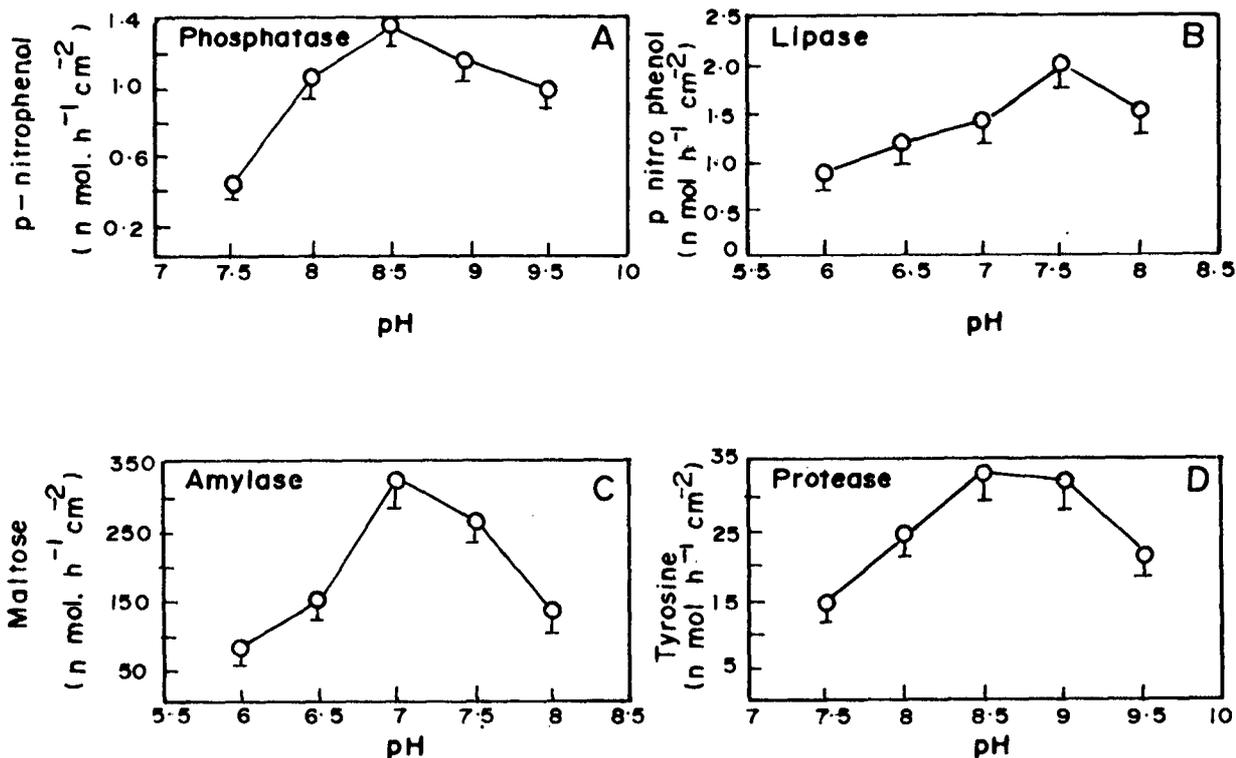


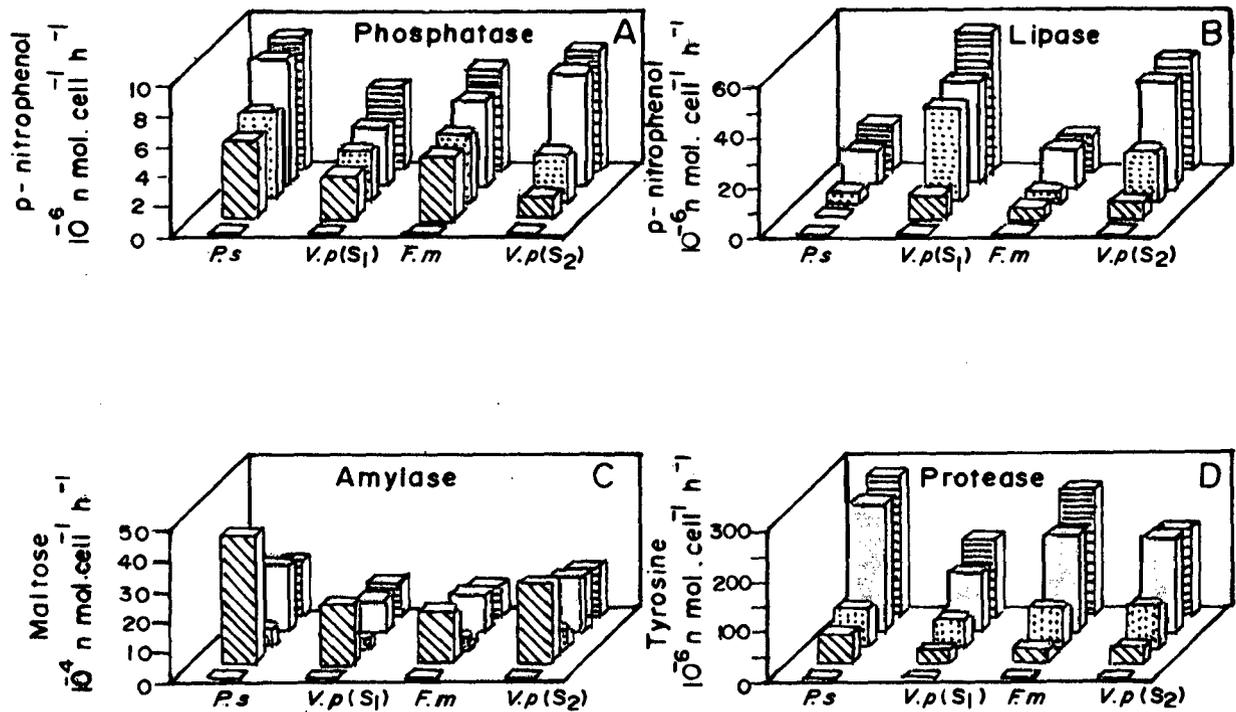
Fig. 4B-3. Effect of pH on A).Alkaline phosphatase , B)Lipase, C)Amylase and D) Protease activity of *V. pelagius* (S₂) cells attached to stainless steel surface . Points are the means of measurements done in triplicate. Error bars represent standard deviation .

IV. Alkaline Phosphatase activity of different bacteria attached to various surfaces : Bacterial cells of the four cultures *P. stutzeri*, *V. pelagius* (S_1) *F. multivorum* and *V. pelagius* (S_2) attached to polystyrene panels showed highest alkaline phosphatase activity. This was followed by the bacterial cells attached to stainless steel. Bacterial cells attached to aluminium and copper panels, however, exhibited relatively lower activity than the cells attached to stainless steel. Free living cells of all the cultures always showed lower activity than the cells attached to the surfaces (Fig 4B.4A).

Of the four cultures, *P. stutzeri* cells attached to all the four surfaces showed higher alkaline phosphatase activity. This was followed by cells of *F. multivorum*. However, cells of *V. pelagius* (S_1) attached to polystyrene and stainless steel showed slightly higher APA than the cells of *F. multivorum* attached to these surfaces (Fig 4B.4A). *V. pelagius* (S_1) cells attached to copper and aluminium surfaces showed higher activity than the cells of *V. pelagius* (S_2) attached to these surfaces. As compared to free cells, cells attached to the surfaces showed higher APA for all the experiments described here.

3.2 Lipase

I Effect of substrate concentration : An increase in lipase activity was observed as the substrate (p-nitrophenyl acetate) concentration increased from 0.002 to 0.006 M (Fig. 4B.1B). Highest



P.s = *P. stutzeri* ; *V.p(S₁)* = *V. pelagius* (*S₁*) ; *F.m* = *F. multivorum* ; *V.p(S₂)* = *V. pelagius* (*S₂*)

- g. 4B. 4. Effect of adhesion of *P. stutzeri*; *V. pelagius* (*S₁*), *F. multivorum* and *V. pelagius* (*S₂*) to copper , aluminium , stainless steel and polystyrene on the activity of A) Alkaline phosphatase B) Lipase C) Amylase and D) Protease. Enzyme activities of free living cells is also shown. Bar values represent the means of 3 determinations. In all cases the standard deviation is less than 10% value of the plotted mean bars.

activity for the *V. pelagius* (S_2) cells attached to stainless steel panels was recorded with the substrate concentration of 0.006 M. No further increase in the activity was observed, when the substrate concentration was further increased to 0.008 and 0.01 M. Therefore, lipase activity was estimated using 0.006 M p-nitrophenyl acetate.

II. Effect of incubation period : Lipase activity increased over an incubation period of 4h (2h - 4h). At longer incubation period (5h & 6h), there was no further increase in the lipase activity (Fig. 4B.2B). Since lipase activity of *V. pelagius* (S_2) cells attached to stainless steel was highest at 4h, further lipase assays were carried out using 4h incubation period.

III. Effect of pH : There was a considerable increase in lipase activity, as pH increased from 6 to 7.5 (Fig. 4B.3B). However, lipase activity decreased with increase in pH from 7.5 to 8. Highest activity was found at pH 7.5.

IV Lipase activity of bacteria attached to various surfaces : After the standardization, the enzyme assay was performed on free and attached bacterial cells. Bacteria *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to various surfaces were used to measure the lipase activity. As observed for the APA, lipase activity was highest with bacteria attached to polystyrene surface (Fig 4B.4B). This was followed by the cells attached to stainless steel. Cells attached to aluminium

panels exhibited relatively lower lipase activity than the cells attached to stainless steel and polystyrene surfaces, when the activity was expressed on per cell basis. Bacteria attached to copper panels showed lowest activity. In all the cases attached bacteria exhibited higher activity compared to their free living counterparts.

V. pelagius (S_1) cells attached to polystyrene, stainless steel and aluminium surfaces showed higher activity than the cells of other cultures attached to the same surfaces. As shown in Fig 4B.4B, not much difference was found in the magnitude of the enzyme activity of cells of *P. stutzeri* and *F. multivorum*. Cells of *V. pelagius* (S_2) attached to various surfaces showed higher activity than *P. stutzeri* and *F. multivorum*. *V. pelagius* (s_1) and *V. pelagius* (S_2) attached to stainless steel surfaces showed similar activity.

3.3. Amylase

I. Effect of substrate concentration : The amount of maltose formed increased non-linearly when starch concentration was increased from 0.25% to 1.5% (Fig. 4B.1C). No further increase in the amount of maltose produced was observed when the starch concentration was increased to 2%. Highest amount of maltose was produced, when starch was used at 1.5% concentration.

II Effect of incubation period : The amount of starch hydrolysed increased with the increasing incubation time, from 2 h to 4 h (Fig. 4B.2C). Highest production of maltose was noted

at 4 h, after which it remained constant over an incubation period of 5 h and 6 h.

III. Effect pH : As the pH increased from 5 to 7, the amount of maltose formed from the hydrolysis of starch also increased (Fig. 4B.3C). However, a considerable decrease in the amylase activity was found when the pH values increased further to 7.5 and above.

IV. Amylase activity of different bacterial cells attached to various surfaces : Standardization experiments suggest that the use of 1.5 % (w/v) substrate concentration, incubation time of 4h and pH 7.0 of the buffer gave highest amylase activity. These conditions were utilized to carry out the enzyme assay using bacterial cells attached to various surfaces. Bacterial cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to copper panels showed highest amylase activity (Fig 4B.4C). The results were reproducible when repeated for a number of times. Bacteria attached to other surfaces including polystyrene and stainless steel surfaces produced relatively lower activity, whereas, cells attached to aluminium surface showed lowest amylase activity (Fig 4B.4C).

Among the four bacterial cultures utilized, *P. stutzeri* cells attached to copper surface exhibited higher activity. (Fig 4B.4C). *V. pelagius* (S_2) cells attached to the four surfaces showed relatively higher activity than the cells of *V. pelagius* (S_1) and *F. multivorum*

attached to these surfaces. Amylase activity of the free cells of these bacteria was always lower than that recorded for their attached counterpart.

3.4 *Protease*

I Effect of substrate concentration : As the casein concentration increased from 0.25 to 1.0%, protease activity increased as was evident from the increasing amount of tyrosine released. Further increase in casein concentration (>1.5%) did not increase the amount of tyrosine liberated in the medium (Fig. 4B.1D). Highest amount of tyrosine was produced when casein concentration was 1.0%.

II. Effect of incubation period : Protease activity increased as the incubation period increased from 2h to 4h. Thereafter, it did not show much increase (Fig. 4B.2D). Highest protease activity of *V. pelagius* (S_2) cells attached to stainless steel was observed when the incubation period was 4 h.

III. Effect of pH : The rate of hydrolysis of casein increased linearly as the pH increased from 7.5 to 8.5 and the protease activity was highest when the pH was 8.5. A small decrease in the protease activity was found when pH of the reaction buffer was more than 8.5 (Fig. 4B.3D). The rate of casein hydrolysis was highest at pH 8.5.

IV. Protease activity of different bacterial cells attached to various surfaces : A substrate concentration of 1.5%, 4 h incubation period and pH 8.5 appear to be optimal for the protease activity of the attached bacteria. All four bacterial cultures, *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) showed highest protease activity when attached to polystyrene panels. Bacterial cells attached to stainless steel also showed relatively high activity. Aluminium panel attached bacteria showed lower activity than the cells attached to polystyrene and stainless steel. Copper attached bacteria gave lowest protease activity. (Fig 4B.4D). As observed for other enzymes, attached bacteria showed higher protease activity than the free living cells.

Protease activity also varied with respect to the bacterial cultures. *P. stutzeri* cells showed higher activity followed by *F. multivorum*. *Vibrio* cultures generally exhibited lowest activity when attached to metal and non-metal surfaces. Small increase in protease activity of *V. pelagius* (S_2) cells attached to aluminium panels as compared to other cultures attached to the same surface was recorded.

3.5 Statistical analysis

It was seen that alkaline phosphatase enzyme was significantly correlated ($p < 0.005$) with other enzymes i.e. lipase, amylase and protease (Table 4B.1). A significant relationship was also found between protease and lipase. However, no such relationship was observed between amylase and protease or lipase ($p > 0.05$).

Table 4B.1

Relationships between various enzymes as assessed by linear regression analysis (n = 20)

Enzymes	Alkaline phosphatase	Lipase	Amylase
Lipase	$r = 0.5182$ $p < 0.05$	X	X
Amylase	$r = 0.4835$ $p < 0.05$	$r = 0.0769$ $p > 0.05$	X
Protease	$r = 0.8956$ $p < 0.001$	$r = 0.5018$ $p < 0.05$	$r = 0.3062$ $p > 0.05$

4. DISCUSSION

ETS and respiratory rates of the free and attached cells of bacteria were compared in the earlier chapter. In the present chapter, extracellular enzymes activities (EEA) of free and attached cells of the four bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) was assessed using four enzymes i.e. alkaline phosphatase, lipase, amylase and protease. These enzymes appear to play an important role in the decomposition of organic matter in natural waters.

Higher enzyme activity was recorded with the cells attached to the surfaces than their free living counterparts. Similar results are reported by other workers. For examples Karner and Rassoulzadegan (1995) observed higher activities of α and β glucosidase attached to particles. Sinsabaugh *et al.*, (1991) found that the activities of phenol oxidase, peroxidase and phosphatase were largely particle bound. Similarly, Hoppe (1991) has reported that the attached bacteria were more active than the free bacteria and played an important role in the production of low molecular weight organic compounds. Furthermore, Jones & Lock (1989) have reported that the attached bacteria have higher protein degrading activities than free living bacteria and they suggested the possible stimulation of peptidase activity within the biofilm. Specific glutamate uptake rates of biofilm bacteria were 26 times greater than those of planktonic bacteria (Ladd *et al.*, 1979).

Several mechanisms have been put forward to explain the higher enzyme activity of the attached bacteria. The ability of the cell to utilize hydrolytic products decreases as the distance of the enzyme from the cell increases (Wetzel, 1991). Solid surfaces accumulate both the cells and the enzymes, thus avoiding the dilution of the enzymes as well as the hydrolytic products into the bulk water. Further, substrate molecules are also more easily available to the cells attached to the substratum than the free living cells.

Moreover, enzyme substratum complex may help in protection of enzyme active sites as well as their activities. These interactions avoid competitive interactions that would retard the functions of enzymes in biofilm (Wetzel, 1991). Stimulation of bacterial activity by colloids have been reported in a number of cases (Estermann & Mc Laren, 1959).

It is also suggested that the enzymes attached to the particles may be relatively more resistant to degradation and denaturation than are free enzymes in solution (Nannipieri *et al.*; 1982, Monsan & Combes, 1988; Arnosti, 1998). Such results are reported during studies of soil associated enzymes and investigations of the properties of immobilized enzymes (Ensminger & Giesecking, 1942).

Alternatively, the different conditions present at the solid/liquid interface than the bulk water may contribute towards the higher

enzyme activities of the attached bacteria. For example, the pH on the solid surface may be different due to the accumulation of ions or charged molecules on the surface. Further, adsorption of macro-molecules may result in conformational changes, thereby making them more accessible to the enzymes (Stotzky, 1972; Bright & Fletcher, 1983). Higher activities of enzymes of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the surfaces as compared to the free cells, might have been caused due to any of these factors or due to a combination of several factors.

Relationships between the activities of various enzymes were assessed using linear regression analysis. Alkaline phosphatase enzyme correlated significantly with other enzymes. Such correlations have been reported by Griffiths *et al.*, (1983). High correlation between protease activity and alkaline phosphatase activity ($r = 0.8956$, $n = 20$, $p < 0.001$) was observed in the present study. There were significant relationships of alkaline phosphatase with lipase ($r = 0.5182$, $n = 20$, $p < 0.05$) and amylase ($r = 0.4835$, $n = 20$, $p < 0.05$). In some other studies, the repression of bacterial alkaline phosphatase was observed with the addition of easily utilizable natural substrates for bacteria such as glucose, cellobiose and amino acids (Chrost *et al.*, 1989). Further, Chrost and Overbeck (1987), observed that alkaline phosphatase may be as important to carbon uptake as phosphorus uptake in aquatic bacteria, because some organic compounds must be dephosphorylated prior to assimilation of the organic moiety. The observed correlation of

alkaline phosphatase enzyme with protease, lipase and amylase in the present study may be due to the dephosphorylation of these organic substrates; which is necessary for their assimilation by the bacteria.

There were marked differences in the enzyme activity of the cells attached to different surfaces. Highest activity was observed by the cells attached to polystyrene surfaces, followed by stainless steel. Cells attached to aluminium surface showed lower activity. Lowest activity was shown by cells attached to the copper surfaces.

Copper surface is known to be toxic to the microorganisms (Rogers *et al.*, 1994) Alkaline phosphatase are shown to be sensitive to copper ions (Reuter, 1983). Inhibition of the activity of extracellular proteases by copper ions has also been described (Little *et al.*, 1979). Hence, it was not surprising that the bacterial cells attached to the copper surface showed minimum activity. However, cells attached to copper surface gave highest amylase activity. At present it is not known why cells attached to a toxic material like copper could exhibit higher activity for amylase. It is likely that the presence of copper ions may induce the production of certain enzymes like amylase.

The enzyme activity of the cells attached to aluminium was low as compared to that of the cells attached to polystyrene and stainless steel surfaces. This decrease could be due to large number of cells attached to aluminium panels, which may decrease the surface area available to the individual cells.

Cells of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) attached to the polystyrene surface showed highest activity. Polystyrene is a hydrophobic surface. Dalton *et al.*, (1994) have observed differences in both the distribution and morphologies of a marine bacterium on hydrophilic and hydrophobic substrata. Organisms at hydrophobic surfaces were characterized by the formation of tightly packed biofilm consisting of single and paired cells whereas those at hydrophilic surfaces exhibited sparse colonization on the surface and the formation of chains anchored at the surface only by the terminal cells. This suggests that all the cells present at the hydrophobic surfaces can express the enzyme activity, as the substratum area is available to all the cells, whereas, only the terminal cells present at the hydrophilic surfaces will be in a position to utilize the substratum surface area for the expression of enzyme activity. It is possible that similar formation of biofilm structures might have taken place on the surfaces during present study. In such a case, the observed increase in the enzyme activity of cells attached to the polystyrene surface in the present study may be due to the higher biofilm packing density. Moreover, polystyrene is a non-metal. Toxic effects of the metal ions on the enzyme activity are not present in case of polystyrene surface. Hence, it is possible that higher enzyme activity was observed with enzymes of *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) cells attached to the polystyrene surface. Furthermore, the differences in

the enzyme activity were also observed with respect to the different cultures. Dalton *et al.*, (1994) suggest that there are many types of biofilm structures, depending on both the organism and the nature of the substratum, which may influence the activity of the enzymes.

Such differences in biofilm structures on different substrata may provide an explanation for the differences in the enzyme activity, of bacteria attached to various surfaces observed in the present study.

CHAPTER 5

*Role of bacteria in
corrosion of metals*

1. INTRODUCTION

The phenomenon of microbiologically induced corrosion is widespread. The corrosion of metals is well documented in a number of studies (Thomas *et al.*, 1988; Little *et al.*, 1991; Bremer & Geesey, 1991; Gouda *et al.*, 1993; Deshmukh, 1996). Microorganisms may induce metal corrosion by enhancing electrochemical reaction that generally occurs on metal surfaces immersed in marine waters. Similarly, production of organic acids and exopolysaccharides by biofilm bacteria can also influence the metal corrosion (Thomas *et al.*, 1988; Dexter, 1993). Bacterial exopolymers have metal ion binding properties which can affect the metal surface. Cathodic depolarization by sulphate reducing bacteria may also lead to corrosion. The uneven thickness of biofilm may create oxygen concentration cells on the metal surface. This may lead to local damage such as pitting corrosion. Degradation of corrosion inhibitors and protective paints by bacteria also enhances corrosion of the metals.

Contrary to these findings a few workers have reported corrosion inhibition by bacteria (Videla & Guiamet, 1987; Ford *et al.*, 1988; Soracco *et al.*, 1989; Pedersen & Hermansson, 1989, 1991). Therefore the present chapter is designed to elucidate the effect of fouling bacteria on corrosion of various metals immersed in artificial sea water.

2 MATERIALS AND METHODS

2.1 *Test surfaces*

Commercial grade mild steel, aluminium, copper and stainless steel were chosen for the experimental studies. The composition of these metals is given in Table 5.1. These metals were chosen because of their extensive use in the marine environment. All the coupons were of the same size (3.5 x 1.5 cms) and were cleaned and sterilized as described earlier (Chapter 2B). Before sterilization the coupons, were mechanically polished on zero emery paper and finally degreased in acetone.

2.2 *Bacterial cultures and growth conditions*

The four isolates *Pseudomonas stutzeri*, *Vibrio pelagius* (S_1), *Flavobacterium multivorun*, and *Vibrio pelagius* (S_2) were used to study their effect on corrosion of mild steel, stainless steel, aluminium and copper.

The effect of bacteria on corrosion of metals was studied using nutrient broth (Table 2A.1) prepared in BSS (Table 2A.2).

2.3 *Effect of Nutrient enriched Medium*

Flasks containing sterilized nutrient broth prepared in BSS were inoculated with 18 h old bacterial cultures individually, previously grown in the same medium. For each culture, 11 flasks were used for each metal. In each flask, 3 metal coupons were kept suspended using nylon thread. Thus a total of 44 flasks were

Table 5.1

Composition of Mild steel, Copper, Aluminium and Stainless steel used for the corrosion experiments.

Surface	Composition
Mild steel	C-0.2%, Si-0.5%, Mn-0.26%, P-0.05%, S-0.04%, Fe-rest
Copper	Ni-0.05%, Cu-rest
Aluminium	Si-0.15%, Mn-0.8%, Al-rest
Stainless steel	C-0.02%, Cr-20%, Ni-25%, Cu-0.4%, Mo-4.5%, Si-0.4%, Mn-1.7%, Fe-rest

used for assessing the effect of each culture on 4 metals. Flasks were incubated at room temperature ($28 \pm 2^{\circ}\text{C}$) under static condition over a period of 13 d. Two flasks (i.e. 6 metal panels) of each culture was removed at d 1, 4, 7, 10 and 13 following inoculation. Of these, three panels were used to estimate weight loss and other three were used to quantify the viable cell count of attached bacteria using crystal violet method. Electrochemical measurements were carried out on the test coupons removed at d 13 of the immersion period only. At the time of sampling, pH of the growth medium was measured using a pH meter.

2.4 Effect of growth phase

A. Exponential phase : To assess the effect of growth phases, all the four bacterial cultures were inoculated in nutrient broth (Table 2A.1) prepared in BSS (Table 2A.2). After incubating for 18 hrs at room temperature on a shaker, these isolates were inoculated in fresh nutrient broth. Two flasks were used for each culture. The cells from one set of flasks were harvested in mid-exponential phase (i.e. after 7 h of incubation), washed thrice in BSS to remove any traces of nutrients and suspended in the starvation medium (i.e. BSS). Optical density of the bacterial suspension was adjusted to 0.2 at 400 nm which corresponds to 1.82×10^9 CFU. ml^{-1} . In each of the four bacterial culture suspension, fifteen coupons (preweighed) of each metal were kept suspended. Each flask had three coupons of any one metal. A total of 5 flasks were employed for each bacterial culture.

B Stationary phase : Cultures were grown as above and the stationary phase cultures were harvested at the end of 18 hrs. The cell pellets were washed thrice with BSS and suspended in starvation medium (i.e. BSS) to give an OD of 0.2. Cell suspension was dispensed in conical flasks. Three coupons (pre-weighed) each were deployed in each flask. A total of 5 flasks were employed for each culture. Flasks were removed at day 7 and 13 of the immersion period. Triplicate metal panels were used to estimate corrosion rate by weight loss and to estimate viable cell count of the attached bacteria. Electrochemical measurements were carried out on 13 d. Viable cell count of the attached bacterial cell was analyzed by the crystal violet method (Chapter 2B).

C. Controls : During the course of the above corrosion experiments, flasks containing only growth medium, test panels and no bacterial cultures were treated as control. The test coupons from the control flasks were treated in the same manner as that of the test coupons.

2.5 Measurements of corrosion rates :

A. Weight loss method : At each sampling, test and control panels were retrieved and the corrosion products were carefully removed using a sharp knife. The panels were cleaned with acid and washed with tap water followed by distilled water and dried in an oven. The panels were cooled in a desiccator and

weighed on a Mettler balance to estimate the weight loss. The corrosion rates were calculated using the formula

$$C = \frac{W_1 - W_2}{At}$$

Where C is the corrosion rate (mg. dm⁻²d⁻¹); W₁ and W₂ are the weights in milligrams of the metal panels before and after suspending into the media, respectively. A is the area of the panels (dm²) and t is the period of immersion in days.

B. Electrochemical measurements : Potentiodynamic polarization experiments were conducted using a computerised corrosion measurement system (Potentiostat/Galvanostat, Princeton Applied Research Corporation (PARC), Model EG & G 273 A). The scan rate was 0.5 MVS⁻¹. The working electrode had a fixed exposed area of 1.0 cm². The cathodic and anodic runs were conducted on the same specimens. Potentials were measured against a saturated calomel electrode (SCE), with an agar-agar/KCl bridge. Corrosion rate was calculated using SOFTCORR software.

2.6 X-Ray diffraction

Corrosion products were collected during the experiments carried out to assess the effect of growth phase of bacteria on the corrosion of metals and alloys. Corrosion products were thoroughly washed with the distilled water, filtered onto Whatman filter paper (41) and dried in an oven. Corrosion products were then analyzed by the XRD spectrometer (Philips model 1840). The scan rate

was 0.02. Analysis was carried out over a range of 5×10^3 CRS and $10 - 60^\circ 2\theta$. The corrosion products of aluminium, mild steel and copper coupons were subjected to XRD. However, stainless steel did not give enough corrosion products for XRD analysis and so XRD data for stainless steel could not be obtained.

3. RESULTS

3.1 *Corrosion of metals/alloys in nutrient rich medium*

A Corrosion rates : The corrosion of all the four metal surfaces was accelerated in the presence of bacteria compared to the control coupons. Although, corrosion rate, expressed as mdd ($\text{mg.dm}^{-2}.\text{d}^{-1}$) decreased during 13 d immersion period, in the presence of bacterial cells, it was relatively higher than that observed for the control coupons. Corrosion rate of mild steel, aluminium and copper surfaces was relatively very high at day 1 following incubation. It decreased substantially on day 4. For example, in the presence of *P. stutzeri* cells corrosion rate of mild steel was 31.0 ± 1.20 mdd on day 1 of the immersion period (Fig 5.1 A). It decreased to 16.25 ± 1.95 mdd on day 7. However, a small increase in corrosion rate was evident at day 10 and 13 of the immersion period especially for stainless steel.

Among the four surfaces studied, mild steel coupons showed highest corrosion, especially in the presence of *P. stutzeri* cells. This was followed by aluminium (Fig 5.1 B) and then the copper (Fig 5.1 C). Stainless steel was most resistant to corrosion in the

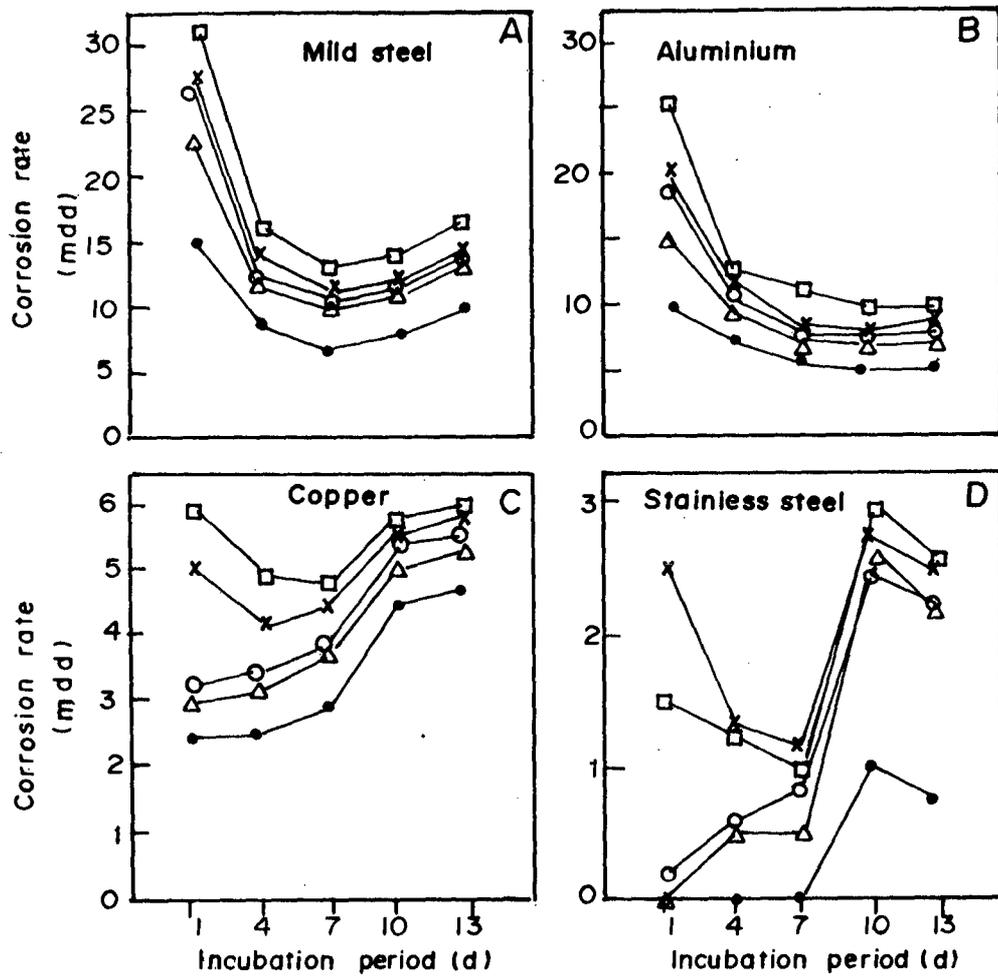


Fig. 5.1. Corrosion rates of A, Mild steel; B, Aluminium; C, Copper and D, stainless steel in the presence of *P. stutzeri* (—□—), *V. pelagius* (S₁) (—△—), *F. multivorum* (—x—) and *V. pelagius* (S₂) (—○—) in nutrient rich medium. Corrosion rates of control panels (—●—) are also shown.

presence of bacteria (Fig 5.1 D). A similar trend was shown by *V. pelagius* (S_1). However, the corrosion rate of all the metals i.e. mild steel, aluminium, copper and stainless steel in the presence of *V. pelagius* (S_1) cells was relatively much less than that observed in the presence of *P. stutzeri* cells. Corrosion rates of metals in the presence of *F. multivorum* and *V. pelagius* (S_2) were also lower than the corrosion rates observed in the presence of *P. stutzeri* cells. Nevertheless, the trend was similar i.e. mild steel had highest corrosion rates followed by aluminium. Copper had lower corrosion rates than the aluminium. Lowest corrosion was recorded with stainless steel panels or coupons which showed almost negligible corrosion rates.

B. Viable cell count of the attached bacteria : The number of bacterial cells attached to metal surfaces increased during the 13 d period of incubation (Fig 5.2). Bacterial cells attached to aluminium increased until day 7 followed by a small decrease on 10th day of the cultivation period. However, bacterial cell numbers increased once again on day 13. Highest cells of *P. stutzeri* were attached to aluminium coupons. *P. stutzeri* cells attached to other metals increased steadily over the 13 d period of cultivation (Fig 5.2 a).

The number of *P. stutzeri* cells attached to mild steel surfaces was relatively less than that attached to aluminium. While number of *P. stutzeri* cells attached to stainless steel were

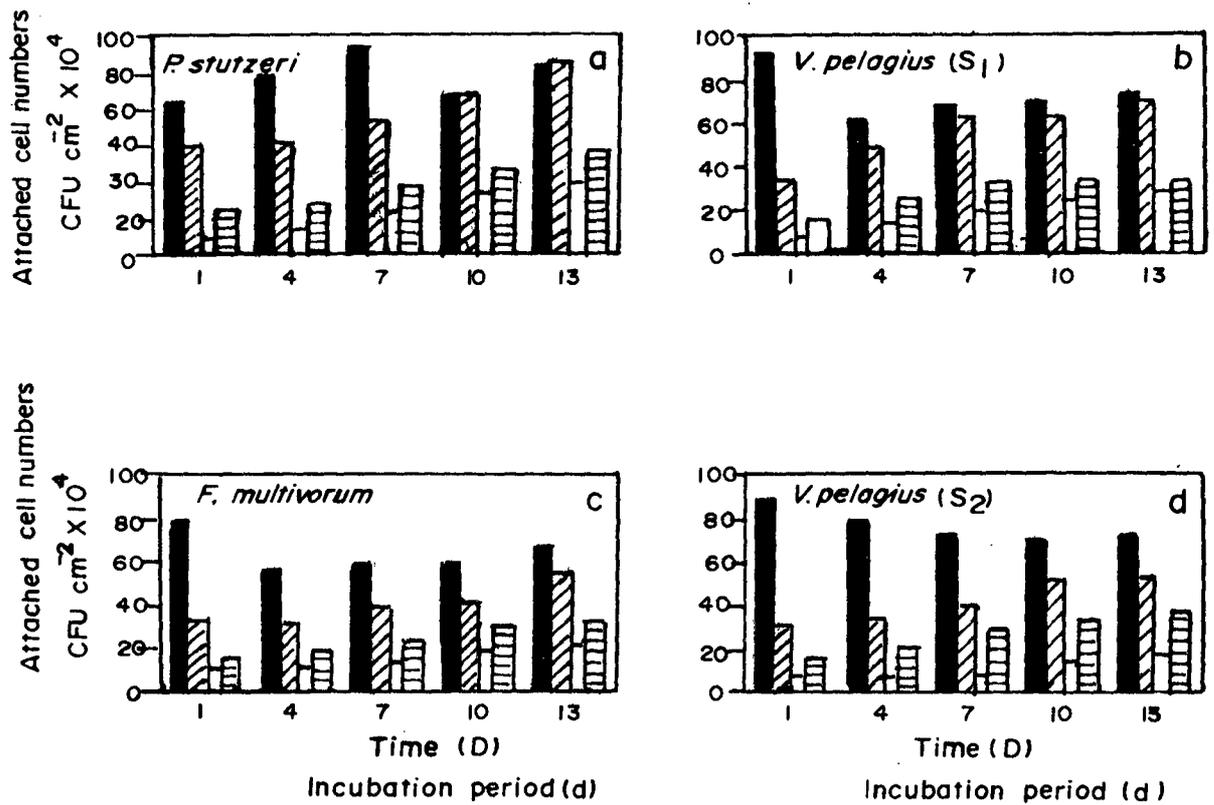


Fig. 5.2. The number of bacterial cells a, *P. stutzeri*; b, *V. pelagius* (S₁); c, *F. multivorum* and d, *V. pelagius* (S₂) attached to aluminium ■, mild steel ▨, copper □ and stainless steel ▤ in nutrient rich medium during the 13 days immersion period. Bar values are the means of 3 determinations.

higher than those attached to copper. Relatively less bacterial cells were attached to copper.

V. pelagius (S_1) cells attached to aluminium were highest at day 1 followed by a decrease on day 4 of the immersion period. Cells attached to aluminium increased again at d 7 and thereafter showed small changes during the period of incubation. The number of *V. pelagius* (S_1) cells attached to mild steel, copper, and stainless steel showed consistent increase over the 13 d period of incubation (Fig 5.2 b).

F. multivorum cells attached to all the surfaces increased with the incubation period (Fig 5.2 c). Cells attached to aluminium surface decreased at day 4 and increased again thereafter. In contrast to this *V. pelagius* (S_2) cells attached to surfaces other than aluminium generally increased over the period of incubation (Fig 5.2d).

C. pH measurement : Changes in pH of the culture broth was monitored while assessing the effect of the bacteria on the corrosion of metals (Fig 5.3). After an initial decrease in pH on day 1, pH generally increased over the 13 d period of cultivation. Culture medium containing stainless steel coupons did not show any appreciable change in pH. Almost similar trends were found for all four bacterial cultures (Fig 5.3 a, b, c, d).

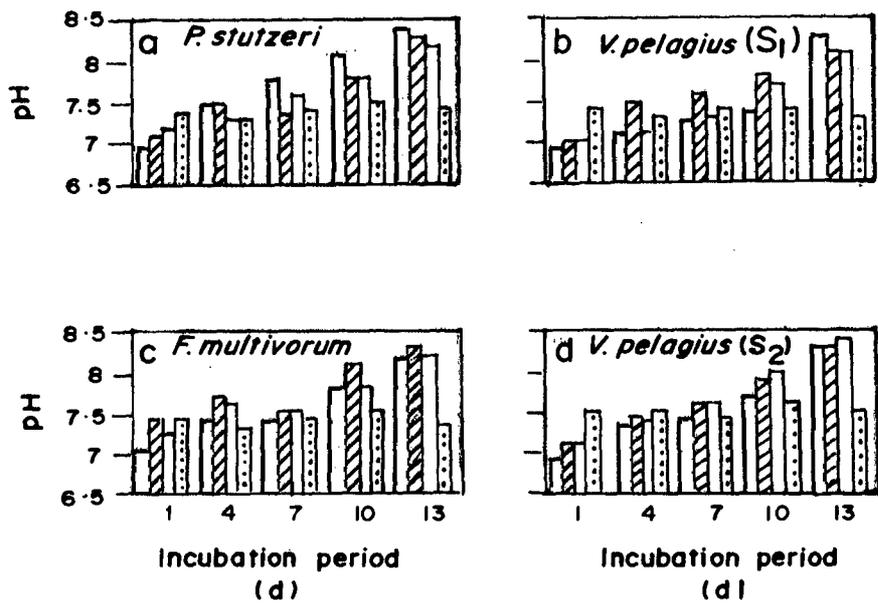


Fig.5.3. Changes in pH of the medium containing aluminium \square , mild steel ▨ , copper ▩ and stainless steel ▤ surfaces in the presence of a, *P. stutzeri*; b, *V. pelagius* (S₁); c, *F. multivorum* and d, *V. pelagius* (S₂).

3.2 Effect of growth phases

Exponential phase : The corrosion rate of all four metals was higher in the presence of exponential phase bacterial cells than the control panels. Highest corrosion rate of mild steel was recorded in the presence of *P. stutzeri* cells (Fig 5.4 Ia) followed by *F. multivorum*. Corrosion of mild steel was relatively low in the presence of *V. pelagius* cells. Lowest rate of corrosion was recorded in the presence of *V. pelagius* (S_1) cells. Corrosion of mild steel was higher at 13 d than at 7 d.

Corrosion rates of aluminium were higher than copper and stainless steel but lower than mild steel. Corrosion rates increased over the period of incubation (Fig 5.4 Ib). Corrosion of aluminium was highest in the presence of *P. stutzeri* cells followed by *F. multivorum*.

Copper corrosion generally increased as function of incubation period (Fig 5.4 Ic). Highest corrosion rate was recorded in presence of *F. multivorum* on the 13 d followed by *P. stutzeri*. Lowest rate of corrosion was observed in the presence of *V. pelagius* (S_1) cells.

Corrosion rates of stainless steel were lowest among the four metals. The rates were higher on d 13 than at d 7 (Fig 5.4 Id). On d 7 corrosion rates of stainless steel were negligible. However, at d 13 considerable corrosion of stainless steel in the presence of bacteria was observed.

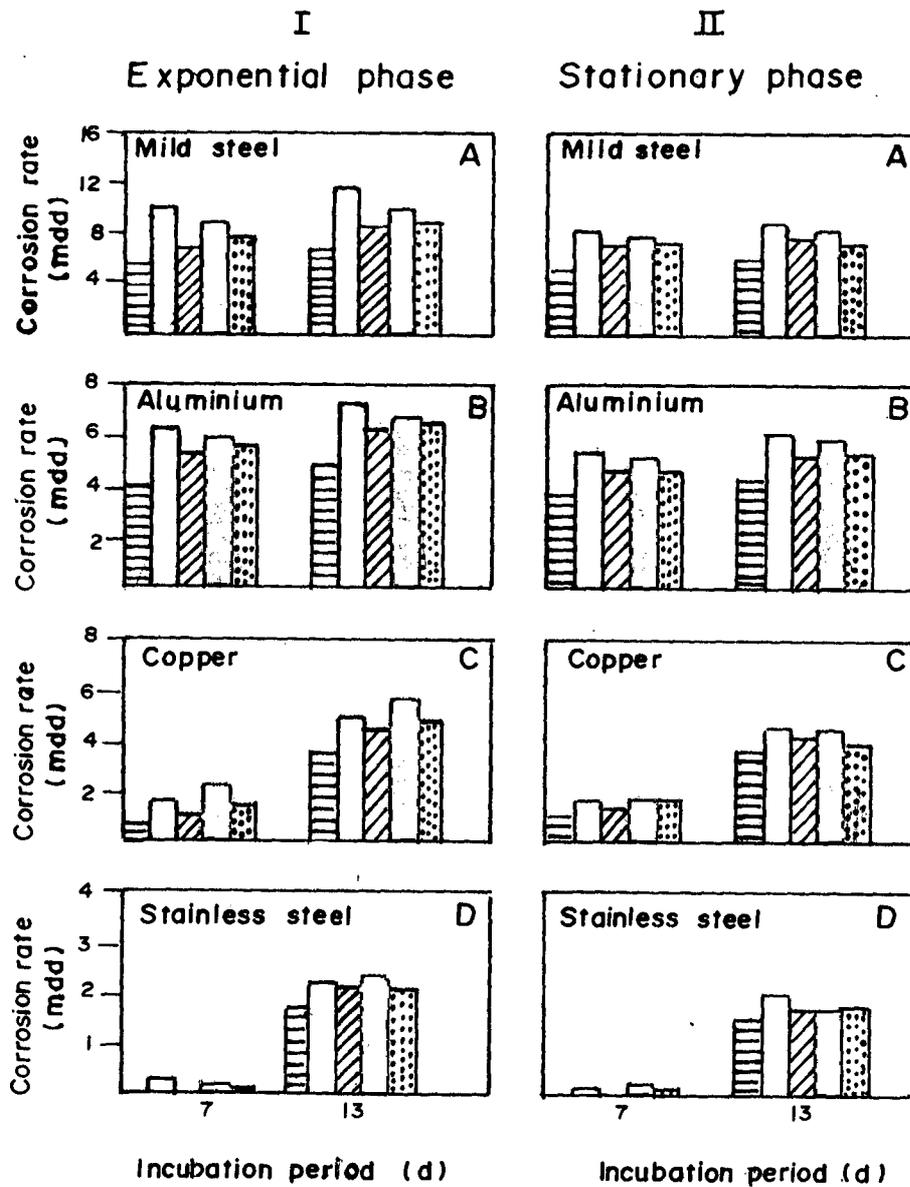


Fig. 5.4. Corrosion rates of A, mild steel; B, aluminium; C, copper and D, stainless steel in the presence of I, exponential and II, stationary phase cells of *P. stutzeri* (□); *V. pelagius* (S₁) (▨) *F. multivorum* (□) *V. pelagius* (S₂) (▨) in BSS. Corrosion rates of control panels in the absence of bacteria (▨) is also shown.

Stationary phase : Corrosion rates of all four metal surfaces were lower in the presence of stationary phase cells of *P. stutzeri* (Fig 5.4 II a) *V. pelagius* (S_1) (Fig 5.4 II b), *F. multivorum* (Fig 5.4 II c) and *V. pelagius* (S_2) (Fig 5.4 II d) than the exponential phase cells of the same cultures. However, the trend recorded for the corrosion rates of the four metals in the presence of exponential phase cells of the four bacterial cultures remained same even in the presence of stationary phase cells.

Number of attached cells : Growth phase of the culture influenced the attachment to surfaces. *P. stutzeri* cells of the stationary phase exhibited relatively higher attachment ability than that of the exponential phase cells (Fig 5.5 a). The number of cells attached to the metal surfaces were higher on 13th as compared to 7th day of incubation. Similar trend was found with the other three cultures i.e. *V. pelagius* (S_1) (Fig 5.5 b), *F. multivorum* (Fig 5.5 c) and *V. pelagius* (S_2) (Fig 5.5 d).

3.3 Potentiodynamic polarization

Electrochemical measurements by potentiodynamic polarization showed a trend similar to that obtained by weight loss method though the absolute values for both the methods varied. Bacteria accelerated the corrosion of metals (Tables 5.2, 5.3, 5.4). Mild steel panels exhibited highest rates of corrosion in the presence of *P. stutzeri* cells followed by *F. multivorum* cells. In the presence of *V. pelagius* cells the corrosion of mild steel was inhibited in

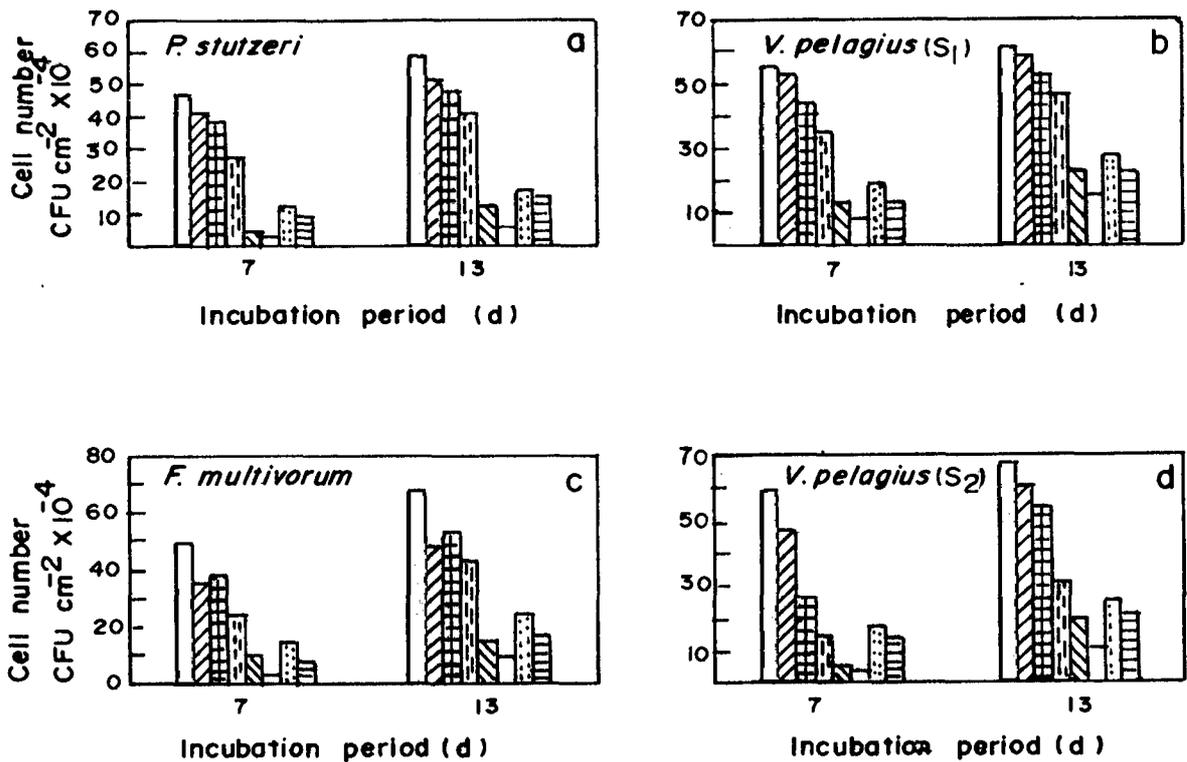


Fig. 5.5. Number of bacterial cells a, *P. stutzeri*; b, *V. pelagius* (S₁), c, *F. multivorum* and d, *V. pelagius* (S₂) attached to the four metal surfaces i.e. aluminium (□ stat and ▨ exp), mild steel (▩ stat and ▪ exp), copper (▧ stat and ◻ exp) and stainless steel (▤ stat and ▥ exp) during the two growth phases stationary (stat) and exponential (exp) in basal salt solution.

Table 5.2

E_{corr}, I_{corr}, corrosion rate and polarization resistance (R_p) of different metals in the presence of bacterial cultures grown in nutrient broth and BSS.

Bacterial cultures	Surface	E _{corr}	I _{corr}	Corr rate (mpy)	R _p
<i>P. stutzeri</i>	Mild steel	- 388	58.25	23.44	0.4587
	Aluminium	-	-	-	-
	Copper	134	9.05	8.34	8.4500
	Stainless steel	172	0.41	0.15	258.5700
<i>V. pelagius (S₁)</i>	Mild steel	- 421	10.21	4.71	2.4698
	Aluminium	187	4.92	2.13	1.6616
	Copper	44	2.43	2.24	21.2400
	Stainless steel	235	0.12	0.045	184.0800
<i>F. multivorum</i>	Mild steel	- 418	45.63	21.04	0.6652
	Aluminium	- 436	39.35	17.03	1.8380
	Copper	46	5.65	5.21	3.3563
	Stainless steel	124	1.29	0.4927	73.6800
<i>V. pelagius (S₂)</i>	Mild steel	- 513	20.95	9.66	1.5062
	Aluminium	- 334	18.25	7.9	3.7923
	Copper	164	4.00	3.69	8.1200
	Stainless steel	52	0.16	0.0614	358.9000
Control	Mild steel	- 470	23.22	10.71	0.8583
	Aluminium	- 405	36.11	15.63	1.7303
	Copper	30	1.21	1.11	4.9844
	Stainless steel	46	0.07	0.027	538.21

- Data not available

E_{corr} - Corrosion potential

mpy - milli inch per year

I_{corr} - corrosion current

Table 5.3

E_{corr}, I_{corr}, corrosion rate and polarization resistance (R_p) of different metals in the presence of exponential phase bacterial cells inoculated in Basal Salt Solution.

Bacterial culture	Surface	E _{corr}	I _{corr}	Corr rate (mpy)	R _p
<i>P. stutzeri</i>	Mild steel	- 600	60.83	29.86	0.6778
	Aluminium	- 425	4.42	1.92	16.6900
	Copper	- 70	16.92	15.60	1.4155
	Stainless steel	60	2.87	1.10	7.6400
<i>V. pelagius (S₁)</i>	Mild steel	- 510	107.55	52.80	0.3873
	Aluminium	- 418	11.07	4.79	5.9900
	Copper	- 81	25.20	23.23	0.9287
	Stainless steel	- 14	1.43	0.546	13.3200
<i>F. multivorum</i>	Mild steel	- 602	27.26	13.38	0.9697
	Aluminium	- 538	16.90	7.31	5.4700
	Copper	- 85	37.55	34.62	1.1302
	Stainless steel	- 17	4.34	1.65	4.5576
<i>V. pelagius (S₂)</i>	Mild steel	- 609	22.86	11.22	1.5101
	Aluminium	-	-	-	-
	Copper	- 75	8.85	8.16	2.0915
	Stainless steel	-	-	-	-
Control	Mild steel	- 555	11.45	5.65	1.1241
	Aluminium	- 532	9.55	4.13	11.7800
	Copper	1.34	2.11	1.94	28.71
	Stainless steel	- 54	0.08	0.0292	283.35

- Data not available

E_{corr} - Corrosion potential

mpy - milli inch per year

I_{corr} - Corrosion current

Table 5.4

E_{corr}, I_{corr}, corrosion rate and polarization resistance (R_p) of different metals in the presence of stationary phase bacterial cells inoculated in Basal Salt Solution

Bacterial culture	Surface	E _{corr}	I _{corr}	Corr rate (mpy)	R _p
<i>P. stutzeri</i>	Mild steel	- 343	43.25	19.95	0.3607
	Aluminium	- 455	10.81	4.21	7.7800
	Copper	- 56	7.40	6.87	1.8963
	Stainless steel	-	-	-	-
<i>V. pelagius (S₁)</i>	Mild steel	- 445	16.28	7.51	0.9068
	Aluminium	- 467	16.76	7.26	5.6900
	Copper	- 61	5.41	5.02	2.4398
	Stainless steel	- 15	4.08	1.56	5.3200
<i>F. multivorum</i>	Mild steel	- 528	30.16	14.81	0.7271
	Aluminium	- 517	138.48	59.94	2.6145
	Copper	- 46	19.54	18.01	1.1481
	Stainless steel	- 12	1.49	0.5627	14.5700
<i>V. pelagius (S₂)</i>	Mild steel	- 570	7.83	3.84	2.1685
	Aluminium	- 504	27.43	11.87	3.0880
	Copper	- 43	9.23	8.5	2.4843
	Stainless steel	- 43	2.90	1.09	7.4900
Control	Mild steel	- 295	6.07	2.98	2.1633
	Aluminium	- 382	8.68	3.75	11.7800
	Copper	- 74	5.10	4.7900	2.1500
	Stainless steel	- 45	1.02	0.389	7.2000

- Data not available

E_{corr} - Corrosion potential

mpy - milli inch per year

I_{corr} - Corrosion current

nutrient rich medium. Corrosion rates of aluminium were lower than the mild steel. There was a little increase in the corrosion rate of aluminium in the presence of *F. multivorum* cells, whereas, corrosion of aluminium was inhibited in the presence of *V. pelagius* cells. Corrosion of copper was enhanced in the presence of all cultures. Highest rate of corrosion for copper was recorded for *P. stutzeri* cells followed by *F. multivorum*. Lowest corrosion rate was observed in the presence of *V. pelagius* (S_1) cells.

Growth phases of bacteria also seemed to influence the corrosion of metals. Though corrosion of metals was enhanced in the presence of bacterial cells, aluminium corrosion was found to be inhibited by *P. stutzeri* cells. Highest corrosion rates were recorded for mild steel. In most of the cases higher corrosion rates were observed for the exponential cells.

In the presence of stationary phase cells the corrosion rates of all the metals were higher than the control. Highest corrosion rates for mild steel were observed in the presence of *P. stutzeri* and then *F. multivorum* cells, whereas, the corrosion rates for aluminium were high in the presence of *F. multivorum* and *V. pelagius* (S_2) cells. Highest corrosion of copper was recorded in the presence of *F. multivorum* cells.

Corrosion current (I_{corr}) and consequently the corrosion rate was enhanced in the presence of bacteria (Table 5.2, 5.3 & 5.4). Corrosion potential (E_{corr}) values shifted towards more negative

values in the presence of bacteria. Polarization resistance (R_p) data was also consistent with these results. In general, R_p decreased in the presence of bacteria. Presence of bacteria resulted in the stimulation of anodic reaction whereas cathodic reaction was not much affected (Fig 5.6, 5.7, 5.8).

3.4 XRD analysis

Microbiologically induced corrosion can be explained by the nature of the corrosion product. The corrosion products formed were different in the presence of bacteria (Table 5.5). Furthermore, corrosion products were slightly different in the presence of exponential and stationary phase cultures, at least for the mild steel and copper. In the absence of bacteria, the only corrosion product detected for mild steel was FeO(OH) . However, Fe_2O_3 was major corrosion product when exponential phase bacterial cells were employed. While in the stationary phase, cells of all the bacteria produced Fe_2O_3 and FeO(OH) .

In the absence of bacteria, corrosion of copper in the BSS produced different corrosion products i.e. $\text{Cu}_2\text{Cl(OH)}_2$, Cu_2S , CuS , $\text{Cu(OH}_2\text{Cl)}_2 \cdot 2\text{H}_2\text{O}$, $6\text{CuOCu}_2\text{O}$. However, in the presence of bacteria, $\text{Cu}_2\text{Cl(OH)}_2$ and Cu_2S were the main products, though some minute quantities of other products were also detected. Cu_2S remained the major product at least in the stationary phase of bacterial cultures.

In the case of aluminium, Al(OH)_3 was the only product detected both in the presence and absence of bacteria. All the four

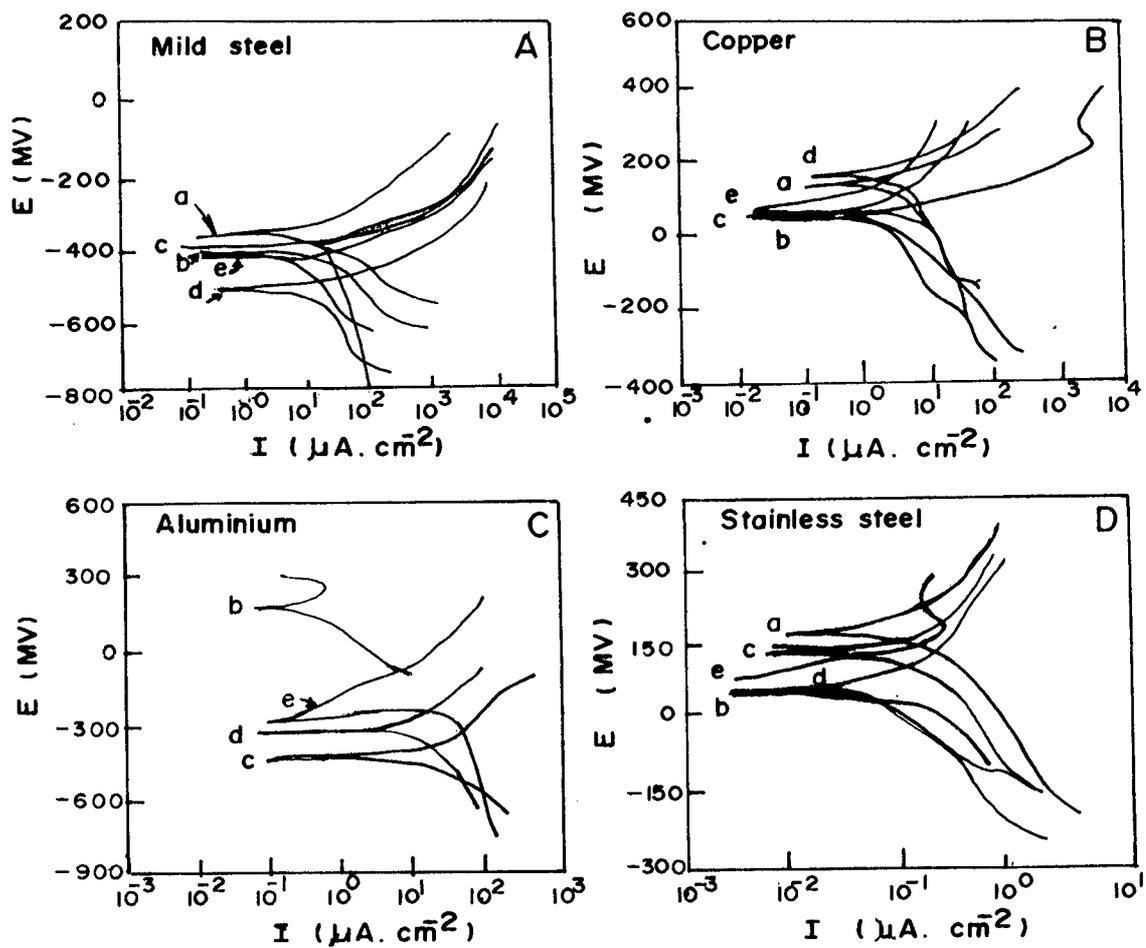


Fig. 5.6. Polarization curves for A, Mild steel; B, Copper; C, Aluminium and D, Stainless steel after 13 d exposure to nutrient rich medium inoculated with a) *P. stutzeri*; b) *V. pelagius* (S_1); c) *F. multivorum* and d) *V. pelagius* (S_2); uninoculated control e) is also shown.

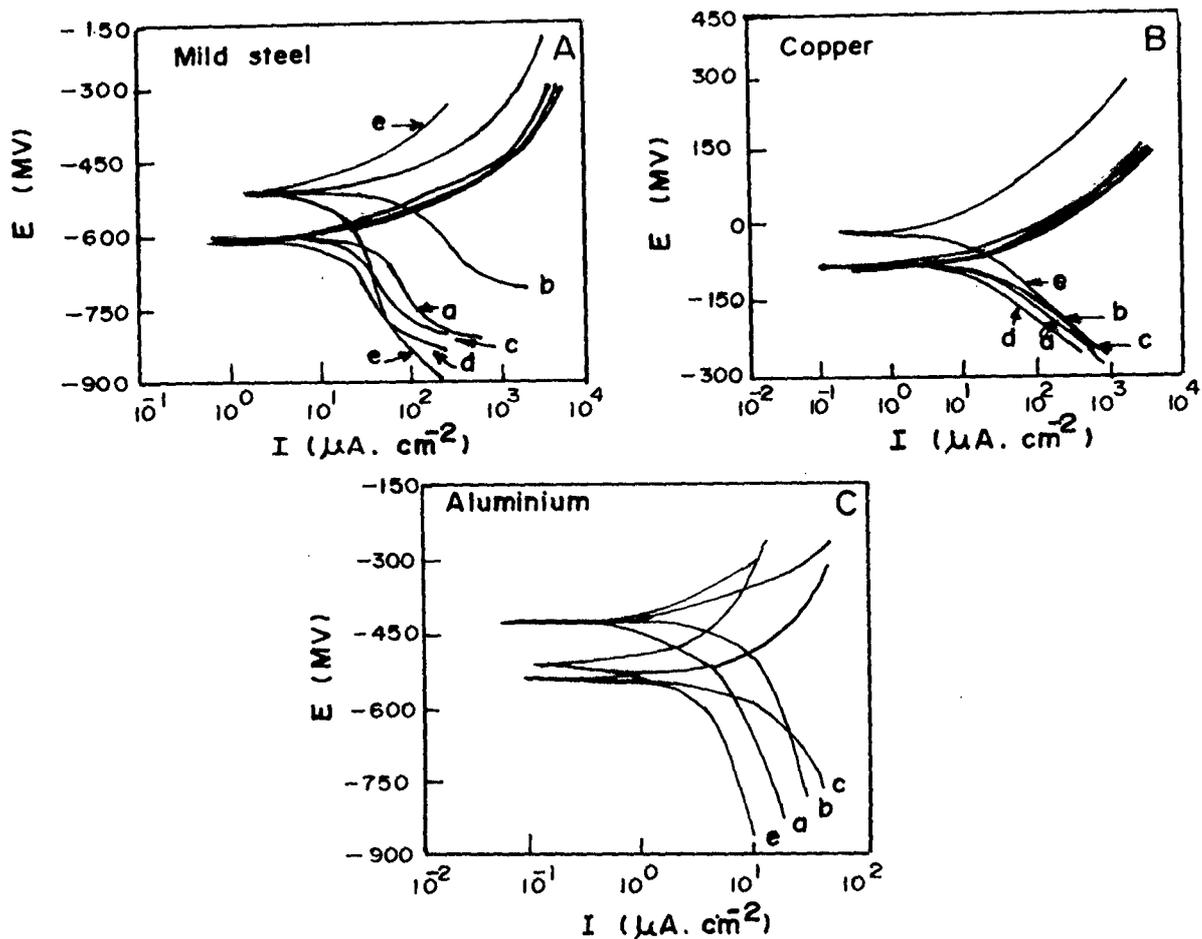
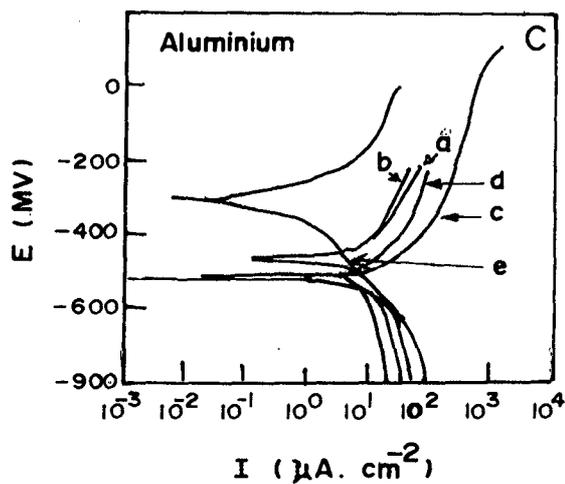
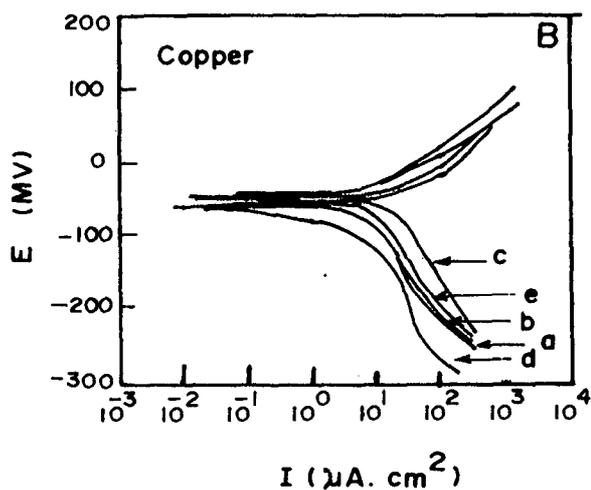
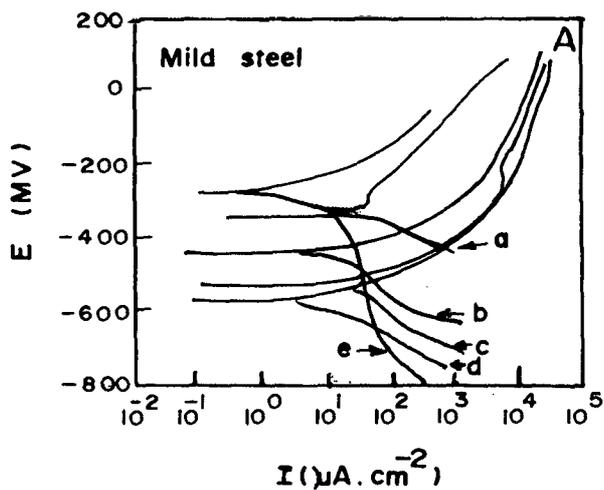


Fig. 5.7. Polarization curves for A) Mild steel, B) Copper, C) Aluminium after 13 days exposure to basal salt solution inoculated with exponential phase bacterial cultures a) *P. stutzeri*, b) *V. pelagius* (S_1), c) *F. multivorum*, d) *V. pelagius* (S_2) and e) uninoculated control



5.8. Polarization curves for A, Mild steel; B, Copper; C, Aluminium after 13 days exposure to basal salt solution inoculated with stationary phase bacterial cultures of a) *P. stutzeri*, b) *V. pelagius* (S₁); c) *F. multivorum*, d) *V. pelagius* (S₂) and e) uninoculated control.

Table 5.5

Composition of corrosion products formed during the corrosion of metal coupons in the presence of exponential and stationary phase bacterial cultures.

Surfaces				
Bacterial cultures	Growth phase	Mild steel	Copper	Aluminium
Control	-	FeO(OH)	Cu ₂ Cl(OH) ₂ Cu ₂ S Cu ₂ S, Cu(OH ₂ Cl) 2H ₂ O 6CuOCu ₂ O	Al(OH) ₃
<i>P. stutzeri</i>	Exponential	Fe ₂ O ₃ FeO(OH)	Cu ₂ Cl(OH) ₂ Cu ₂ S	Al(OH) ₃
	Stationary	FeO(OH)	Cu ₂ S Cu ₂ Cl(OH) ₂	Al(OH) ₃
<i>V. pelagius</i> (a)	Exponential	Fe ₂ O ₃ FeO(OH)	Cu ₂ Cl(OH) ₂ Cu ₂ S	Al(OH) ₃
	Stationary	FeO(OH)	Cu ₂ S, Cu ₂ Cl(OH) ₂ 6CuO Cu ₂ O	Al(OH) ₃
<i>F. multivorum</i>	Exponential	Fe ₂ O ₃ FeO(OH)	Cu ₂ S Cu ₂ Cl(OH) ₂	Al(OH) ₃
	Stationary	FeO(OH)	Cu ₂ S Cu ₂ Cl(OH) ₂ CuS	Al(OH) ₃
<i>V. pelagius</i> (b)	Exponential	Fe ₂ O ₃ FeO(OH)	Cu ₂ S, Cu ₂ Cl(OH) ₂ 6CuOCu ₂ O	Al(OH) ₃
	Stationary	Fe ₂ O ₃	Cu ₂ S, Cu ₂ Cl(OH) ₂	Al(OH) ₃

bacterial cultures gave the same product. The growth phase of the culture did not influence the composition of the corrosion products of aluminium.

4. DISCUSSION

Microbiologically induced corrosion has received growing attention in the recent years. Involvement of microorganisms in the corrosion process has been successfully demonstrated by a number of workers (Thomas *et al.*, 1988; Bremer & Geesey, 1991; Little *et al.*, 1991; Deshmukh *et al.*, 1992; Gouda *et al.*, 1993; Maruthamuthu *et al.*, 1993). Most of the research on MIC is concentrated on sulphate reducing bacteria (SRB) (Thomas *et al.*, 1988; Deshmukh *et al.*, 1992; Gouda *et al.*, 1993; Lee *et al.*, 1993). Although sulphate reducing bacteria play an important role in the corrosion of metals, especially in the anaerobic environment, the role of other aerobic bacteria in MIC cannot be neglected. Therefore, in the present investigation, marine bacteria isolated from the biofilms formed on aluminium panels were used to study their effect on corrosion. These bacteria appeared to enhance the corrosion of at least three metals, i.e. mild steel, aluminium and copper. Stainless steel was not much effected. Similar results were reported by other workers who suggested that the presence of biofilms increase corrosion of metals in seawater (Compton, 1970; Lagutine *et al.*, 1970; Deshmukh, 1996). Similarly, in most other investigations bacteria have been reported to accelerate corrosion of

metals (Scotlow *et al.*, 1985; Nivens *et al.*, 1986; Obueckwe *et al.*, 1987; Ford *et al.*, 1987; Dexter & Goa 1988; Mollica *et al.*, 1989). Corrosion rates expressed as mdd decreased during the 13 d incubation period. This implies that the early stages are decisive for the corrosion rates. Nevertheless, the corrosion rates were higher in the presence of bacteria than that of the control coupons.

The accelerated corrosion in the presence of bacteria, as found in the present study, could be due to the enhanced oxygen reduction by bacteria (Little *et al.*, 1991). *P. stutzeri* and *F. multivorum* were found to be more aggressive than *V. pelagius* (S_1 and S_2) cultures. The possible mechanism could be the formation of differential aeration cell. The oxygen concentration under any microbial colony becomes depleted due to uptake of oxygen by the microbial colony. This poorly aerated area on the surface becomes the anode of the cell. The better aerated regions away from the deposits provide the balancing cathodic reaction. This type of mechanism is reported for bacterial species of *Pseudomonas* and *Flavobacterium* (Tiller, 1982). Pitting type of corrosion which is characteristic of this mechanism was observed on metal coupons in the presence of these bacteria in the present study. The enhanced corrosion in the presence of bacteria, as observed for *P. stutzeri*, *V. pelagius* (S_1) *F. multivorum*, and *V. pelagius* (S_2) can also be attributed to the production of corrosive metabolites. In some other studies, exopolymers of bacteria have been shown to enhance corrosion (Nivens *et al.*,

1986). Increased corrosion rate, observed in the present study, in the presence of bacteria suggests the role of exopolysaccharides in the corrosion process. Furthermore, different types of exopolyaccharides may be produced during different growth phases. Such results are reported by Christensen *et al.*, (1985) and Wrangstadh *et al.*, (1990) who observed that the composition of EPS of a marine *Pseudomonas* sp. varies during different growth phases of the bacteria. The observed increase in the corrosion rate by the bacteria, may be due to the effect of EPS (Chapter 6) produced by these cells.

The number of cells attached to the metal surface did not appear to have direct correlation with corrosion rates. Number of cells attached to the metal coupons showed an increasing trend during the 13 d incubation period, though the corrosion rate showed a decreasing trend. The number of cells attached to aluminium were more, though mild steel appeared to show maximum corrosion. Further, though copper was most resistant to bacterial attachment, the corrosion of copper was higher than that of stainless steel. More cells were present on stainless steel than on copper. Similarly, number of stationary phase cells present on the metal coupons, was more than the exponential phase cells, though corrosion rates was higher with exponential phase bacteria.

Changes in pH are very important for metallic corrosion. However, higher pH values were found during this experiment. The

hydroxides formed during the corrosion process may render the medium alkaline. Furthermore, pH may be acidic beneath the microbial colony, though the medium becomes alkaline (Pope *et al.*, 1988). Kasahara and Kajiyama (1988), while studying the role of sulphate reducing bacteria in corrosion of buried pipes (SRB) in soil found a similar increase in pH. However in another study, Pedersen and Hermansson (1989) did not find any correlation between pH and corrosion.

Further, electrochemical potentiodynamic polarisation studies also confirm that the presence of bacteria enhanced corrosion of metals. Anodic reaction was generally accelerated in the presence of bacteria. It is likely that the anodic process controlled overall corrosion rate. This agrees well with earlier studies. For example, Westlake *et al.* (1988) found that *Pseudomonas* sp- 200, present in fluids from oil fields activated the corrosion process by anodic depolarisation. The cathodic reaction was not affected by this organism. Gouda *et al.* (1993) have also reported that, in their experiment, microbially induced corrosion was controlled essentially by anodic process.

In the present experiments, copper showed passive regions at higher corrosion currents, indicating the formation of protective layers on the copper surface. Anodic polarisation curves obtained for mild steel with stationary phase cells of *P. stutzeri* and *F. multivorum* also showed the presence of a passive region, which thereafter diminished, with increasing I_{corr} .

Presence of large quantities of Fe_2O_3 in the medium suggests dissolution of Fe metal in the solution. This Fe_2O_3 film must have been removed from the surface of the metal by the bacteria. It is possible that during the metabolic activities of bacteria (especially in the exponential phase) or the chemical breakdown of Fe_2O_3 layer by bacteria i.e. during the reduction of ferric iron, the protective layer of Fe_2O_3 is mechanically removed from the metal surface into the medium. This accounts for higher weight loss of the metal in the presence of bacteria. Such removal of the protective gamma Fe_2O_3 film by using the ferric oxide as a terminal electron acceptor during the growth of *Pseudomonas* sp-200 has been shown by Westlake *et al.* (1988). Removal of this Fe_2O_3 film by *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum*, and *V. pelagius* (S_2) increased the anodic depolarisation and subsequently the corrosion rate in the present study. The other corrosion product of mild steel FeO(OH) is commonly found on the tubercles formed by iron oxidising bacteria on water distribution pipelines (Ainsworth *et al.*, 1978; Tiller, 1982). Stainless steel is known to passivate readily when exposed to seawater due to the formation of a protective corrosion product film. Corrosion product of stainless steel could not be subjected to XRD, as very minute quantities of the corrosion products were formed. It is suggested that the protective films are formed at low ferrous iron concentrations while at concentrations sufficiently high to precipitate all the bacterially produced sulphide as iron sulphide, no protective films are formed and the rate of corrosion increases

markedly (King *et al.*, 1973, Tiller, 1982).

Formation of passive films on steel embedded in the concrete has been reported by Maruthamuthu *et al.*, (1997). They suggest that there may be enhancement of passivation due to the production of organic complexes by bacterial metabolism. Further, in another study, it is reported that the excess carbon and phosphorus which bacteria consume, is released in the form of glucose-6-phosphate. Since glucose anions are negatively charged, they are attracted towards the steel and form a protective layer on the surface (Maloney *et al.*, 1990).

In the absence of bacteria copper formed many corrosion products like $\text{Cu}_2\text{Cl}(\text{OH})_2$, CuS , Cu_2S , $\text{Cu}(\text{OH}_2\text{Cl})_2$, H_2O , 6CuO , Cu_2O . Efirid (1976) reported that cuprous oxide layer is toxic to microorganisms. He attributed the fouling resistance of copper alloys in seawater to this toxic layer. However, in the present experiment, sulphides and hydroxychlorides were the major products formed in the presence of bacteria. Sulphides are known to be corrosive. The hydroxychlorides formed are not toxic to the organisms. But this layer can be easily removed. The fouling, therefore, is not very adherent (Walch, 1986). This can explain the low number of cells on copper coupons, despite its corrosion.

Changes in the metal surface exposed to bacteria have been reported by earlier workers. For example, Gomes de Saravia *et al.* (1990) found that uneven distribution of bacteria and their EPS,

CHAPTER 6

*Studies on bacterial extracellular
polysaccharide production*

1. INTRODUCTION

Surfaces immersed in water are colonized by bacteria. While colonizing surfaces, microorganisms may produce exopolysaccharides. Definite evidence for the involvement of exopolysaccharides in bacterial adhesion was provided by Fletcher & Floodgate (1973). Exopolysaccharides appear to play important role in adsorption of nutrients, protection from desiccation, motility and maintenance of biofilm. These compounds may also inhibit or accelerate metal corrosion and induce the settlement of invertebrate larvae.

Growth condition and nutrient status and growth phase influence the production and chemical composition of exopolysaccharide. Further, each adherent microorganism may produce unique exopolysaccharides.

Despite the important role of exopolysaccharides in fouling and corrosion, we know little about its synthesis, production and composition. Therefore, aims of this chapter were to study the production of EPS by fouling bacteria over the 30 d immersion period, assess the effects of several factors on the EPS production and to understand the role of EPS and proteins in the adhesion of bacteria to surfaces.

2. MATERIALS AND METHODS

2.1 *Bacterial cultures*

Bacterial cultures (90) were isolated from the aluminium panels immersed in Mandovi estuary over a period of 30 d.

2.2 *Screening of bacterial cultures for EPS production*

Bacterial cultures which were isolated over the immersion period of 30 d were grown to stationary phase for 48 h in Basal Salt Solution (BSS) and trisodium citrate (0.3%) as the sole carbon source. The inoculum used was 1% of the 18 h old bacterial culture, grown in the same medium. The cultures were harvested after 48 h, by centrifugation and the supernatants were filtered through 0.22 μm millipore filters. Aliquot (0.1ml) was used for assessing EPS production by the phenol-sulphuric acid method (Dubois *et al.*, 1956). D-glucose was used as the standard. Based on this initial screening, four cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were selected for further studies on EPS production.

2.3 *Growth and EPS production by bacterial cultures*

Four bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum*, *V. pelagius* (S_2) were studied for their growth pattern and EPS production using BSS medium supplemented with trisodium citrate as a sole source of carbon and energy.

Four flasks containing BSS were supplemented with trisodium

citrate (0.3%) as the sole carbon source. Flasks were inoculated with 18 h old bacterial cultures previously grown in the same medium.

The inoculated flasks were incubated at room temperature ($28 \pm 2^\circ\text{C}$) on the rotary shaker, at 150 rpm. At regular intervals (3 h), a five ml aliquot was removed. Turbidity was measured at 420 nm using Beckman DU 64 Spectrophotometer. The sample was then centrifuged at 5000 rpm for 30 mins and then filtered through 0.2 μm filter paper. The cell free filtrate (1 ml) was used for assessing the EPS production by the phenol-sulphuric acid method (Dubois *et al.*, 1956), using D-glucose as standard. These measurements were performed until the cultures attained the stationary phase.

2.4 Effect of yeast extract & glucose on EPS production

All four bacterial cultures i.e. *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum*, *V. pelagius* (S_2) were inoculated in two different media 1) BSS containing 1% yeast extract (BSSY) and BSS containing 1% glucose (BSSG). In both cases, 50 ml of the medium (BSSY or BSSG) in 250 ml flasks were inoculated with 18 h old cultures, previously grown in same medium. The flasks were incubated for 48 h at room temperature ($28 \pm 2^\circ\text{C}$), on a rotary shaker at 150 rpm. At the end of incubation period, the media were centrifuged at 5000 rpm for 30 mins to separate the supernatant and the cells. The supernatant was studied for extracellular polysaccharide production (EPS) and the bacterial cells

were used to study capsular polysaccharides production (CPS). Supernatant was filtered through GF/F filter paper followed by 0.22 μm filter paper. EPS was isolated by precipitation with three volumes of cold ethanol added to the cell free supernatant while constantly stirring. The precipitate was dissolved in water and reprecipitated with ethanol. This precipitate was then dried. EPS was dissolved in 3 ml of distilled water and dialysed against distilled water at 4°C to eliminate any low molecular weight sugars and salts until contaminating carbohydrate material was undetectable by the phenol-sulphuric acid method. Before use, dialysis tubings (MW cut off 10000) were pretreated with 0.01 M EDTA in 1% (W/V) sodium carbonate followed by washings with distilled water.

To extract the CPS, the cell pellet was vortex mixed with 5 ml NaCl (2 M) and then centrifuged for 30 mins. Supernatant containing CPS was then treated as above and the CPS was precipitated using cold ethanol. CPS was collected by centrifugation at 5000 rpm and dried at 50°C. The dried CPS was dissolved in distilled water and dialysed as above. Dialysed CPS was then used to estimate total carbohydrates by the phenol sulphuric acid method. Exopolysaccharide results were expressed as $\mu\text{g mg}^{-1}$ DW cells.

As compared to CPS, all the cultures produced higher amounts of EPS. Of these culture, *V. pelagius* (S_1) produced highest amount of EPS. Therefore, this culture was used for further studies on EPS production.

2.5 Effect of carbon source on EPS production by *V. pelagius* (S_1)

Flasks with BSS medium containing (0.5%) of either glucose, galactose, fructose, sucrose, maltose or melibiose as the sole carbon source were inoculated with 18 h old *V. pelagius* (S_1) culture (1 ml) which was grown in the same medium. The flasks were kept on rotary shaker (150 rpm) at room temperature for 48 h. At the end of 48 h, the total viable cell count was noted by spread plate method. *V. pelagius* (S_1) cell suspensions were centrifuged (5000 rpm) for 30 mins. The supernatants were passed through 0.2 μ m filter papers. The aliquots (8 ml) of the cell free filtrates were dialysed against distilled water (4°). After the dialysis, the volume of the dialysed filtrates was adjusted to 10 ml and aliquots (1 ml) were used for the analysis of carbohydrate using the phenol-sulphuric acid method. The EPS produced per ml of medium was calculated for different carbon sources. This method of EPS production evaluation was followed for all other experiments. EPS produced was expressed as ng cell⁻¹.

2.6 Growth pattern of *V. pelagius* (S_1) on glucose and EPS production by *V. pelagius* (S_1)

The BSS with glucose as the sole carbon source was inoculated with 18 h old *V. pelagius* (S_1) culture (1% inoculum) which was grown in the same medium. The flask was kept on the rotary shaker at 150 rpm. At regular intervals of 3 h, 5.0 ml aliquot

was removed. Turbidity was measured at 420 nm using Beckman DU-64 spectrophotometer. Cells were centrifuged at 5000 rpm for 30 mins. The supernatant was used for the estimation of EPS following the procedure described earlier. This experiment was carried out over a period of 96 h.

2.7 Effect of carbon concentration

Effect of carbon concentration on the production of EPS was assessed with varying concentrations of glucose (0.5% - 10% W/V). Glucose was selected as carbon source, as the culture produced highest amount of EPS when grown on glucose.

Flasks (100 ml) containing BSS medium were sterilized. Various concentrations of glucose (0.5 to 10% W/V) were added to these flasks. The flasks, after inoculating with *V. pelagius* (S_1) culture were incubated at room temperature for 48 hrs on a rotary shaker at 150 rpm. At the end of incubation period EPS, was measured as above.

2.8 Effect of nitrogen concentration on EPS production

To study the influence of nitrogen concentration, various amounts of NH_4Cl (0.05 to 2.0% W/V) were added to the BSS. Flasks containing BSS with glucose (1.0%) as the sole carbon source were inoculated with 18 h old *V. pelagius* (S_1) culture grown in the same medium. Flasks were inoculated with *V. pelagius* (S_1) cells and incubated at room temperature ($28 \pm 2^\circ\text{C}$)

on a rotary shaker at 150 rpm for 48 h. At the end of 48 hrs, the bacterial culture supernatant was assessed for EPS production as above.

2.9 Effect of divalent cations on EPS production

Effect of divalent cations (calcium and magnesium) on the production of EPS by *V. pelagius* (S_1) culture was studied. Various concentrations of CaCl_2 (0 - 0.5 % W/V) were added to BSS. These media containing various concentrations of CaCl_2 in BSS with glucose (1.0%) as the sole carbon source were inoculated with 18 h old *V. pelagius* (S_1) culture grown in the same media. Flasks were incubated at room temperature for 48 h on a rotary shaker at 150 rpm and EPS was estimated as described earlier. Similar experiments were performed to assess the effect of magnesium sulphate concentration on EPS production. Flasks containing basal salt solution with various concentrations of MgSO_4 (0 to 1.0% W/V) were inoculated with the *V. pelagius* (S_1) culture. EPS production was assessed as described earlier.

2.10 Effect of starvation on EPS production

Flasks containing nutrient broth (Table 2A.1) prepared in BSS were inoculated with 18 h old *V. pelagius* (S_1) culture grown in the same medium. Flasks were incubated at room temperature on a rotary shaker at 150 rpm for 24 h. At the end of the 24 h, *V. pelagius* (S_1) cells in the stationary phase, were harvested by centrifugation and washed thrice with BSS and resuspended in the

BSS medium. OD of the suspension was adjusted to 0.2. This corresponds to 1.94×10^9 cells ml^{-1} . Aliquots of the suspension were removed at regular intervals and used to measure turbidity, total viable cell count and EPS production. Turbidity was checked at 420 nm using Beckman DU-64 spectrophotometer. Total viable cell count of the *V. pelagius* (S_1) culture was monitored using spread plate technique on ZMA Petri dishes. Appropriate dilutions were plated on ZMA Petri dishes. Petri dishes were incubated at room temperature for 24 h. Colony counts were noted after 24 h incubation. EPS was measured using the method described earlier.

2.11 Effect of surfaces on EPS production

Five flasks containing basal salt solution with glucose (1%) as sole carbon source were inoculated with *V. pelagius* (S_1) culture. Four panels each of aluminium, stainless steel, polystyrene and copper (3.5 x 1.5 cm) were suspended in these flasks. Each flask contained four panels of each surface. One flask was treated as control without the panels. The flasks were kept on rotary shaker at room temperature ($28 \pm 2^\circ\text{C}$). The speed of the shaker was maintained at 150 rpm. At the end 48 h panels were removed. Suitable aliquots of the media from various flasks were filtered through 0.22 μm millipore filters. Aliquots (in triplicate) were used for EPS estimation (0.5 ml) as described earlier.

The cells from the control flasks were also harvested after 48 h and filtered through 0.22 μm filter. Aliquots (0.2 ml) of the filtrate were used for estimation of EPS as above.

2.12 Role of EPS and protein on the adhesion of bacteria to surfaces

a) Periodate treatment

Cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) were grown in nutrient broth prepared in BSS for 18 h. The cells in the stationary phase were harvested and washed thrice with BSS. These cell pellets were suspended in 1% (w/v) sodium periodate solution. The suspension was incubated at room temperature for 60 min. At the end of this incubation period, the cell pellet was again washed thrice in BSS and the cells suspended in BSS were then allowed to attach to various surfaces for one hour. The cells attached to the surfaces were estimated using the crystal violet method as described in Chapter 2B. Bacterial cells without the pretreatment with sodium periodate were used as experimental control.

b) Protease treatment

The washed cell pellets obtained as above were also treated with protease (0.1mg.ml^{-1}). The cell pellets were incubated for 60 min. in the BSS containing protease (0.1mg.ml^{-1}). At the end of 60 min, these cells were again washed in BSS, and suspended in BSS. The cells suspended in BSS were then allowed to attach to various surfaces for one hour. At the end of one hour, panels were removed and rinsed with BSS. Crystal violet staining was carried out to enumerate the number of bacterial cells attached to various

surfaces (Chapter 2B). The bacterial cells without protease treatment were used as control.

3. RESULTS

3.1 *Effect of immersion period*

Based on the EPS production, the bacterial cultures were divided into five groups, for the ease of presentation. Group A cultures showed lowest EPS production ($< 5 \mu\text{g ml}^{-1}$). All the five groups, A ($< 5 \mu\text{g ml}^{-1}$), B ($5 - 10 \mu\text{g ml}^{-1}$), C ($11 - 15 \mu\text{g ml}^{-1}$), D ($16 - 20 \mu\text{g ml}^{-1}$) and E ($> 20 \mu\text{g ml}^{-1}$) did not show any particular trend for EPS production with respect to the period of immersion (Table 6.1).

3.2 *Growth and EPS production of bacterial cultures*

Growth pattern of all four bacterial cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) was studied using trisodium citrate as the sole carbon source. EPS production was also monitored simultaneously. All the cultures showed a characteristic sigmoidal growth curve (Fig 6.1). The lag phase in citrate medium was longer than that in nutrient broth (Chapter 2A). The active exponential phase followed the lag phase. All four bacterial cultures attained stationary phase within 48 h. *V. pelagius* (S_1) cultures grew faster and produced more cell yield than *P. stutzeri* and *F. multivorum* (Fig 6.1). The carbohydrate measurements showed that the EPS was released into the medium during the exponential phase. EPS production was maximum at the

Table 6.1

EPS production by bacterial cultures isolated from aluminium panels immersed in Mandovi estuary as a function of time.

EPS	Immersion period (d)				
$\mu\text{g.ml}^{-1}$	1	7	16	22	30
> 5	7	5	8	8	6
5 - 10	5	6	8	4	7
11 - 15	4	3	2	2	1
16 - 20	1	1	-	3	-
> 20	1	1	-	-	1

Numbers indicate number of isolates giving positive results

- = Negative results

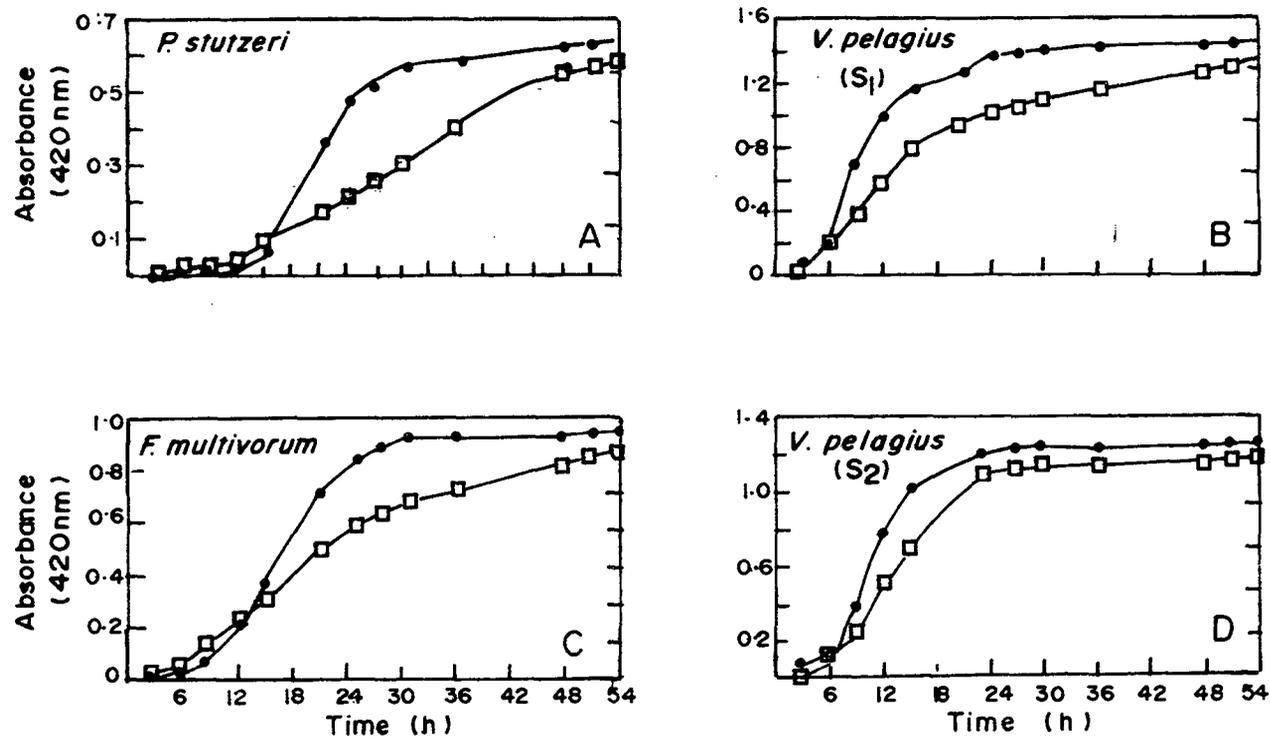


Fig. 6.1. Growth pattern (—●—) and EPS production (—□—) by the four bacterial cultures A, *P. stutzeri*; B, *V. pelagius* (S₁); C, *F. multivorum* and D, *V. pelagius* (S₂) using BSS medium containing trisodium citrate as the sole carbon source.

end of exponential phase. Amongst the four bacterial cultures, *V. pelagius* cultures showed highest EPS production (Fig 6.1).

3.3 *EPS and CPS production*

EPS and CPS production was assessed when cultures were grown on either Yeast extract or glucose. EPS production was much higher than the CPS production under both growth conditions studied. Nevertheless, CPS production was very low when glucose was the sole carbon source. Yeast extract grown cells produced relatively higher amounts of CPS (Table 6.2).

3.4 *Effect of carbon source*

Different carbon sources were used to study the influence of carbon source on EPS production by *V. pelagius* (S_1) culture. Higher polysaccharide production was observed when glucose or sucrose was used as carbon source (Table 6.3). Hence for all other experiments, glucose was used as a carbon source. Intermediate EPS production was observed on maltose, melibiose and galactose. Lowest EPS production was observed with fructose.

3.5 *Growth pattern and EPS production by V. pelagius (S_1) using glucose as sole carbon source*

Growth pattern and EPS production by *V. pelagius* (S_1) culture was studied using glucose as sole carbon source. The culture showed sigmoidal growth curve as was observed for the citrate grown cells (Fig.6.2). However, growth was faster when

Table 6.2

Production of EPS and CPS (expressed as $\mu\text{g.mg}^{-1}$ cell dry wt) by bacterial cells grown in YE & BSS and glucose & BSS

Bacteria	Carbon Source			
	Yeast Extract		Glucose	
	CPS	EPS	CPS	EPS
<i>P. stutzeri</i>	14.349	26.373	6.354	19.745
<i>V. pelagius (S₁)</i>	31.58	46.638	6.211	92.227
<i>F. multivorum</i>	9.362	41.198	8.30	56.258
<i>V. pelagius (S₂)</i>	10.962	49.001	5.50	86.043

Table 6.3

Effect of carbon source on the production of EPS by *V. pelagius* (S_1) culture

Carbon source	EPS (ng cell ⁻¹ x 10 ⁻⁹)
Glucose	322.66 ± 14.35
Sucrose	300.74 ± 5.52
Maltose	220.96 ± 7.35
Melibiose	208.26 ± 11.72
Galactose	202.24 ± 9.17
Fructose	167.23 ± 8.07

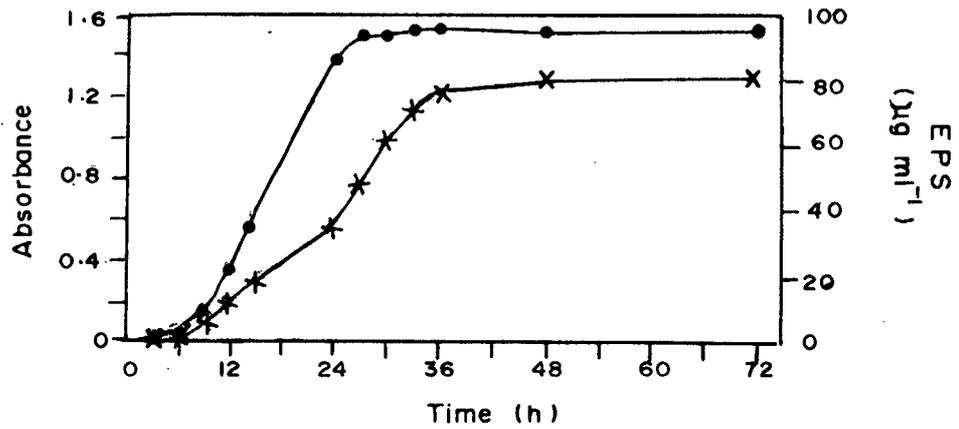


Fig. 6.2. Growth pattern (—●—) and EPS production (—x—) of *V. pelagius* (S_1) as a function of time.

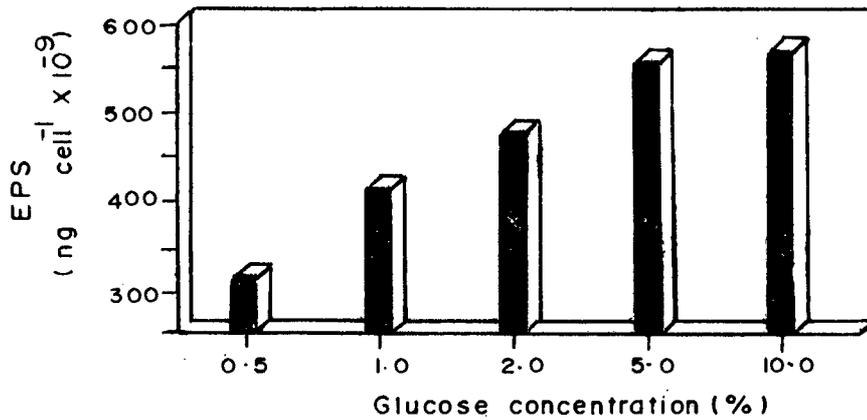


Fig. 6.3. Effect of glucose concentration on the production of EPS by *V. pelagius* (S_1).

glucose was used as carbon source as compared to the citrate. The stationary phase was attained within 30 h (Fig 6.2). EPS production started during exponential phase and increased with incubation period. EPS production was highest in the stationary phase. EPS production continued for 36 h, though the stationary phase was attained within 30 h (Fig 6.2).

3.6 Effect of carbon concentration

Production of EPS by *V. pelagius* (S_1) culture increased as the concentration of glucose increased. The concentration of 5% glucose in the medium produced highest amounts of EPS. No further increase in EPS production was observed when glucose concentration was higher than 5% (Fig 6.3).

3.7 Effect of nitrogen concentration

EPS production by *V. pelagius* (S_1) was higher when nitrogen concentration was low (0.05 to 0.1% NH_4Cl). Further increase in nitrogen content, however reduced the EPS production by the culture (Fig 6.4).

3.8 Effect of calcium and magnesium

Presence of calcium in the medium enhanced the EPS production by *V. pelagius* (S_1). Calcium concentration had a direct influence on EPS production. The production of EPS increased as the calcium concentration increased to 0.1% (as CaCl_2). After this, further increase in calcium concentration had no effect on EPS production (Fig 6.5A).

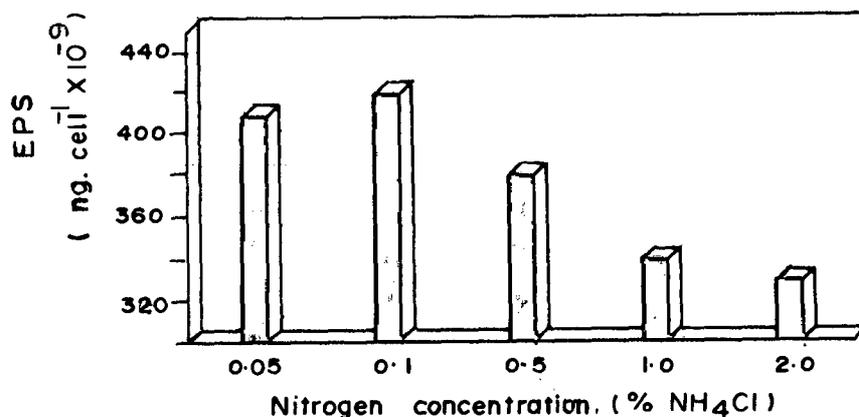


Fig. 6.4. Effect of nitrogen concentration (% NH₄Cl) on the production of EPS by *V. pelagius* (S₁).

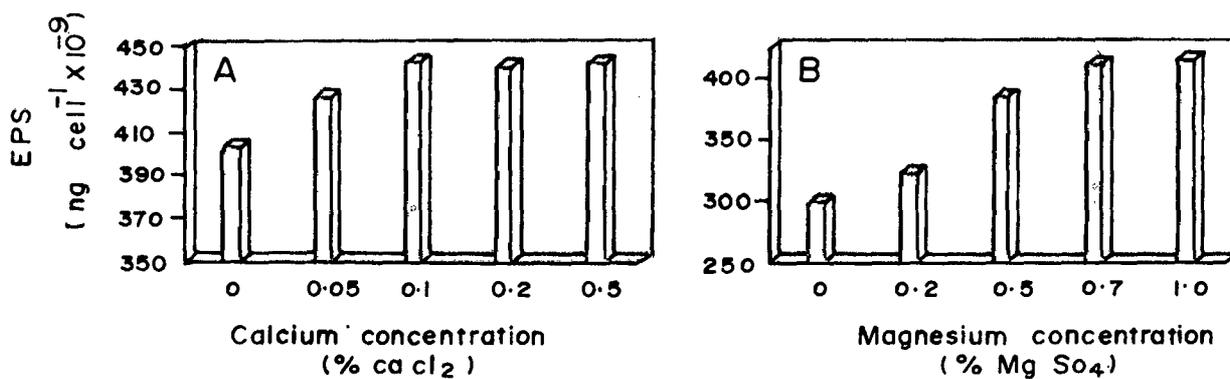


Fig. 6.5. Effect of divalent cations A, calcium concentration and B, magnesium concentration on the EPS production by *V. pelagius* (S₁).

Magnesium concentration also had a similar effect. Magnesium sulphate concentration of 0.7% produced highest amount of EPS by *V. pelagius* (S_1). On further increase in magnesium sulphate concentration, however, no increase in EPS production was noticed (Fig 6.5B).

3.9 Effect of starvation

As the period of starvation increased, production of EPS by *V. pelagius* (S_1) culture also increased. EPS production increased continuously until 144 h (Fig 6.6).

3.10 Effect of surfaces

When *V. pelagius* (S_1) cells were grown in the presence of surfaces, more production of EPS was induced as compared to control cells (Table 6.4). EPS production was highest in the presence of copper panels followed by stainless steel. In the presence of aluminium, lower amount of EPS was produced than the stainless steel and copper. Lowest EPS production was found in the presence of polystyrene and the control flask containing only bacterial cells.

3.11 a Periodate treatment

Treatment of bacterial cells with periodate produced variable effect on the adhesion of bacteria to surfaces. Adhesion to aluminium surface was most affected (Fig 6.7). The adhesion of *V. pelagius* cells to the four surfaces was most affected by periodate treatment whereas the adhesion of *F. multivorum* cells to the surfaces had minimum effect of periodate treatment.

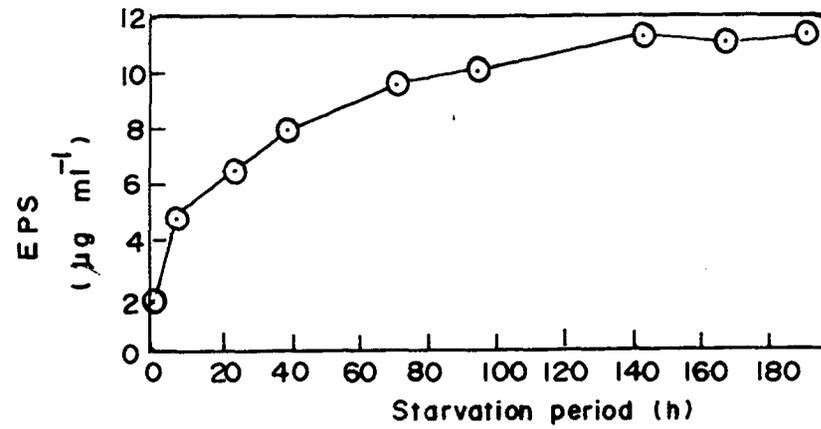


Fig. 6.6. Effect of starvation on the EPS production by *V. pelagius* (S_1)

Table 6.4

Production of EPS by *V. pelagius* (S_1) culture in the presence of surfaces

Surface	EPS ($\mu\text{g ml}^{-1}$)
Aluminium	112.38
Stainless steel	128.48
Polystyrene	94.86
Copper	146.24
Control	80.64

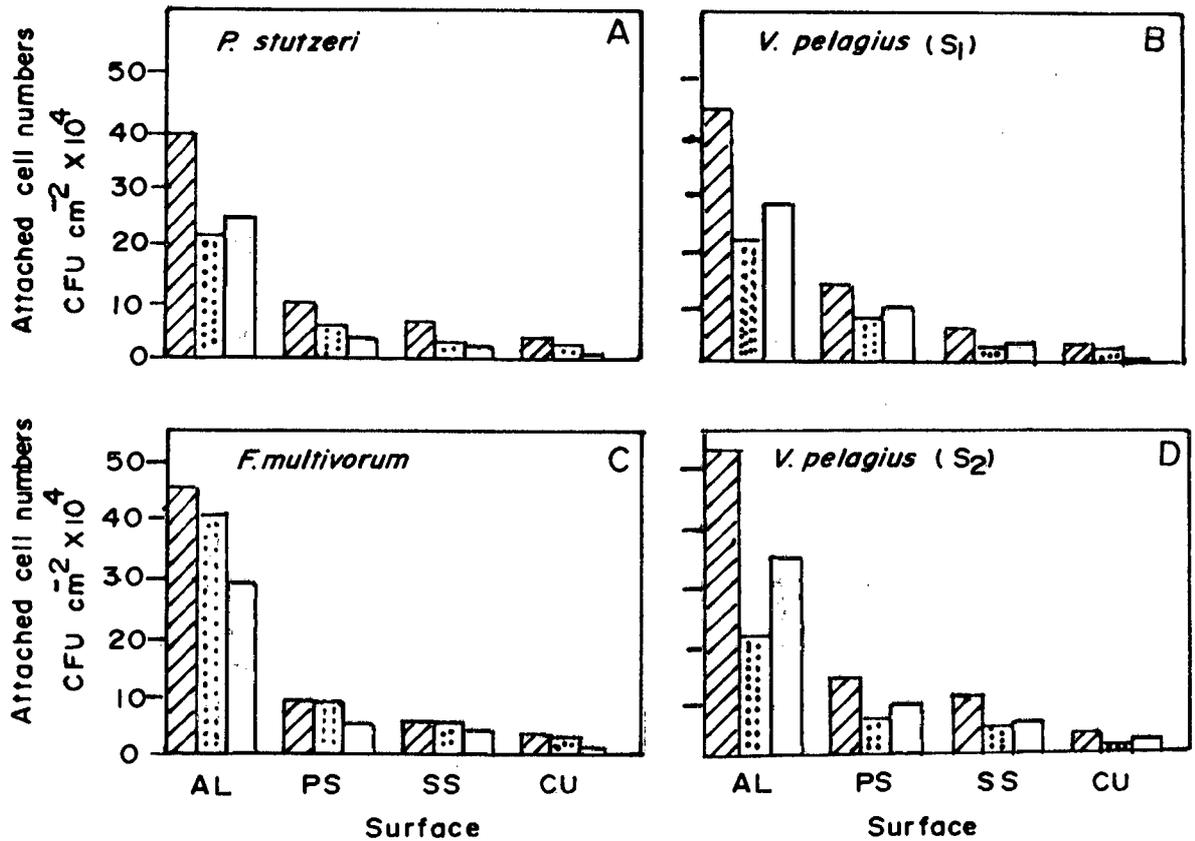


Fig. 6.7. Effect of sodium periodate (▨) and protease (□) treatment on the adhesion of A, *P. stutzeri*; B, *V. pelagius* (S₁); C, *F. multivorum* and D, *V. pelagius* (S₂) to aluminium (AL); polystyrene (PS); stainless steel (SS); and copper (CU). Control (▧) is also shown in the figure.

b Protease treatment

Protease treatment also affected cell adhesion. The adhesion of all the four cultures *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) was affected by protease treatment (Fig 6.7).

4. DISCUSSION

Surfaces immersed in marine environments are colonized by micro-organisms including bacteria. While colonizing the surfaces bacteria may produce exopolysaccharides (EPS). These polysaccharides help bacteria to form firm adhesion to surfaces. In our study most of the bacterial isolates produced exopolysaccharides in varying amounts during the period of immersion. This suggests the role of EPS in biofilm formation on the surfaces.

Bacteria may produce CPS and EPS. Relative distribution of these two depend on the nutrient levels and type. Very high nutrient media favoured the production of CPS. This was evident from higher amount of CPS production by the culture while growing on YE as compared to glucose. Production of CPS was relatively higher with yeast extract than with glucose. On the contrary, EPS production was higher in presence of glucose than in yeast extract medium. Bonet *et. al.*, (1993) reported that the yeast extract concentration had a pronounced effect on the nature of polysaccharide produced by *Aeromonas salmonicida*. In their experiments, total exopolysaccharides remained constant throughout the experiment; but the relative yield of EPS and CPS were strongly

associated with the yeast extract concentration. Contrary to this finding, Stringfellow *et. al.*, (1991) while working with capsular polysaccharide of *Staphylococcus aureus* found that the production of CPS was linked to energy source and its availability and not to the carbohydrate concentration or carbon/nitrogen ratio.

Production of EPS by the bacterial cultures employed for this study, was influenced by several factors. For example, growth phase of the bacteria affected the production of EPS (Rodrigues & Bhosle, 1991; Titus *et. al.*, 1995). EPS production was recorded in the early exponential phase and continued until the stationary phase. Highest EPS production occurred during the stationary phase. Similar results are reported by other workers (Mian *et. al.*, 1978; Rapp *et. al.*, 1979; Rodrigues & Bhosle, 1991; deSouza & Sutherland, 1994; Titus *et. al.*, 1995). Furthermore, Wrangstadh *et. al.*, (1990) showed that the composition of the EPS of a marine *Pseudomonas* sp. varied with growth phase. It appears that the EPS formed during the exponential phase directly or indirectly helps in adhesion of bacteria to surface while EPS formed during the stationary phase may help in its detachment from the surface. (Wrangstadh *et. al.*, 1990).

EPS production was highest with glucose as the carbon source. Rodrigues & Bhosle (1991) also found that when glucose was used as carbon source, EPS yield was higher. Cerning *et. al.*, (1994) also reported that glucose gave higher EPS yield.

Furthermore, EPS yield increased when the sugar concentration in the medium increased. When the glucose concentration was increased from 0.5 to 5.0% (W/V), EPS production was increased four fold. However, further increase in glucose to 10.0%, did not increase EPS production. Bacterial cultures in the presence of excess carbon and energy source and low nutrients such as N, S or P favour synthesis of exopolysaccharide (Sutherland, 1990; Dawes, 1991; deSouza & Sutherland, 1994; Cerning *et. al.*, 1994). It is suggested that the bacterial cells modulate the rate of EPS synthesis to match the rate of glucose access to the bacterium (Linton, 1990). Under nitrogen limiting conditions with excess glucose, excess of energy that exists over that needed for growth and other essential cell processes might have been utilized for the synthesis of EPS (deSouza & Sutherland, 1994).

Nitrogen concentration affected the EPS production. EPS production decreased as the nitrogen concentration in the medium increased. It is documented that nitrogen limitation enhances EPS synthesis in many bacteria (Sutherland, 1977; Nishikawa & Oi 1990; Novak *et. al.*, 1992; Meade *et. al.*, 1994). Titus *et. al* (1995) also found increased EPS production with the decrease of nitrogen concentration in the growth medium. Thus the decrease in EPS production with increasing N concentration, found in the present study could be attributed to the cell growth and other essential cell processes in the presence of nitrogen.

Cations such as Ca^{+2} and Mg^{+2} are essential for adhesion of cells to surfaces (Marshall *et al.*, 1971; Fletcher & Floodgate, 1973). These cations especially Ca^{+2} can form bridges between negatively charged substrata and microorganisms, stabilize the structure of EPS or cause precipitation of EPS in the space between the cell and substrata (Characklis & Cooksey, 1983). Roger *et al.*, (1990) found that *Ruminococcus flavefanices* required presence of Ca^{+2} and Mg^{+} for its adhesion. The mechanism involved in adhesion may be the interaction between the glycocalyx and the divalent cations Ca^{+2} and Mg^{+2} . Exopolysaccharides that combine ions are adhesive because they form strong metal bridges (Sutherland, 1983). Ion uptake by bacterial EPS has been reported by other workers (Geddie & Sutherland, 1993).

Starvation of bacterial cells increased EPS production. Some workers (Lappin-Scott & Costerton, 1989) have reported decreased EPS production by starved cells. It is possible that the EPS production serves as a survival mechanism for the cells during the adverse environmental conditions.

It is suggested that solid surfaces stimulate EPS production by bacteria (Vandevivere & Kirchman, 1993). Wrangstadh *et al.*, (1989) found that the presence of a solid surface accelerated the production of exopolysaccharides during the initial stages of starvation of *Pseudomonas* sp. strain S9. In the presence of the solid surface, EPS synthesis in the medium also increased. It was found that there was

higher production of EPS by *P. stutzeri*, *V. pelagius* (S_1), *F. multivorum* and *V. pelagius* (S_2) in the presence of surfaces than in the control flask. EPS synthesis in the medium was highest in the presence of copper. Several workers (Beech *et. al.*, 1991; Spencely *et. al.*, 1992) have observed that bacteria embedded in a biofilm produce markedly larger amounts of EPS than bacteria in suspension. However, Lappinscott & Costerton (1989) observed that polysaccharide production occurred when the adhesion rates were lower and was absent when the adhesion rates were higher. The role of EPS in heavy metal adsorption has been demonstrated by a few workers (Brown & Lester, 1979, 1982; Rudd *et. al.*, 1984; Geddie & Sutherland, 1993). Larger amounts of exopolysaccharides in the presence of copper surfaces observed in the present study may protect the bacterial cells against the toxicity of copper ions. EPS may be synthesized as a response to environmental stress and may help the cells in removing the toxicity of the metal ions. Vandevivere & Kirchman (1993) also reported that surfaces enhance exopolysaccharide synthesis. Cooksey (1992) suggested that adhesion in itself stimulates the synthesis of EPS. Direct influence of solid surfaces on bacterial activity at the level of gene transcription have also been demonstrated (Belas *et. al.*, 1986; Dagostino *et. al.*, 1991; Davies *et. al.*, 1993). Davies *et. al.*, (1993) showed that activation of a gene (alg C) for the exopolysaccharide alginate was higher for *Pseudomonas aeruginosa* attached to a teflon mesh than for unattached bacteria.

The treatment of bacterial cells with sodium periodate and protease reduced the bacterial adhesion to surfaces. Sodium periodate denatures exopolysaccharides at cell surfaces. Reduced adhesion of bacteria after periodate indicates that EPS may be involved in the attachment of bacteria. This was very conspicuous for *V. pelagius* culture attached to the surfaces. On the other hand, adhesion of *E. multivorum* was not much affected suggesting that polysaccharides may not contribute significantly to its adhesion to surfaces.

Protease destroys the cell surface proteins. Protease treatment inhibited adhesion of all four isolates, thus suggesting the involvement of a protein in the adhesion. The association of cell proteins with adhesion of the cells to the substratum has been indicated by a number of workers (Danielsson *et. al.*, 1977; Conway & Kjelleberg, 1989; Wadstrom *et. al.*, 1987; Henriksson *et. al.*, 1991; Imam *et. al.*, 1993). Inhibition of bacterial adhesion to the surfaces, observed in the present study, could be due to the degradation of some cell proteins associated with cell adhesion. Although treatments with periodate and protease reduced the adhesion, it did not completely inhibit the bacterial adhesion. This implies that some other factors were also involved.

It seems likely that both proteins and exopolysaccharides are involved in the bacterial attachment to surfaces. However, the lack of attachment inhibition after protease or periodate treatment as in

F. multivorum does not necessarily indicate that proteins or polysaccharides are not involved in adhesion. Periodate does not degrade all carbohydrates and it can also be excluded by highly charged polymers (Rainbourg, 1971). The different types of proteolytic enzymes used in the attachment studies have been found to affect attachment differently (Corpe, 1974). Our data suggest that in addition to proteins and EPS, some other components not specific to periodate and protease might have been associated with the adhesion of bacteria to surfaces employed in this study.

CHAPTER 7

Summary

Solid surfaces exposed to marine environment are subjected to fouling. The microbial colonization of surfaces follows a pattern almost independent of the type of surface and consists of five stages such as 1) conditioning of films 2) transport of microbial cells to the surfaces 3) reversible sorption of microbial cells 4) irreversible adhesion of microbial cells and 5) development of secondary microflora. Transport of the microbial cells to the conditioned surface is supposed to be the rate-determining step. Bacterial fouling may provide a basis for the subsequent attachment and growth of macrofouling organisms.

Attachment of bacteria to solid substrata confers some advantages to bacteria. Firstly they do not have to spend energy searching for the food as biofilms capture nutrients from the bulk liquid. The metabolic waste products are also removed by the water flowing over the substratum. The substratum also provides shelter to the bacteria. Biofilm also has a protective function for the cells within it. Many conventional biocides are capable of killing planktonic cells, yet they do not kill sessile cells at the same biocide concentration and contact time.

Biofouling causes significant financial losses to the manmade systems. The fouling of ships increases the drag on the vessels and in

turn increases fuel consumption. Industrial cooling towers and heat exchangers may also be adversely affected by biofilms. Tap water distribution network, food and canning industry, medical prostheses inserted in the patients are also affected by biofouling. Another problem associated with microfouling is microbiologically induced corrosion (MIC). In recent years, MIC has emerged as an important economic problem.

Implications of surface biofouling are tremendous. A better understanding of bacterial interaction with surfaces will help in developing better techniques to control biofouling. With this goal, various factors affecting adhesion were studied during the present research work.

The development of biofilm on aluminium was studied with respect to the succession of bacteria over an immersion period of 30 days in the estuarine water. Aluminium panels were deployed in Mandovi estuary. These panels were retrieved in triplicate at 1, 8, 16, 22 and 30 days. They were scraped using a nylon brush and filter sterilized estuarine water. Microfouling biomass was estimated as organic carbon, dry weight and total viable bacterial count.

Microfouling biomass (dry-weight and organic carbon) on aluminium panels immersed in Mandovi estuary increased over the period of immersion. Total viable cell count of the biofilm bacteria also showed an increasing trend over the period of immersion. This suggests that the observed increase in microfouling biomass could be due to the enhanced settlement and/or growth on the aluminium panels.

Hundred colonies were selected and purified using Zobell Marine Agar (ZMA) plates. Characterization of these cultures was done using standard biochemical tests. Most of the isolates found on aluminium panels were gram negative rods. *Pseudomonas*, *Alkaligenes* and *Chromobacterium* were the most abundant biofilm bacteria at least on the first day following immersion. The bacterial community structure changed, as the period of immersion increased. *Vibrio* was the most abundant in biofilm on aluminium. This change was evident from the abundance of various bacteria during the period of immersion. This is also shown by the changes in the Shannon Diversity indices. The apparent succession of bacterial types suggests an alteration of the surface by those bacteria that are sorbed initially.

Based on their abundance during the period of deployment and their ability to produce extracellular polysaccharides (EPS), four cultures which were tentatively identified as *Pseudomonas stutzeri*, *Vibrio pelagius* (S1), *Flavobacterium multivorum* and *Vibrio pelagius* (S2) were selected for further research work.. Growth pattern of these bacteria was studied using nutrient broth. All the four cultures attained stationary phase within 16 h of inoculation.

The existing methods like ATP, DNA, total count to estimate bacterial adhesion are time consuming, laborious and uneconomical. Therefore, a simple adhesion assay based on crystal violet staining of the attached cells was developed to evaluate bacterial adhesion to surfaces. The viable cell count of the attached cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) and the absorbance of crystal violet stained cells attached to metal and non-metal surfaces gave highly significant positive linear relationships ($r = 0.9994$, $p \leq 0.001$, $n = 6$). This method was then used for the estimation of bacterial adhesion to various surfaces throughout the course of this study.

Adhesion of bacterial cells to surfaces is influenced by several factors such as temperature, pH, salinity and nutrients. The effect of these

parameters on the adhesion of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) to various surfaces was studied. The surfaces selected were aluminium, stainless steel, copper and polystyrene. It was found that with increase in temperature from 4° to 37 °C, adhesion of bacteria to surfaces increased. However, when the temperature was increased further to 50 °C, the bacterial adhesion to all four surfaces decreased. Temperature may influence adhesion by affecting the physiology of the organisms. Secondly, higher temperatures (within limits) favour chemisorption. However, relatively higher temperatures (50°) may influence the viscosity of adhesive polymers thereby reducing the bacterial adhesion to surfaces.

pH of the medium also influenced adhesion of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) to the surfaces. Highest adhesion was found when pH varied from 7 to 8. The effect of pH on adhesion could be due to the changes in the polymer viscosity. Nevertheless, pH could also influence other factors important for permanent adhesion e.g. electrical double layer, thickness and the dissociation of charged groups on the solid and the cell surfaces.

The presence of nutrients also had a marked effect on adhesion of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) to various surfaces. Bacterial adhesion was very poor in the absence of nitrogen and phosphate. The presence of nutrients may change the liquid surface tension, thus affecting the adhesion.

Concentration of sodium chloride was found to affect adhesion. Increased adhesion of bacteria was found with the increasing concentration of sodium chloride (upto 35 g.l⁻¹). Increasing the ionic strength of the aqueous solution has been experimentally shown to increase bacterial adhesion to various surfaces by a number of workers. Increased concentration of electrolyte is believed to compress the electrical double layer and according to DLVO theory, it is expected that the surfaces of like charges can attract one another. However, beyond a certain limit continued addition of electrolyte does not compress the double layer any further and hence the adhesion of bacterial cells to the surface is also affected.

The substratum surface texture and/or topography can influence biofilm development. Maximum number of bacterial cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) were found

attached to aluminium followed by polystyrene. Minimum attachment was found on copper. The number of bacterial cells attached to stainless steel was less than polystyrene but more than copper. It is generally observed that rough surfaces are more conducive to bacterial adhesion than smooth surfaces. Rough surfaces may shield the cells from the bulk flow and secondly, more substratum area may be available for the biofilm on a rough surface. Aluminium, being a rough surface, maximum number of cells attached to aluminium. Substratum wettability is an important factor in bacterial adhesion. Numbers of bacterial cells initially attached to hydrophilic and hydrophobic surfaces appear to be different. High number of bacterial cells on polystyrene surfaces may thus be explained by the hydrophobicity of the polystyrene surface. Lower number of bacterial cells on stainless steel surface could be due to the toxic effects of chromium ions. Minimum attachment found on the copper surface could be due to environmental stress caused by toxicity of copper corrosion products or due to the unstable corrosion product films.

Hydrophobicity of the bacterial cells is known to be associated with the adhesion of the bacterial cells to various surfaces. Ninety colonies isolated from aluminium panels immersed in estuarine waters over a period of 30d were screened for the hydrophobicity. Most of the

cultures were hydrophilic with less than 40% of absorbance in hexadecane suggesting that hydrophilic bacteria are more abundant on hydrophilic surface. This was further substantiated by a significant positive correlation between the hydrophobicity of bacteria and their adhesion to aluminium.

Bacteria appeared to become relatively more hydrophobic with the increase in the immersion period. Several factors including optimal growth conditions, growth phase, temperature, carbon source and concentration, nitrogen content and starvation may influence cell surface hydrophobicity of bacteria. Some of these factors were studied during the course of this study. Increase in temperature lowered the hydrophobicity of cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2). Carbon source also influenced hydrophobicity of cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2). Glucose grown cells were more hydrophobic than citrate grown cells. Increased hydrophobicity of *V. pelagius* (S1) was observed with sucrose, melibiose, galactose, maltose, fructose and glucose. It was observed that all the four isolates were more hydrophobic in the stationary phase than in the exponential phase implying the effect of growth phase. It appears that the capsular polysaccharides of the mid-exponential cells interfered

with adherence to hexadecane. There was a sharp rise in the hydrophobicity of cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) after 12 hrs of starvation. Starving bacteria with a higher cell surface hydrophobicity may adhere to surfaces and scavenge the adsorbed nutrients, thereby enhancing their chances of survival in aquatic environment. Furthermore, hydrophobicity increased with the concentration of the nitrogen in the growth medium, whereas, it decreased with the concentration of the carbon in the medium.

The physiological activity of bacteria attached to surfaces may differ from that of free living cells. Electron Transport System (ETS) is one of the functions determining physiological activity of bacteria. ETS activity of free living and the attached bacteria was assessed using bacterial cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) and all the four surfaces. Attached bacteria showed greater activity than their free living counterparts. Growth phases of bacteria were also shown to have an effect on respiration rate and ETS activity. Since respiration is not a linear function of ETS, R:ETS ratio also changed with the various growth phases. During the exponential phase, R:ETS ratio was almost similar for both free living and attached bacteria. However, R per cell and ETS per cell were higher for attached bacteria as

compared to the free-living cells. Cell specific respiration rate and ETS activity decreased during the stationary phase for both attached bacteria and their free-living counterparts. R:ETS ratio also decreased from exponential phase to stationary phase. R:ETS ratio of the free-living cells was less than that of the attached cells, during the stationary phase. This is because the decrease in respiration rates was more in case of free living cells than in the attached cells. The decrease in respiration rates was more than the decrease in ETS activity.

Further, exoenzyme accumulation is often proposed as an explanation for high metabolic activity of biofilms. When the free and attached bacterial cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) were studied for the enzyme activity with respect to four enzymes i.e. alkaline phosphatase, lipase, protease and amylase, bacteria attached to the surfaces were found to have higher enzyme activity than the free-living cells. Extracellular enzymes have the potential to become decoupled from the organisms that produced them. Biofilm matrix promotes accumulation of these exoenzymes either by physical enmeshment by glycocalyx, hindered diffusion, adsorption or by cation bridging. The advantage of accumulation could be diversion of resources from exoenzyme synthesis to microbial metabolism which may

contribute to the high specific activity rates observed for attached bacteria.

One of the costly problems associated with the microfouling of metal surfaces is microbiologically induced corrosion (MIC). Bacterial cells of *P. stutzeri*, *V. pelagius* (S1), *F. multivorum* and *V. pelagius* (S2) appeared to accelerate the corrosion of mild steel, aluminium and copper. *P. stutzeri* and *F. multivorum* were found to be more aggressive than *V. pelagius* cultures. The increased corrosion could also be due to the production of aggressive metabolites. Pitting type of corrosion was evident on these metal coupons, especially on mild steel and aluminium coupons.

XRD analysis showed that FeO(OH) and Fe_2O_3 were major products of mild steel corrosion. Copper formed many corrosion products in the absence of bacteria e.g. $\text{Cu}_2\text{Cl(OH)}_2$, CuS , Cu_2S , $\text{Cu(OH}_2\text{Cl)}_2\text{H}_2\text{O}$, 6CuO , Cu_2O etc. However, in the presence of bacteria, sulphides and hydroxychlorides were the major products formed. Aluminium hydroxide was the only product detected from aluminium coupons, both in the presence or absence of bacteria. All the four cultures used in this study did not have any effect on the corrosion of stainless steel.

Bacteria attached to solid surfaces in natural environments are often embedded in exopolymers. Bacterial cells may produce EPS during adhesion to the surface. However, EPS production by bacterial cultures isolated during different immersion periods did not show any particular trend with respect to their period of isolation.

Growth phases of bacteria were also found to influence EPS production. EPS production started in exponential phase and continued during the stationary phase. Maximum production occurred at the end of exponential phase. When the bacterial isolates were studied for capsular polysaccharide production (CPS), it was found that the production of CPS was better with yeast extract than with glucose.

Various parameters were studied to assess the influence on the production of EPS. EPS yield was found to be maximum, when glucose was used as carbon source. Production of EPS increased with the increase in the concentration of carbon source. However, nitrogen concentration was found to have an opposite effect. EPS production decreased as the nitrogen concentration in the medium increased. Nitrogen limitation enhances EPS synthesis. It is possible that under nitrogen limiting conditions with excess glucose, excess of energy that

exists over that needed for growth and other essential cell processes might have been utilized for the synthesis of EPS.

Calcium and magnesium cations also seemed to influence EPS production. As the calcium and magnesium concentration increased, EPS production was also found to increase. Ca^{++} is necessary for the adhesion of cells to the surfaces. The results suggest that these divalent cations are directly or indirectly involved in EPS production.

EPS synthesis was found to be more in the presence of surfaces. Several workers have observed that bacteria embedded in a biofilm produce markedly larger amounts of EPS than bacteria in suspension. EPS may be synthesized as a response to environmental stress. Larger amounts of EPS may protect the cells against the toxicity of metal ions.

Bacterial isolates were treated with sodium periodate and protease to study the biochemical contents involved in adhesion. It seems likely that both proteins and exopolysaccharides are involved in the bacterial attachment to surfaces. This was shown by an inhibitory effect on bacterial adhesion after the treatment of the bacterial cells with sodium periodate and protease. Sodium periodate denatures exopolysaccharides at cell surface and protease denatures cell surface proteins. The

inhibition of bacterial adhesion by the treatment with sodium periodate and protease suggests involvement of proteins and polysaccharides in the bacterial adhesion.

It is believed that these studies provide some useful basic data on biofilm development on surfaces, which in turn may help to develop techniques to control biofouling and microbiologically induced corrosion.

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APPENDIX

Biochemical test media

All the media were prepared following standard methods (Cruickshank *et al*, 1975) using basal salt solution - BSS.

Sterilization 121°C for 15 min.
Incubation Room temperature
(28-30°C) two to three days.

Gram's Stain

1. *Crystal Violet*

- a. Crystal Violet Dissolve 20g in 200 ml methylated spirit
- b. Ammonium Oxalate Dissolve 8g in 800 ml of distilled water

Mix both together to make 1000 ml.

2. *Grams Iodine*

Iodine	-	10g
Potassium Iodide	-	20g
Distilled water	-	1000 ml

3. 95% alcohol

4. Safranin (0.5%)

Safranin	-	0.5g
Distilled water	-	100 ml

Smear was prepared using 18 - 24 hr old culture and fixed heating over or flame, stained with crystal violet, washed with water followed by grams iodine rinsed with alcohol and washed with water, counter stained with

safranin and observed microscopically

Violet = Gm +ve ; Pink = Gm -ve

Sugar fermentation

Peptone 10 g
Phenol red (0.2%) 50 ml
Basal salt solution (BSS) 950 ml
pH 7.2
Carbohydrate (10%) 1%

Sterilized separately and added to the tubed medium.

Red = - ; Yellow = +

Gas in Durham tube.

Hugh - Leifson's medium

Peptone 2 g
 K_2HPO_4 0.3g
BSS 1000 ml
pH 7.5
Boromothymol blue 1% aqueous 3 ml
Agar 4 gms
Glucose 1% (added) separately

Tubes stab inoculated in duplicate. One of the tubes covered with sterilized with liquid paraffin for fermentation.

Yellow = + ; Blue = -

Nitrate reductase

Potassium nitrate 0.2 gm
Peptone 5.0 gm
BSS (without nitrogen compound) 1000 ml



Regents

Solution A

8 gm Sulphanilic acid in 1000 ml 5 N acetic acid.

Solution B

5 gm Alpha naphthylamine in 1000 ml of 5 N acetic acid.

Test reagent

Equal volumes of A and B, 0.5 ml test reagent to test culture.

Red colour = + ; No colour = - .

Oxidase

Test reagent

1% Tetramethyl-p-phenylenediamine hydrochloride in distilled water.

Streak young culture on filter paper soaked in test reagent.

Purple colour in 5-10 seconds = +

Catalase

Test reagent

30% hydrogen peroxide

Few drops of reagent added to broth culture.

Effervescence = +

Indole Production

Tryptone	10g
BSS	1000 ml
pH	7.4

Test reagent

Amyl alcohol	150
P - Dimethyl amino benzaldehyde	10g
Con. HCl	50 ml
Add 0.5 ml test reagent		
Red Colour	=	+

H₂S Production

Peptone	20 gm
Sodium thiosulphate	0.8 gm
BSS	1000 ml
pH	7.5
Agar	3.0 gm

Tubes with lead acetate paper stab inoculated. Blackening of lead acetate paper = +.

Gelatin Liquification

Nutrient broth	100 ml
Gelatin	15 gm
pH	7.4

Tube the media, sterilize at 121°C for 10 mins. Make stabs with bacterial cultures. Incubate overnight at room temperature.

Observation

Media in liquid condition at cold temperature indicates a positive test.

Alginase

Nutrient broth with sodium alginate, spot inoculation, clear zone around colonies after 10% cetyl pyridinium chloride = +.

Amylase

Nutrient agar with 0.4% soluble starch spot inoculation, zone around colonies after lugol's iodine = +.