Isolation and characterization of a manganese oxidizing bacterium from the Mediterranean marine sponge *Suberites domuncula*.

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Mugdha Divekar

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Summary

In the present study of sponge-bacterial association, the presence of a marine bacterium which has not seen to be associated previously with the Mediterranean sponge *Suberites domuncula* was investigated. The marine sponge *S. domuncula* was chosen as the subject of investigation, for the identification of potential symbiotic microorganisms, since it can be kept under controlled laboratory conditions for over five years. By the use of specialized media assisting in the growth of a metal oxidizing bacterium, the manganese oxidizing bacterium was isolated from the surface of the marine sponge. The bacterium so isolated was characterized for its growth characteristics by microbiological and biochemical techniques, a detailed analysis of which showed that the bacterium followed a life cycle where the culture showed the presence of spore forming bacteria. This was correlated to the manganese oxidation activity of the bacteria and it was found that both stages are interdependent.

The action of the protein responsible for carrying out the manganese (Mn) oxidation was studied by an in-gel oxidation assay, and the presence of a multi copper oxidase was confirmed by the use of copper chelators in the buffer. In parallel the effect of addition of copper was observed on the manganese oxidation by the bacteria thus supporting the observations. The manganese oxidation reaction by the bacteria was determined in the culture medium and on the surface of the cells, and it could be concluded that the oxidation was facilitated by the presence of the polysaccharides and proteins on the surface of the cells.

Thus the presence of a bacterium capable of oxidizing the manganese from the surroundings was confirmed to be symbiotically associated with the marine sponge *S. domuncula* by monitoring its growth in axenic cultures. The reasons behind this association were studied.This bacterium displays a crucial role in the physiology/metabolism of the sponge by acting as a reversible Mn store in *S. domuncula*. According to this view, the presence of SubDo-03 bacteria is required as a protection against higher, toxic concentrations of Mn in the environment; manganese (II) after undergoing oxidation to manganese (IV), becomes an insoluble ion. Since only minute levels of manganese exist in the surrounding seawater a substantial accumulation of manganese has to arise, or a release by the bacterial-precipitated manganese (IV) is implicated to maintain the reversible balance. The other possible benefits provided by the bacterial association to the sponge could be in preventing cellular oxygen toxicity, help in nutrient scavenging and detoxification.

Chapter 1 - General introduction

1.1 Metazoa: origin and evolution

The siliceous marine sponge *Suberites domuncula* is a member of the most ancient and the simplest phylum of multicellular animals Porifera which have branched off from the common ancestor of all Metazoa. Porifera represent the most simplest of the Metazoan phylum. Since it was interesting to know the evolution and systematics of animal diversity and animal body plan, a lot of work has been done on the evolutionary relationships among the different groups of animals depending upon the morphological characteristics: especially those expressed in the early development, (e.g. the embryological characters).

More recently a significant change in the characterization and its understanding has been brought about by the study of molecules, (in particular the genes and their protein products) contained in the animal cells. The evolution of the Metazoa from unicellular/colonial organisms occurred some 1,300–600 million years (Myr) ago in the pre-Ediacaran period (Conway-Morris, 1998). Morphological contributions to understanding of the transitional stages to the Metazoa suggest a colonial origin of Metazoa (Dewel, 2000).

A deep reorganization of the metazoan phylogenetic tree is presently taking place as as result of input of molecular data, which is represented in the (Fig.1B). The ‘‘traditional’’ animal phylogeny found in major zoology textbooks (Barnes, 1985) as seen in (Fig.1A) is following the work of (Hyman, 1940). It is a good illustration of the long prevailing notion that animal evolution went from simple to complex through gradual steps, with extant animals actually representing grades of intermediate complexity supposed to have been those of their ancestors. New animal phylogeny proposed by Adoutte et al., (2000) is exclusively based on rRNA (Fig.1B). New animal phylogeny leaves us with no evolutionary ‘‘intermediates’’ and suggest a paraphyletic emergence of sponges at the base of metazoan tree, followed by a monophyletic Ctenophora and a possibly paraphyletic Cnidaria.

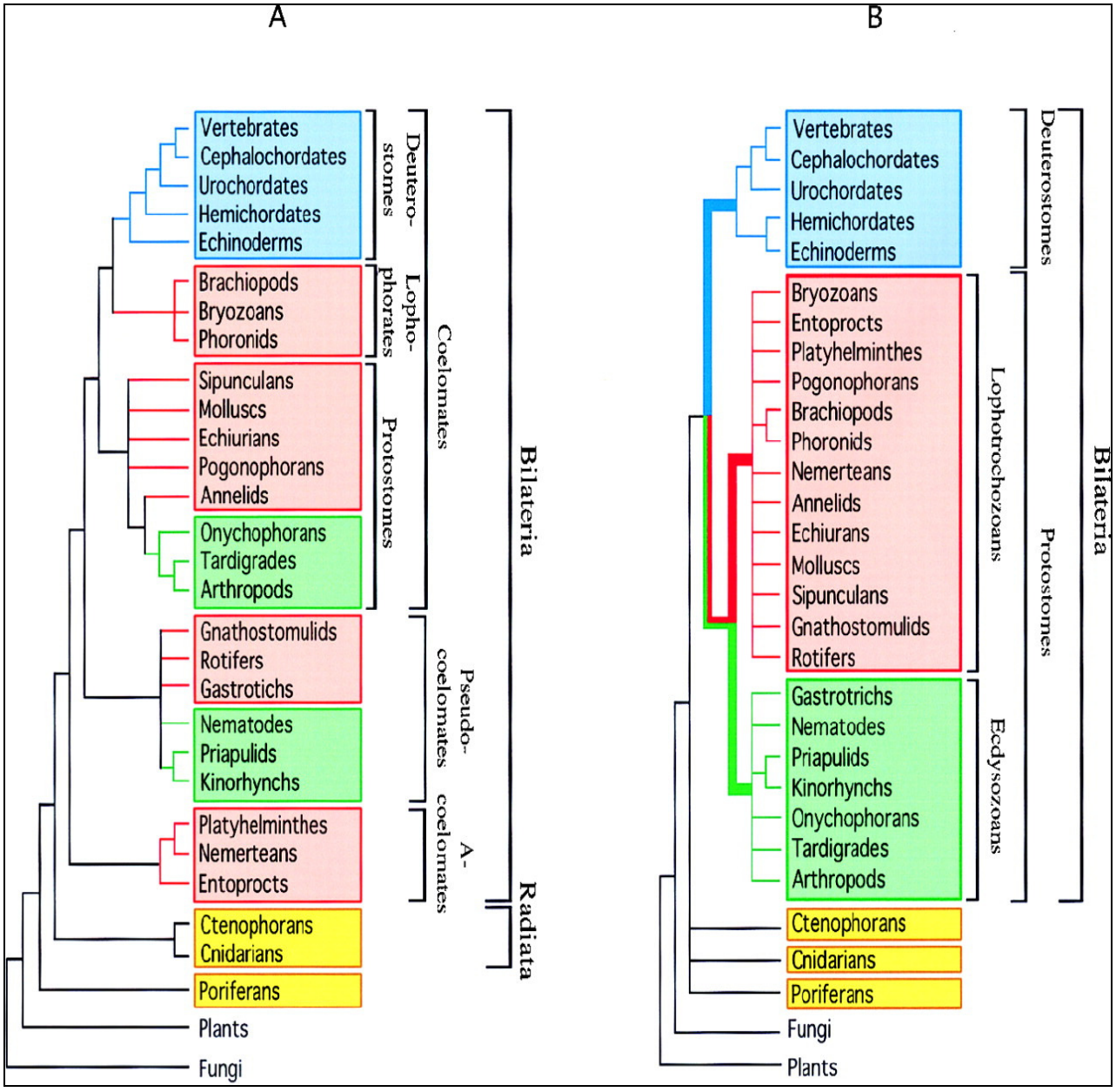


Figure 1: Metazoan phylogenies A; Traditional view of animal phylogeny based on "Grades" in body plan characterized on morphology and embryology *(Hyman, 1940).* ***B; New animal phylogeny based on molecular data*** *(Adoutee et al., 2000).*

Regardless of evolutionary theorizing about Metazoan origin, today it is widely accepted that all metazoan phyla are of monophyletic origin (Borchiellini et al, 2001; Müller, 1995) with the hypothetical common metazoan ancestor, named "Urmetazoa" (Müller, 2001).

Sponges (Porifera) as the phylogenetically oldest metazoan phylum still extant today share the closest relationship to the Urmetazoa. The sponges, as the most basal group in the animal phylogeny, laid the foundation for all animal life to follow. They emerged approximately 600–800 million years ago as calculated on basis on amino acid exchanges (Müller et al., 1994) and since then their overall Bauplan (blueprint) have not changed (Müller, 2001).

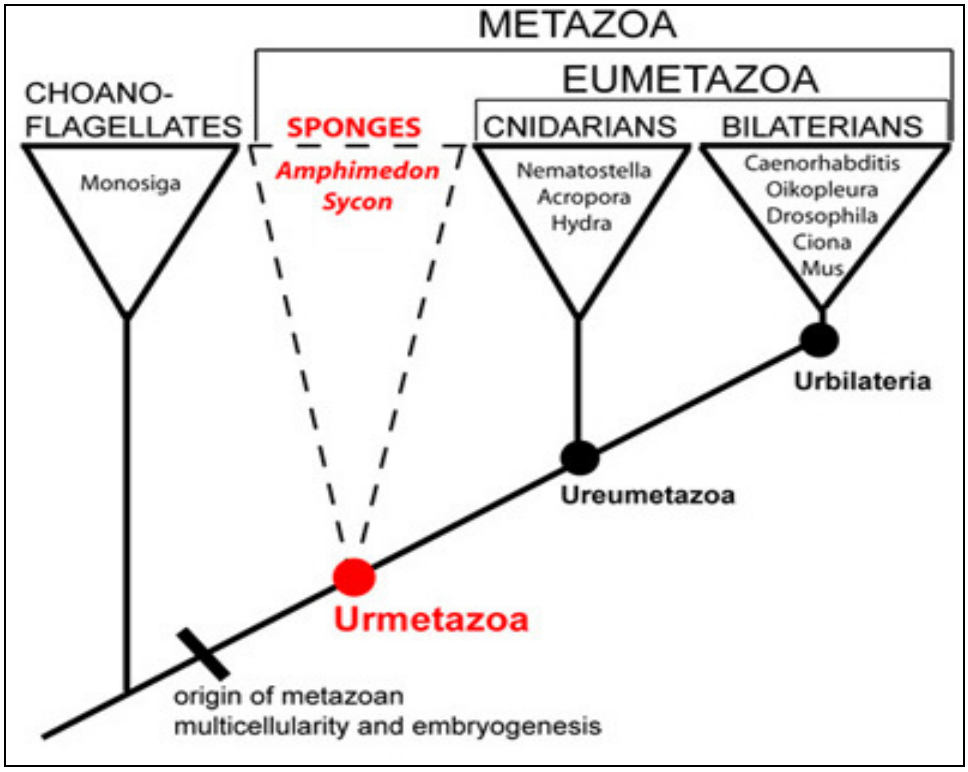


Figure 2: Evolutionalry placement of Porifera monophyletically with other metazoan phyla.
Taken from http://www.sars.no/research/AdamskaGrp.php

1.2 Origin and evolution of sponges

Sponges represent the very base of metazoan evolution and can be regarded as the oldest animal phylum still alive. The spicule record for sponges starts in the Late Proterozoic. The oldest spicules with demosponge affinities were found in (ca. 750 Myr) old Noon Day Dolomite in Nevada, and in the Neoproterozoic Cloudina-Reefs (ca. 555 Myr) of southern Namibia (Reitner & Wörheide, 2002). Chemical traces of early life, or 'biomarkers', showing the presence of demosponges, a class of sponge that accounts for around 90 per cent of sponges living today were found in the South Oman Salt Basin off the South-Eastern edge of the Arabian Peninsula. The biomarkers form a continuous chemical fossil record of marine sponges living in the areas seas during the late Neoproterozoic period, which lasted from 1000 to 542 million years ago, and into the Early Cambrian, which lasted until around 513 million years ago. This implies that the evolution of the Porifera must have taken place in the Precambrian, well before the so called “Cambrian Explosion”. Chemofossil records even indicate the presence of sponges or their direct ancestors already in the Early Proterozoic (McCaffrey et al., 1994).

1.3 Sponges body structure and morphology

Sponges are sedentary filter-feeding organisms, characterized by an unusual body plan built around a system of water canals and chambers. The whole sponge body (Demospongia) is formed by individual cells which stretch from the outside or the dermal membrane to the inside or atrial membrane. The body wall is divided into three regions. The major component occupying the centre of the body wall is the choanosome, which contains the flagellated chambers. Within the chambers, special flagellated cells called choanocytes (collar-cells) produce a water current that enters the sponge body through surface pores (Porifera = bearing pores), and leaves it through larger openings called osculi. An external cell layer (pinacoderm) encloses the sponge mesohyl, a glycosidic matrix containing several cell types which perform a variety of functions. The choanocytes have one long whip-like appendage called a flagellum (or cilium) surrounded by a jelly-like "collar" which traps food particles, like bacteria and other unicellular organisms to take some for their nourishment, while the rest is absorbed and carried

off by amoebocytes, which are motile, totipotent cells that carry their cargo of food to all of the other cells. These ostia empty out into the spongocoel, or central "body cavity" of the sponge. The wastes of the body are carried here, and passed out with the filtered water through the osculum, which is the main large "hole" of the sponge, being entirely ex current, so water only flows out, but at tremendous speeds, so as to prevent the reabsorption of wastes. Sometimes there is more than one osculum. This incredible pump system created by the cooperation of collar cells, porocytes, and myocytes pumps tremendous quantities of water through the sponge body. The mode of sexual reproduction and embryogenesis, production of an extracellular matrix with collagen fibrils, possession of adhesion molecules and receptors for cell-cell contact as well as simple processes for signal transduction and a functional immune system (Müller, 1998) characterize sponges as true metazoan.

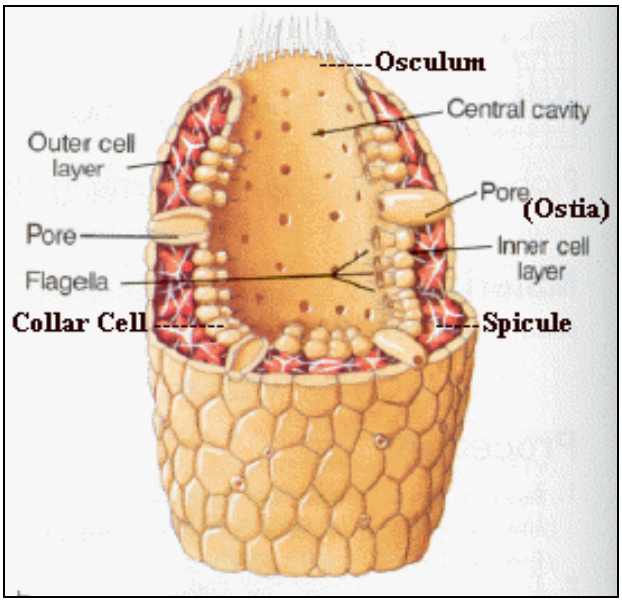


Figure 3: The schematic representation of the components of the sponge body wall.

Taken from http://johnson.emcs.net/life/_derived/invert.htm_txt_sponge.gif

1.4 The sponge skeleton, classification and reproduction

An internal skeleton is present in almost all sponges. Between the pinacoderm and the spongocoel lies the skeleton, which is surrounded by a jellylike mass known as the mesenchyme, or mesohyl. Within the mesenchyme lie the sklerocytes, which produce the

skeleton. The skeleton can be comprised of a few different substances: silica (like all of those classified as the Hexactinellida and Demospongiae); spongin (which is the absorbent protein which makes up the skeleton of the few sponges that can be used for bathing, all of which are included in the Demospongiae); a combination of the two (as with Demospongia); or calcium carbonate (as with those of the Calcarea). The skeleton is made up of rods called spicules, which can be either small (microskleres) or large (macroskleres). The microskleres form the main structure, which is supported by the macroskleres. They show a remarkable ability to communicate on the intercellular level, likely due to chemical perception and production. The spicules function as a kind of mesh work or internal skeleton in some sponges or as a protective device against predation in some others.

Sponges have three different types of body plans, although these morphologies do not define taxonomic groups. Asconoid sponges have the simplest type of organization. They are small and tube shaped sponges with internal part called the spongocoel that contains the collar cells. Syconoid sponges tend to be larger than asconoids and have a tubular body with a single osculum. The syconoid body wall is thicker and the pores that penetrate it are longer, forming a system of simple canals. These canals are lined by collar cells, the flagellae which move water from the outside, into the spongocoel and out through osculum. The third category of body organization is leuconoid. The sponges with this type of the skeleton are the largest and most complex. These sponges are made up of masses of tissue penetrated by numerous canals. Canals lead to numerous small chambers lined with flagellated cells. Water moves through the canals, into these chambers and out via a central canal and osculum.

Reproduction by sponges is by both sexual and asexual means. Asexual reproduction is by means of external buds. Some species also form internal buds, called gemmules, which can survive extremely unfavorable conditions that cause the rest of the sponge to die. Sexual reproduction takes place in the mesohyl. Male gametes are released into the water by a sponge and taken into the pore systems of its neighbors in the same way as food items. Spermatozoa are "captured" by collar cells, which then lose their collars and transform into specialized, amoeba-like cells that carry the spermatozoa to the eggs. Some sponge species (sp.) are monoecious; others are dioecious. In most sponges for which developmental patterns are known, the fertilized egg develops into a blastula, which is released into the water (in some species, release takes place right after fertilization; in others, it is delayed and some

development takes place within the parent). The larvae may settle directly and transform into adult sponges, or they may be planktonic for a time. Adult sponges are generally assumed to be completely sessile, but a few studies have shown that adult sponges in a variety of species can crawl slowly (Bond & Harris, 1988).

Sponges have recently gained wider interest for three major reasons. Firstly they are a rich source of a broad variety of bioactive compounds (reviewed in Sarma et al., 1993) that are useful to them e.g as a chemical defense against predators such as carnivorous fish (reviewed in Proksch, 1999). Secondly the elucidation of the genome composition and organization of sponges as "Living fossils" allow insight into the early evolution of Metazoa prior to the Cambrian explosion (reviewed in Mueller, 1998). Thirdly the cultivation of the marine sponges in bioreactors (Osinga et al., 1998) and in vitro (Mueller et al., 1999) has become possible for the first time.

1.3 Sponge bacterial association

Host specificity involves the association of organisms with only a few (specialization) or many (generalization) host species. This type of associations is very predominant in the eukaryote ecology as a key determinant of diversity. Bacteria and many other microbes are living in close symbiotic association with a number of higher organisms. Out of those the association between the bacteria and the sponges has been well studied and explored in detail as they are among the most interesting and also the most complex type of associations found in the marine environments.

These associations gained an added interest over the past few years due to the production of the biologically active compounds which were reported. Though convincing evidence exists only in a few cases it is tempting to assume that the microorganisms associated with the animals are responsible for the production of the active compounds. These microbes may contribute to the integrity and defense of their hosts by the production of the active metabolites. Sponge bacterium associations promote mutual proliferations of nutrients and digestion and recycling of materials. The mesohyl of marine sponges represents a unique niche to which a diversity of microorganisms adapts to. The diversity of these sponge associated microbial community profiles are expected to vary according to the existing abiotic factors such as depth, latitude and other biotic conditions such as the host and the secondary metabolite production (Lopez et

al., 1999). The bacterial load in sponges seems proportionally correlated with the irrigation status of the sponge. Sponges with a poor irrigation system contain high bacterial numbers while the well-irrigated sponges have fewer bacteria within their tissues (Wilkinson, 1978). Bacterial distribution follows a general pattern within every sponge.

Microbes associated with sponges can be divided into three categories: exosymbiont, endosymbiont and intracellular symbiont. The exosymbionts are present on the outer layers of the sponge while the endosymbionts are found in the mesohyl. The intracellular symbionts are present within the sponge cells and sometime inside the nuclei of sponge cells (Fuerst et al., 1999; Lee et al., 2001; Vacelet, 1970). As filter feeders sponges are capable of processing large volumes of seawater with microorganisms as the main sponge diet. Whereas most captured microorganisms serve as food particles and are phagocytotically digested, some are retained and form specific associations with sponges. These sponge–microorganism associations become a common phenomenon in nature. In the sponge "microcosmos" we can find unicellular algae (Vacelet & Donadey, 1977), cyanobacteria (Thacker & Starnes, 2003), dinoflagellates (Garson et al, 1998), zooxanthellae (Sara & Liaci, 1964), zoochlorellae (Gilbert & Allen, 1973; Williamson, 1979) and members of the domain Archaea (Preston et al., 1996; Fuerst et al., 1999). Discovery of bacteria in sponges (Vacelet, 1975), that can attribute as much as 40-60% of the total biomass (Wilkinson, 1978), opened a new field of scientific interest. Sponge-associated microbial consortia represent a huge variety from the poorly characterized phyla (Acidobacteria, Chloroflexi, Actinobacteria) the classes (*Alpha, Gamma* & *Deltaproteobacteria*) to recently discovered candidate phylum Poribacteria (Fiesler, 2005; Fiesler et al., 2004).

Immunological studies (Wilkinson, 1981) and biomarker investigations (Thiel et al., 1999; Thiel et al., 2002) indicate that sponge associated bacteria are passed on from generation to generation and over geological time. However, a comparative phylogenetic analysis of microbial symbionts from different sponges (Hentschel et al., 2002) suggests that the sponge microbial consortium contains a mixture of evolutionary ancient permanently associated bacteria and those that are acquired horizontally from the water column. Associated bacteria undoubtedly benefit from the protected and nutrient-rich environment within the sponge tissue. Several lines of evidence indicate that some sponges obtain a significant portion of their nutrition from the symbionts making the symbiosis a true mutualism.

The microhabitats on the surface and internal spaces of sponges serve as unique ecological niches. As water feeders, sponges are exposed to pollutants present in waters, and accumulated impurities from phytoplankton, or other suspended matters. Sponges swirl in a large volume of seawater containing organic particles. Hence, it is reasonable enough to believe that some microbes in sponges and/or sponges themselves produce hydrolytic enzymes to convert these organic matters into nutrients. In contrast to the literature on natural compounds, studies on enzymes with biotechnological potential from microbes associated with sponges are rare. Amylases, carboxymethyl cellulases, proteases, and other hydrolytic enzymes are widely used in the industry for the manufacture of pharmaceuticals, foods, beverages, and confectionery as well as in textile and leather processing, and waste water treatment which have been obtained from the sponge bacterial sources. (Lorenz et al., 2005)

1.4 Cultivation of sponge associated bacteria

Classical microbial techniques and cultivation approaches have been used in a number of studies to analyze the diversity of bacteria within sponges. Although bacterial cultivation is a prerequisite to study the physiological basis of possible symbiotic interactions between bacteria and sponges, its contribution to analyze the bacterial diversity is limited by the cultivation success (Button et al., 1993). Cultivation of bacteria is always highly selective due to the choice of media and culture conditions which usually allows only a small fraction of the bacteria present within a natural sample or sponge to grow and in consequence to be isolated. Special skills are required to include those bacteria that are of interest. No selective cultivation method is available to distinguish bacteria specifically associated with sponges from others, including those bacteria that serve as food particles. Therefore, cultivation approaches can only be part of a more general concept to define the diversity of bacteria associated with sponges and to identify the function they play within these complex associations. Early studies on the diversity of bacteria associated with sponges depended on the cultivation of the microbes as well as the microscopical observations of sponge sections (Wang, 2006).
The culture-based studies from environmental samples are known for their limitations because of “the great plate count anomaly” (Staley & Konopka, 1985). The colonies that are easily grown on plates usually represent less than 1% of all microbial cells present in the sample. Though for sponges there are some rare examples, like in the case of the

sponge *Ceratoporella nicholsoni*, where 3 to 11% of the total bacteria were culturable (Santavy et al., 1990), cultured sponge-associated bacteria generally follow the norm by representing less than 1% of the total sponge microbial cells being recovered as culturable colonies (Webster & Hill, 2001). This last decade saw molecular techniques being applied to the exploration and understanding of sponge-associated microbial communities. The use of community analysis 16S rRNA gene sequencing has overcome limitations associated with culture-based community studies and provided a detailed analysis of the sponge microbial community, revealing the presence of bacterial species never suspected before.

The first use of this culture independent technique on the marine sponge *Discodermia* spp. revealed the presence of novel *Gammaproteobacteria* and other uncultivated strains (Lopez et al., 1999). Another pioneer study using a similar approach was done on the Great Barrier Reef sponge *Rhopaloeides odorabile* and showed the remarkable microbial diversity in this sponge. Members of several bacterial classes constitute the microbial community of *R. odorabile* among which *Betaproteobacteria, Gammaproteobacteria*, *Cytophaga/Flavobacterium*, Actinobacteria as well as euryarcheotes and crenarcheotes (Webster et al., 2001 a, b). A comparative study of the microbial communities of the geographically distant sponges *Aplysina aerophoba*, *Theonella swinhoei* and *R. odorabile* suggested that there is a uniform microbial community among sponges from different oceans (Hentschel et al., 2002). It is important to emphasize that sponge-associated microbial communities have been shown to be very diverse and very different from those of the surrounding water column. The identification of bacteria present in sponges and the attempt to recognize specific relationships between these bacteria and the sponges forms the basis of a number of studies. Due to the selectivity of culture conditions, in many studies bacterial isolates with special physiological properties were obtained. These include aerobic chemoheterotrophic bacteria (Wilkinson et al., 1981), nitrogen-fixing bacteria (Shieh & Lin, 1994), methane-oxidising bacteria (Vacelet et al., 1996), phototrophic cyanobacteria (Wilkinson, 1978d; Simpson, 1984), and anoxygenic phototrophic bacteria (Imhoff & Truper, 1976). However, caution is indicated with all conclusions on a specific association of bacteria with sponges that result from culture experiments and identification of isolated bacteria, because the high selectivity of culture media would allow similar or identical bacteria to be specifically obtained from different

sources and at different times. Even if the selected bacterial strains are minor components in the sponge, they may become the dominant forms under the selected culture conditions. In addition to the more general limitations of the cultural approach, limited taxonomic information is yet another shortcoming as the bacteria are assigned to genera, but not identified on the species level. This is a serious handicap in considerations on specific associations between bacteria and sponges.

Because of the inherent selectivity of culture media and growth conditions, it is basically difficult to conclude from culture experiments on the specific association of bacteria with sponges. In fact, sponges represent a special habitat for bacteria that are significantly different from the surrounding seawater and may include nutrient-rich and even microoxic niches. The different environmental conditions could give rise to a specific but non-symbiotic association of comensalistic manner and could explain the dominance of different bacteria in and on sponges compared with the surrounding seawater.

1.5 Research questions and aim for the study

The basic aim of our study is to find out if the marine organisms like sponges are host to any microbes capable of carrying out enzymatic metal oxidation reactions by the application of microbial culture techniques and molecular techniques. The reason behind the presence of these microbes in the marine organisms is very intriguing and forms the main source of motivation behind the study.

S. domuncula: The species is suitable for the identification of potential symbiotic micro organisms, since it can be kept under controlled laboratory conditions for over five years (LePennec et al., 2003). The enzyme responsible for the oxidation of the manganese in the medium was thought to be located on the surface of the spore like bacteria which was observed in the SubDo-03 cultures. The growth phases of the bacterium when studied also showed a lowering in the lag phase by the addition of manganese to the medium and an increase in the number of spore forming bacteria. The outermost layer of the spore forming bacteria, harboring the enzyme when subjected to protein analysis classified the enzyme to the family of multicopper oxidases, identified by their copper binding site motifs (Alexandre & Zhulin, 2000; Claus, 2003). Multicopper oxidases (MCOs) use multiple copper atoms and are classified into three types of copper (Cu) binding sites as cofactors required for the coupled

oxidation of not a restricted range of substrates (Dick et al., 2008).They have been implicated in the oxidation of both organic compounds and metal ions, e.g Fe (II) and Mn (II).This has been also proved in the species under consideration by determining the metal oxidation in the presence of divalent copper as a coenzyme. Until now no biochemical analysis of Mn (II) oxidase(s) had been possible. Therefore, comparative molecular biological investigation might help to solve the question on the physiological relevance of multicopper oxidase in Mn (II)-oxidizing bacteria in general and also of those bacteria as symbionts for their host.

Thus the presence of a bacterial species which is able to oxidize divalent metal ion manganese to its tetravalent form in the marine environment, if detectable in the marine Mediterranean sponge *S. domuncula* was the fundamental reasons behind this research. The most interesting aspect would be to determine the reasons behind the symbiotic association of the organism with the marine sponge *S. domuncula*. This would shed light on the possible environmental influences on the symbiotic microbial-host associations in the marine environment.

Chapter 2-Microbial assisted mineral deposition

2.1 The biogenic view of mineralization

Biomineralisation is a frequently used term in nanotechnology, astrobiology, geology and medicine. It is the process by which minerals are synthesized from simple compounds. The living organism provides a chemical environment that controls the nucleation and growth of unique mineral phase. Biomineralisation is widespread in the biosphere and hundreds of different materials are produced or assisted by a variety of organisms from the bacteria to the humans. One of the most significant findings of these studies is that in virtually all cases of biomineralisation, macromolecular structures composed of lipids, proteins, and or polysaccharides are intimately associated with the minerals phase and serve vital roles in their crystallization. Mineralisation is observed in nature by a few prokaryotes such as the magnotactic bacteria (MTB). They have been studied to form magnetosomes which are membrane bound, single crystal nanoparticles on the order of 30-120 nm in diameter formed within the bacterial cell. These bacteria have been isolated by applying a magnetic field to sediments from a freshwater swamp and are capable of reducing nitrate without accumulating nitrite and produced ammonia during growth.

2.1.1 Marine bacteria as bio-seeds for polymetallic nodules

Sponges which live in the deep-sea have been studied to harbor a large number of symbiotic and asymbiotic microbes, among which chemoautotrophic microorganisms capable of oxidizing inorganic energy resources can be considered. These chemoautotrophic bacteria can be speculated to form the bio-seeds for the mineralized phases in the marine environment. Very recently and by the application of high resolution-scanning electron microscopy (HR-SEM) approaches it has been possible to demonstrate that within the mineralized deposits of polymetallic nodules from the deep sea floor, and not on their surfaces, traces of bacterial imprints can be observed (Wang & Mueller, 2009; Wang et al, 2009 a, b).Thus this findings imply that bacteria had been buried alive during Mn deposition within the growing nodules.

Furthermore support that bacteria are involved as bio-seeds in (at least) the initial phases of nodule formation came from the studies which showed that the imprints of bacteria in the polymetallic nodules are decorated with S-layer structures (Wang et al., 2009 b).
It has been proposed that polymetallic nodules which are found on the deep sea floor at a depth of around 4,000-6,000 m serve as concentrators for Mn(II), and in turn can serve as energy source to Mn(II) oxidizing bacteria living inside the nodules (Ehrlich, 2002). The biogenesis of marine manganese nodules is till today a very interesting topic of research.
Various theories have been put forth for explaining this phenomenon. The biological involvement in marine manganese nodule development has been proposed by several researchers and it follows a common feature. Mn is accreted to nodules in the form of manganese oxide. The Mn is first sorbed by the nodules in the form of Mn^{2+} and only subsequently oxidized by certain bacteria to Mn(IV) oxides (Ehrlich, 1963, 1966 & 1968). The hydrated MnO_2 (H_2MnO_3) being of weak acidic properties, undergoes a proton displacement which is not catalyzed by bacteria (Ehrlich, 1981). In a subsequent reaction, the sorbed Mn^{2+} undergoes an oxidation reaction to form Mn(IV) which is bound to the nodule matrix, ready to react with another Mn^{2+}, now catalyzed by specific kinds of bacteria which are found on the nodules and the surrounding environment.

$$\mathbf{H_2MnO_3 + Mn^{2+} \rightarrow MnMnO_3 + 2\,H^+} \quad \text{… (Reaction i)}$$

$$\mathbf{MnMnO_3 + 0.5\,O_2 + 2\,H_2O \rightarrow 2\,H_2MnO_3} \quad \text{… (Reaction ii)}$$

Equation 1 Accretion of Mn^{2+} to nodules followed by its oxidation by manganese oxidizing bacteria. (Ehrlich, 1963).

It is observed that reaction (ii) is slow when compared to reaction (i) and therefore, the rate controlling step in the overall uptake by the manganese nodules. It is catalysed by specific kinds of bacteria, which contain a Mn^{2+} oxidizing enzyme system (Ehrlich, 1968). Even more solid evidence exists that biogenic Mn oxides are the most abundant and highly reactive Mn oxide phases in the environment, since they mediate redox reactions with both organic and inorganic compounds and sequester/deposit a variety of metals (Tebo et al., 2004).

2.1.2 Manganese bio-oxides.

Manganese (Mn) oxides are highly reactive minerals that play an important role in the elemental biogeochemical cycle, controlling the speciation and availability of many metals and organic compounds. In nature the Mn oxidation is influenced by physical factors such as pH and redox potential (EH). In general Mn(II) occurs as a cation in solution and is thermodynamically favorable under anoxic or reducing conditions.

Mn(IV) occurs as solid phase Mn oxides or hydroxide minerals and is the thermodynamically favored form under oxic conditions at circumneutral pH. Mn(III) is thermodynamically unstable and disproportionates to Mn(II) and Mn(IV) in the absence of organic compounds that chelate and stabilize Mn(III). For the same reason Mn(III) was not thought to be abundant in nature, however the recent data suggest it is more abundant than previously recognized in the form of intermediated formed in the biological oxidation reactions. It is also found in various suboxic zones in appreciable quantities where the Mn cycling is prevalent. And it has been detected to be having a high affinity complex for the siderophores which are produced by the Mn(II) oxidizing organisms.

Mn is an environmentally abundant transition element that rapidly cycles between reduced and oxidized forms in dynamic biogeochemical systems. Mn(II) is soluble, highly mobile, and initially released into the environment from hydrothermal fluids or weathering of primary silicates and carbonates. However, upon oxidation to Mn(III) or Mn(IV), Mn precipitates as a series of chemically and structurally diverse oxides.The chemistry and geochemical cycling of Mn in natural waters is thought to be controlled to a large extent by redox transformations between soluble Mn(II) ions and insoluble manganese oxides (Stumm & Morgan, 1981).The chemical transformations observed in the environment are kinetically slow and as a result the microbes are thought to act catalyze most of the Mn oxidation reactions (Cowen et al., 1986; Cowen et al., 1990; Fuller & Harvey, 2000; Harvey & Fuller, 1998; Kay et al., 2001; Mandernack & Tebo, 1993; Marble, 1998; Marble et al., 1999; Nealson et al., 1988; Tebo, 1991; Tebo & Emerson,1985; Tebo & Emerson, 1986; Tebo et al, 1984; Tebo et al., 1985; Tipping, 1984; van Cappellen et al., 1998; Wehrli et al., 1995). Hence most of the naturally occurring Mn oxide minerals are thought to be the result of microbial activities (Tebo et al., 2004). Microorganisms that oxidize Mn(II) to Mn(III/IV) are widespread in the nature and they include both the classes of bacteria and fungi.

The initial oxidation and mineralization of Mn is considered to be microbially controlled, because numerous isolated microorganisms have been shown to catalyze Mn(II) oxidation by up to five orders of magnitude relative to abiotic conditions. As a result, Mn oxides are commonly formed within hydrothermal plume particles, reaction rims on the surfaces of weathered mafic rocks (e.g basalt), layers within ferromanganese nodules, coatings on sedimentary rocks such as desert varnish, and as ubiquitous surface coatings on minerals in subsurface sediments and aquifer materials. Because the properties of the oxides (e.g. structure, surface area, and reactivity) are critically important in the sorption and ion exchange of major cations and trace element species from aqueous solutions, there has been a growing interest in determining the mechanisms where by bacteria oxidize Mn and characterizing the biominerals that form.

2.2 Microbial oxidation of manganese in the environment

All bacteria require low, submicromolar levels of Mn for growth and oxidation. Bacilli in general require more Mn for sporulation than during their vegetative growth period (Charney J, Fisher & Hegarty, 1951). Since the time of Beijerinck (1913); it has been known that bacteria can catalyze the oxidation of soluble divalent Mn (Mn^{2+}) to their tetravalent insoluble forms as (MnO_2), although the exact mechanism remains unknown. Till date three different classes of bacteria have been found out from the marine sources which could oxidize the divalent forms of manganese from the environment to its tetravalent forms.This broad phylogenetic diversity mirrors the physiological diversity of Mn(II)-oxidizing bacteria, as demonstrated by the well-studied model organisms: the gram positive spore-forming *Bacillus* sp. strain SG1 (De Vrind, 1986), the *Gammaproteobacteria Pseudomonas putida* MnB1 and GB-1 (Okazaki, 1997), and the Betaproteobacterium sheath-forming *Leptothrix discophora* strain SS-1 (Ghiorse, 1987).The mechanism by which the oxidation was carried out by the bacteria was found to be influenced by the Mn oxidizing enyme which was found in all the three phylogenetically different classes of *Gammaproteobacteria, Betaproteobacteria* and gram positive bacteria containing lower guanine/cytosine content. All the three different classes oxidize the manganese Mn(II) enzymatically on an exoplymer matrix surrounding the cell. The enzyme responsible for the oxidation was genetically determined to be similar to the family of

multicopper oxidases, identified by their copper binding site motifs (Alexandre & Zhulin, 2000; Claus, 2003).The studies of the Mn binding and oxidation by the bacterial cells have been observed under dormant conditions with mature spores suspended in natural seawater(Rosson & Nealson, 1982). The precipitated Mn is generally but not always found outside the cell (Nealson & Tebo, 1980), often associated with acidic cell surface polysaccharides or sheaths which may contain acidic polysaccharides (Van veen, 1973). Studies show that fungi are also able to achieve Mn oxidation (Thompson et al., 2005) in addition to bacteria. The *Acremonium*-like Ascomycete strain KR21-2, studied by (Miyata et al., 2004), is one model organism for fungal oxidation of manganese. A lag phase before the onset of Mn oxidation can also be observed in this species (Miyata et al., 2004).The fungal Mn oxidizing factor is an extracellular protein with laccase activity, which resembles the multi-copper oxidase of manganese oxidizing bacteria.

Yet another means of biogenic manganese oxidation in the environment is by indirect oxidation by the phytoplankton *Chlorella* sp. via alteration of pH in the vicinity of the algal aggregates where the reaction occurs (Richardson et al., 1988). The generation of elevated pH and oxygen in the vicinity of pelagic aggregates of photosynthesizing cells can be considered, in terms of physicochemical hydrodynamics, as a heterogeneous reaction at the surface of a particle suspended in a turbulent or laminar flow (Levich, 1962). Visible light is important in catalyzing Mn oxidation by driving photosynthetic removal of CO_2, with concomitant increases in pH. In response to pH changes, rates of oxidation increase by many orders of magnitude in the presence of light. Interestingly, light can also catalyze the photo reduction of oxidized Mn by dissolved organic humic acids (Sunda et al., 1983). Clearly in surface waters the role of light in the Mn cycle may be very important and will vary depending on the quantities and types of organic matter and phytoplankton present.

Although the function of biogenic Mn(II) oxidation remains enigmatic, the widespread presence of Mn in myriad ecological niches suggests that the diversity of microbial Mn(II) oxidizers will continue to increase. In addition, as our understanding of the mechanism of Mn(II) oxidation improves, our greater ability to detect bacteria that oxidize Mn(II) will probably lead to the discovery of new phylotypes.

Chapter 3 - Materials

3.1 Chemicals used

3- phenylphenol	Sigma, Taufkirchen
Ammonium persulfate	Sigma, Taufkirchen
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Albumin fraktion V	Roth, Karlsruhe
Bactopeptone	Roth Karlsruhe
Bromphenolblue	Roth, Karlsruhe
Copper chloride anhydrous	Roth, Karlsruhe
Chloroform	Serva, Heidelberg
Crystal violet stain	Sigma, Taufkirchen
Ethanol	Peqlab Biotechnologie GmbH,
Ethidium bromide	Roth, Karlsruhe
EDTA (Ethylene diamine tetracetic acid)	Boehringer, Mannheim
Formaldehyde	Roth, Karlsruhe
Formamide	Roth, Karlsruhe
Gelatine	Roth, Karlsruhe
Gelcode blue stain reagent	Serva, Heidelberg
Glycerol	Pierce, Bonn
Glutaraldehyde	Roth, Karlsruhe
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma, Taufkirchen
Hydrochloric acid	Roth, Karlsruhe
Fount india	Pelikan, Hanover
Isopropanol	Roth, Karlsruhe
Leucoberberlin blue	Merck, Darmstadt
Lysozyme	Sigma, Taufkirchen
Maltose	Sigma, Taufkirchen
Malachite green oxalate	Merck, Darmstadt
Manganese chloride tetrahydride	Roth, Karlsruhe
Mn Agar	Sigma, Taufkirchen

N-Morpholino-ethanesulfonic acid (MES)	Sigma, Taufkirchen
Nitrate test strips	Merck, Darmstadt
Phenanthroline	Roth, Karlsruhe
Oxidase test strips	Merck, Darmstadt
Phenol	Fluka,Taufkirchen
Phenyl methane sulfonyl fluoride (PMSF)	Sigma, Taufkirchen
Potassium permanganate	Roth, Karlsruhe
Proteinase inhibitor cocktail, complete-mini	Roth, Karlsruhe
Roti-Quant R250	Roth, Karlsruhe
Saffranin-O	Roth, Karlsruhe
Seawater	Sigma, Taufkirchen
Sodiumdihydrogenphosphat-2-hydrate	BIOZYM, Hess.Oldendorf
Sodiumdodecylsulfat (SDS)	Roth, Karlsruhe
Sulfamic acid	Roth, Karlsruhe
Sulfuric acid	Roth, Karlsruhe
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Triton X-100 (Octylphenolpoly(ethylenglycolether)n)	Roth, Karlsruhe
Tryptophan medium	Sigma,Taufkirchen
Tween 20 Poly(oxyethylen)$_n$-sorbitan-monolaurat	Roth, Karlsruhe
Wheat germ agglutinin	Invitrogen, Karlsruhe
Yeast extract	Roth, Karlsruhe

3.2 Equipments used

Centrifuge: sorvall RC 5B	DuPont, Bad Nauheim
Eppendorf centrifuge 5402	Eppendorf, Hamburg
Gemini Leo 1530 HR-SEM	Carl Zeiss, Oberkochen
Heraeus Biofuge fresco	Kendro,Hanau
Electrophoresis apparatus	Bio-Rad, München
Homogenizer 24	Precellys, Erlangen
Heatblock Thermostat 5320	Eppendorf, Hamburg
Heatplate	IKA Labortechnik, Staufen
Laminar Flow	Sterilbank Slee, Mainz
Light microscope	Olympus AHBT3, Hamburg
Mini Protean II	Bio-Rad, München
Pipettes (2, 10, 20, 100, 200, 1000 µl)	Gilson, Frankreich
Perkin Elmer spectrophotometer (model zv 5500)	Rodgau,Jügesheim
pH-Meter Typ CG 840	Schott, Mainz
Pharmacia LKB-Ultrospec III	Pharmacia, St. Louis, USA
Spectorphotometer SmartSpec Plus	Bio-Rad, München
Spectorphotometer Titertek Multiskan Plus	Bartolomey Labortechnik, Rheinbach
Sunrise microplate reader	TECAN, Männedorf, Switzerland
Thermomixer comfort	Eppendorf, Hamburg
UV-Crosslinker Stratalinker 1800	Stratagene, La Jolla, USA
Vortexer	Labotech, Wiesbaden

3.3 Media

M19 BMS-N medium (Helmke & Weyland, 1984)	
$(NH_4)_2HPO_4$	1 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	25 g
Marine salts mixture	16.7 g
Yeast nitrogen base medium	10 ml
Distilled water	990 ml
pH	7.2

M20 BMS Medium (Helmke & Weyland, 1984)	
KH_2PO_4	0.5 g
K_2HPO_4	1.5 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	25 g
Marine salts mixture	16.7 g
Distilled water	1000 ml
pH	7.2

Modified K Medium (Rosson & Nealson, 1982)	
Bactopeptone	2 g
Yeast extract	0.5 g
Manganese chloride	0.019 g
75% Natural seawater	1 l
pH	6.8

M1 LB Medium modified	**Cat no. X968.3 (Roth, Karlsruhe)**
LB Medium dissolved in 50% artificial seawater	
pH	7.2

M23 BHI Medium modified	**Cat no.299070 (Difco, Augsburg)**
Brain heart infusion medium (BHI) dissolved in 50% artificial seawater	
pH	7.2

Chapter 4 - Methods

4.1 Collection of animals and bacterial isolation

Specimens of the marine sponge *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) had been collected in the Northern Adriatic Sea near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 17° C for more than five years.

Tissue samples (200 mg) were taken and minced with seawater (1:1; wt/vol). Serial dilutions of that sponge extract (10^{-1} to 10^{-5}) were prepared and aliquots of 0.1 ml were placed onto manganese agar plates (Ghiorse, 1984). Plates were incubated at 28 °C for 48 up to 72 h a period during which bacterial species, oxidizing Mn(II) ions, could be detected and stored at 4 °C for further use. The brown colonies of Mn(II)-oxidizing bacteria were picked and grown at 28 °C, while shaking, in liquid medium (Fig.5,6), a modified K-medium pH 6.8; consisting 1 l of 75 % natural seawater, 2 g bacto-peptone, 0.5 g yeast extract, and 0.019 g $MnCl_2$, as described by Rosson & Nealson, (1982). In controls, the microorganisms were cultivated onto manganese agar plates (Fig.8) or in K medium, lacking Mn (Fig.7). For subculturing a 1:10 dilution of the stock culture with modified K-medium was performed. The bacterial strains so isolated on the Mn agar plates were numbered SubDo-01 to 08. The Mn(II)-oxidizing microorganism, isolated from *S. domuncula*, has been designated as strain SubDo-03.

4.1.1 Maintaining pure cultures and general storage of the isolate

The bacterial colonies showing manganese oxidizing activity which were isolated on the Mn agar plates (Fig.8) from the *S. domuncula* extracts were picked up and separated from the other colonies to be maintained as pure cultures by streaking. Every month new agar passages/transfers of the bacteria had been performed. These cultures were stored at 4 °C in plates sealed with Para film and used as stock cultures. The colonies were inoculated in K-medium as described above for starting a new culture during every experiment.

4.2 Identification of the bacterial strains

The bacterial strain so isolated from *S. domuncula* designated SubDo-03 was identified for physiological and biochemical analysis by performing the following studies.

4.2.1 Morphological characterization of SubDo-03

Morphological characterization of SubDo-03 was carried out on the strains which were grown upto 72 h on the manganese agar plates. Several characteristic features were examined such as surface characteristics, consistency, type of margin and elevation. Whole colony formation and colour were determined with the naked eye.

Gram staining was examined under a light compound microscope. Gram reaction of a smeared colony was done by using 3% KOH as well as by staining with crystal violet; decolourisation with ethanol and counterstaining with safranin (Süßmuth et al.,1999). Phase contrast microscopy was carried out to determine the presence of endospores in the culture as described under (4.3.3).

4.2.2 Biochemical and physiological characterization

All the physiological and biochemical characteristics for SubDo-03 were studied using standard procedures (Baumann et al., 1972; Süβmuth et al., 1999). To perform the biochemical analysis 10 ml of test media was inoculated in sterile test tubes. An incubation temperature of 28 °C and test media containing equivalent salts content to half strength seawater had been chosen for the following tests.

Catalase activity was determined using 3% H_2O_2, presence of cytochrome oxidase was tested using oxidase test strip Bactident® oxidase,(Merck, Darmstadt). Reduction of nitrate was determined in M_1 medium (Roth, Karlsruhe) supplemented with 0.1% KNO_3. Formation of nitrite was detected with nitrate-test strips (Merck, Darmstadt) after 3, 7 and 14 days. Production of H_2S gas and indole were tested using M_{23} medium (Helmke & Weyland, 1984). Indole production was detected with Kovac's reagent strips (Fluka, Taufkirchen) on tryptophan medium (Roth, Karlsruhe). Production of acid from different carbohydrates was done as described by Helmke & Weyland (1984) in M_{19} and M_{20} medium respectively.

4.3 Growth characteristics of SubDo-03

The bacteria isolated were studied for their growth characteristics under varying conditions of medium composition, temperature and in varying concentrations of substrates in order to determine its relation to the enzyme activity.

4.3.1 Growth curves in medium with and without Mn

Bacterial cultures were grown at 28 °C in shake flasks with modified (as described in 4.1) K-medium at a pH constant of 6.8 for a period of 72 h. The inoculation volumes of the cultures were maintained throughout for all the experiments at a rate of 1:10. The medium was modified by supplementing Mn^{2+} in the form of 0.1 mM $MnCl_2.4H_2O$, to determine the difference in the growth rate of the cells. Optical density of the culture was measured at 600 nm on a Biorad Smart Spec 3000 UV/Vis spectrophotometer over a period of 72 h at different time intervals. Cultures grown in medium supplemented with Mn^{2+} were compared with those grown in the medium lacking it to determine a growing kinetics of the SubDo-03 culture.

4.3.2 Spore production by the bacteria and development in response to the addition of Mn^{2+} to the medium

The bacterial cultures were grown in modified K-medium at standard incubation temperature and pH conditions for a period of 72 h supplemented with 0.1 mM $MnCl_2.4H_2O$. After the cells enter the exponential growth phase, the spore production by cells was observed by staining the cells as described under (4.3.3). In parallel the cultures grown in medium devoid of any supplemented Mn^{2+} were also stained for the spores at similar time intervals.

4.3.3 Spore staining and observation

The bacterial cultures which were grown in modified K-medium supplemented with 0.1 mM $MnCl_2.4H_2O$ for a period of up to 72 h were used for preparation of the slides for spore staining.

The cultures were smeared onto glass slides, air dried, heat fixed and stained for the presence

of spores, applying the procedure described by Schaeffer & Fulton (1933). Visualization of the spores was achieved by staining with 7.6% malachite green (in distilled water) and counterstaining with 0.5% safranin. Microscopic analysis had been performed with an AHBT3 light microscope (Olympus, Hamburg).

4.3.4 Electron microscopic analysis

Scanning electron microscopy (SEM) analyses were performed to visualize the morphology of the spores. Bacterial colonies developed on Manganese agar plates were picked and allowed to grown overnight in modified K medium at 28 °C in the absence of presence of Mn(II). These overnight cultures were washed twice with sterile water and resuspended in 1x PBS (phosphate buffered saline). Samples were fixed in 0.1% glutaraldehyde and buffered in 50 mM HEPES (pH 7.9; in 75% seawater) overnight. The experimental control samples which were grown in medium without added Mn(II) were also treated similarly and used for the microscopic analysis. The samples so treated, were mounted onto aluminum stubs (SEM-Stubs G031Z; Plano, Wetzlar) and SEM was performed with a Gemini Leo 1530 high resolution field emission scanning electron microscope (Carl Zeiss, Oberkochen).
In addition samples grown in medium supplemented with manganese (II) for over 72 h were also analyzed for the change in the morphology of the spores, if any.

4.4 Manganese oxidation by SubDo-03 detection and quantification

Detection of manganese oxidation by the SubDo-03 species was carried out by three procedures. The identification was facilitated by in situ detection of the enzyme activity in a SDS gel. Further for the manganese oxidation quantification, the direct enzyme activity was measured using an optical and a spectrophotometeric test system using atomic absorption spectroscopy (AAS).

4.4.1 Isolation and purification of spores from the bacterial cultures

For the detection of the manganese oxidation by the in-gel oxidation assays, the isolation of the spores from the bacterial culture was required, as they were supposed to contain the enzyme responsible. Cultures were prepared in modified K medium from the brown colonies,

developed on Mn agar plates, by inoculation of the colonies in the medium for 24 h at 28 °C. During that period the cultures developed spores (Goldman & Tipper, 1978; Rosson & Nealson, 1982).These spore containing cultures were used for the in gel oxidation experiment as decribed in (4.4.8.2). The bacteria were pelleted by centrifugation (10,000 x g; at 4 °C), washed with deionized water and suspended in 10 mM Tris buffer (pH 7.5). The cell pellets were treated with lysozyme (50 µg/ml; 30 min at 37 °C) to lyse the remaining vegetative cells. The resulting suspension was washed once with 1 M NaCl containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) and subsequently five-times with deionized water in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) and 5% (w/v) phenylmethane sulphonyl fluoride (PMSF; pH 7.5) to inhibit the protease activity. The resulting spore samples had been collected und used for the determination of the Mn (II)-oxidizing activity**.**

4.4.2 Measuring protein concentration

The Bradford dye-binding assay is a colorimetric assay for measuring total protein concentration (Bradford, 1976). It involves the binding of Coomassie Brilliant blue to protein. Roti quant 5x (Roth, Karlsuhe) is diluted with water in a ratio of 4:1. For each sample (25 µl) 1 ml of this dilution is added and incubated at room temperature (RT) for 5 min. Samples are placed in 96-well plate in duplicates (100 µl per well) and set on the multiplate reader. Absorbance was measured at a wavelength of 580 nm. Samples to be measured are diluted appropriately and if the concentration of the protein is unknown several samples of various dilutions are made. Protein standards are prepared in the same buffer as the samples to be assayed. A convenient standard curve is made using bovine serum albumin (BSA) with concentrations of 0, 62.5, 125, 250, 500 and 1000 µg/ml for the standard assay. Using protein standards a graph of absorbance at 580 nm vs. protein is prepared. The Bradford assay gives a hyperbolic plot for absorbance versus protein concentration, but within a range of relatively low protein concentrations, the hyperbolic curve can be approximated reasonably well by a straight line. The results are used to graph the standard curve from which unknown protein concentration is determined.

The Bradford method is based on the noncovalent binding of the anionic form of the dye Commassie Blue G-250 with protein (Mikkelson & Cortón, 2004). The dye reacts chiefly with arginine residues which have positively charged side chains, but slight interactions have also

been observed with basic residues(histidine and lysine) and aromatic residues (tyrosine, tryptophan and phenylalanine). In the absence of protein the dye reagent is pale red which immediately turns to blue upon binding to proteins with an absorbance maxima at 590nm.

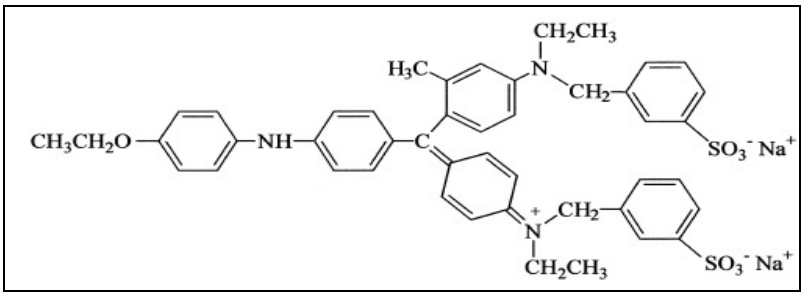


Figure 4: Structure of Commassie blue G-250 used in the Bradfords total protein assay.

Adapted as a reference from Spectroscopic methods for matrix characterization, (Mikkelsen & Corto´n, 2004)

The Bradfords assay is a very popular method for the colorimetric protein assay as it involves single addition of the dye reagent to the sample. It also provides a non-linear calibration curve of A_{590} against concentration over the 0.2-20 µl range of total protein contained in the sample volume of 20 µl (10-1000µg/mL). Negative deviation from linearity occurs with this method as with Lowry and Smith methods. In the Bradfords assay, curvature is due to depletion of free dye at high protein concentration and a better approximation of linearity can be achieved by plotting A_{595}-A_{465} against concentration to take dye depletion into account.

4.4.3 *In situ* detection of manganese oxidation activity

To determine the manganese oxidizing activity by the in-gel oxidation experiment (Francis et al, 2001; Francis & Tebo, 2002) from the cultures, the treated bacterial extracts as described (in isolation and purification of spores from bacterial cultures) above were homogenized at 6,500 rpm in Precellys 24 homogenizer (Peqlab, Erlangen) and solubilized in 2X Laemmli sample buffer with β-mercaptoethanol (Wiens et al., 2005). The enriched spores had been subjected to a Precellys 24 homogenizer (6500 rpm for 3min) to obtain a stripped spore fraction and the outermost spore layers, containing most of the enzyme activity, was collected by centrifugation at 24,000 x *g* (8 min). The supernatants were assayed for Mn (II)-oxidizing activity by sodium dodecyl sulphate polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis has a number of uses, which include the establishment of protein size, protein identification, determination of sample purity, identification of disulfide bonds, quantification of proteins and blotting applications. SDS, sodium dodecyl (lauryl) sulphate, is an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform negative charge, so that they can be separated solely on the basis of their size.

The SDS has a high negative charge, which overwhelms any charge of the proteins giving them an approximately equal negative charge. (Al-Tubuly, 2000). The number of SDS molecules that bind to a protein is proportional to the number of amino acids in the protein. SDS also disrupts the forces that contribute to protein folding (tertiary structure), ensuring that the protein is not only uniformly negatively charged, but then linear. SDS is present in the loading dye, the gel and the running buffer. All proteins migrate to the anode. Furthermore, β-mercaptoethanol is also present in the loading dye, so that the tertiary structure of the proteins is destroyed by cleavage of disulfide bonds, thus breaking the only known covalent bond between the polypeptide chains. The following denaturation at 95 °C for 5 min is responsible for an unfolded state and an elongated conformation of the proteins. The migration distance during the electrophoresis is then linearly dependent on the logarithm of the molecular size (Weber & Osborn, 1969). Through using protein of known molecular mass, it is possible to elevate the molecule mass of the polypeptide chains.

In a discontinuous system, used in this study, a non-restrictive large pore gel (stacking gel) is layered on top of a separating gel (resolving gel). Each gel is made with a different buffer and the tank buffers are different from the gel buffers. In the presence of the radical former ammonium persulfate and the catalyst tetramethylendiamine, acrylamide can polymerize into long chains which are linked to form a net-like porous gel structure by N,N'-methylenbisacrylamide. The size of the pores of the matrix is determined by the concentration of acrylamide and N, N'-methylenbisacrylamide so that the range of separation can be selected as required. For performing $NaDodSO_4$-PAGE, vertical electrophoresis tanks from Mini Protean II from Bio-Rad were used (minigels: 7 cm x 8 cm x 0.75 or 1 mm). The gels were used directly or stored under humid conditions at 4 °C until use. Separation of the samples was

done in 10% resolving gels, overlaid with a 5 % stacking gel and staining was performed by Commassie Brilliant Blue.

In situ assay for detection of Mn (II) oxidation activity was determined as described (Francis & Tebo, 2002). The gels were first incubated in 0.5% Triton X-100–10% glycerol (30 min) to remove sodium dodecyl sulfate and then incubated in 10 mM HEPES buffer (pH 7.6), supplemented with 200 µM $MnCl_2$. The process of Mn (II) oxidation was visualized in the gel by the formation of brown Mn oxide bands that appeared after 4 h of incubation (Fig 17).

The sensitivity of manganese (II)-oxidizing activity to copper chelators was confirmed as described by (Francis & Tebo, 2002) by incubating the gels in HEPES buffer (pH 7.6) containing *o*-phenanthroline (50 µM) for 15 min prior to the addition of 200 µM $MnCl_2$.

4.4.4 Colorimetric identification and quantification of manganese oxidizing enzyme activity

In order to identify the presence of precipitated (oxidized) manganese in the medium a colorimetric test assay, using leucoberberlin blue (Rosson & Nealson, 1982) was used. The bacterial culture containing the spores were incubated into modified K-medium supplemented with 50 µM to 200 µM of $MnCl_2.4H_2O$, for 6 h to 24 h. The cultures were centrifuged down to test for the manganese oxidized in the medium. Aliquot of 0.05 to 0.25 ml of cell-free medium had been taken to determine the amount of oxidized manganese by addition of 0.5 ml of a 0.04 % (w/v) leucoberberlin blue (Krumbein & Altmann, 1973); leucoberberlin blue (LBB) which is oxidized in the presence of Mn(III) and Mn(IV) changes its colour from colourless to blue. The color shift was monitored at an optical density of 620 nm. Potassium permanganate was used as a standard to quantify the manganese oxidized in the medium at the end of the incubation period.

4.4.5 Spectrophotometric quantification of the enzyme activity from the SubDo-03 cultures using atomic absorption spectroscopy

To confirm for the oxidation of Mn^{2+} carried out by the bacteria in the liquid media, atomic absorption spectrophotometry (AAS) was performed on the bacterial cultures. The medium containing the bacterial cells were used for the detection of the manganese oxidized. As a result it can be hypothesized that the concentration of the manganese oxidized should be higher than that of the cell free medium. All experiments for the quantitative determination of Mn^{2+} oxidation were carried out with reaction mixture placed into 100 ml Erlenmeyer flasks. To 25 ml of growth medium, 0.3 to 0.9 ml of $MnCl_2.4H_2O$ was added in concentrations ranging from 20 μM to 200 μM. The culture volume added to all the flasks was 4 ml. The samples were allowed to grow at 28 ºC and pH 6.6 for a period of 12 h. A sample without any added culture was used for equilibration. AAS was used to quantitatively determine the amount of Mn^{2+} in the cultures by a Perkin-Elmer (model 5500zl) spectrophotometer (Rodgau, Jügesheim).

4.5 Cell wall containing bacterial polysaccharide

4.5.1 Production and isolation of bacterial cell bound polymer from SubDo-03

In order to produce cell bound polymer for testing of the metal binding capacity by the SubDo-03 cultures, cells were grown in a 2 liter flask containing 1 liter of modified K-medium supplemented with 3% glucose. The culture was grown at the standard incubation temperature of 28°C over a period of 72 h. At the end of the incubation period, the cells were harvested and cell bound polymer was extracted and concentrated following (Underwood et al., 1995). The bacterial cells were harvested by centrifugation at 5,000 rpm at 4 °C for 1 h. The supernatant was collected and the cell pellet was treated with 10 mM EDTA, vortexed for 15 min and re centrifuged at 5,000 rpm at 4 °C for 1 h to extract the exopolymer.

The cell bound polymer was pooled together with the supernatant, filtered through 0.22 μm filters (Schleicher & Schuell) and concentrated using a rotary evaporator. The concentrated polymer was precipitated using chilled ethanol in a ratio of 3:2 (v/v) of the filtrate to ethanol

(Bhosle et al., 1995) and dissolved in small volumes of distilled water; to remove excess salts they were dialyzed using Snakeskin pleated dialysis tubing (7kDa Molecular weight cutoff, Pierce, Bonn) against distilled water for 2 days at room temperature (approx 25 °C). Excess water was removed under vacuum before lyophilisation and the exopolymer was stored at 4 °C until analysis.

4.5.2 Characterization of the cell bound polymer from SubDo-03

4.5.2.1 Colorimetric analysis

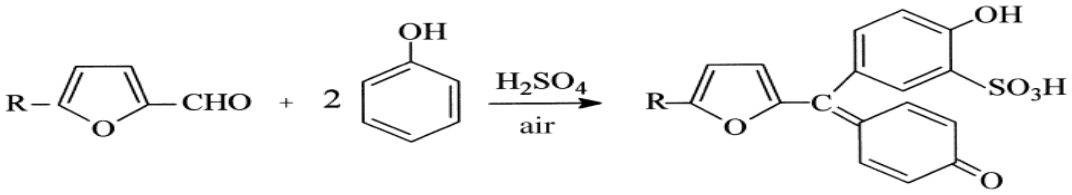
Uronic acid content of the cell bound polymer from SubDo-03 was determined by the m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973; Filisetti-Cozzi & Carpita, 1991). To 40 µl of (1 µg μl^{-1}) polymer solution, 40 µl of 4 mol l^{-1} sulfamic acid was added. After mixing well, 2-4 ml of concentrated sulfuric acid was added, vortexed and heated in boiling water bath for 20 min. After cooling on ice water bath, 80 µl of *m*-hydroxy diphenyl solution was added, mixed well and incubated for 10 min. The sample control was prepared with 80 µl 0.5% NaOH. The contents of the tubes were vortexed three times to ensure that they were mixed well. A pink color develops within 5 to 10 min and is stable for ~ 1 hr after which it fades. The colour that developed was read at 525 nm between 10 min and 1 hr after complete mixture against the reagent control. Standard solution of galacturonic acid was used for comparison (Filisetti-Cozzi & Carpita, 1991).

To prepare the *m*-hydroxydiphenyl solution 0.15 g of 3-phenylphenol was weighed out into a 100-ml volumetric flask, dissolved in <100 ml of 0.5% (w/v) sodium hydroxide, after which the final volume was adjusted to 100 ml with 0.5% sodium hydroxide and the solution stored in a dark bottle (or bottle wrapped in aluminum foil) at 4°C. This solution is stable for ~ 1 month.

Protein content of the cell bound polymer was determined by the Bradford protein assay (1976) using bovine serum albumin as a standard as described in (4.4.2).

The total neutral carbohydrate content was determined by phenol-sulphuric acid method (Dubois et al, 1956). In presence of concentrated sulphuric acid polysaccharides are hydrolyzed to their constituent monosaccharides, which are dehydrated to their reactive

intermediates. In presence of phenol, these intermediates form yellow products with a combined maximal absorbance at 492 nm.



Equation 2: Reactive intermediates from polysaccharides forming yellow products in presence of phenol.
Adapted as a reference from spectroscopic methods for matrix characterization, (Mikkelsen & Corto´n, 2004)

For the estimation of carbohydrate content, to 2 ml of the sample a volume of 0.05ml of 80% phenol was added in cuvettes. To this mixture, 5 ml of concentrated sulphuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the tube. The samples were allowed to stand at room temperature for 10 min and then shaken and placed in a water bath at 25 ° to 30 °C for 10 to 20 min. The absorbance is measured at 490 nm.Blanks are prepared by substituting distilled water for sugar solution. The amount of sugar in the sample was determined by reference to a standard curve of glucose.

All the colorimetric measurements are prepared in triplicates to minimize errors resulting from accidental contamination.

4.5.3 Binding of lectins to the cell wall of SubDo-03 cultures for direct visualization of the polysaccharides on the surface

The presence or absence of extra cellular polysaccharides on the bacterial surface was ascertained by simple negative and positive staining of the liquid cultures. Samples were grown in modified K medium (pH 6.8) for 24 h at 28 °C, and were analyzed for the coating on the surface of the cell. The samples so cultured were mixed with India black ink and observed under a light microscope at 10x magnification. The black ink provides a negative background to highlight the presence of polymer structures on the cell surface and precipitated Mn(IV) oxides, if any.

Lectins are carbohydrate binding proteins which can be used along with their fluorescent conjugates to stain the bacterial cell bound polysaccharide. Wheat germ agglutinin (WGA) is a widely common lectin, due to its binding specificity to the N-acetyl glucosamine and N-acetly muramic acid residues of the bacterial cell wall, and is used for the staining of the bacterial cell wall bound polysaccharides from the SubDo-03 cultures. Wheat germ agglutinin conjugated with Alexa Flour 488 (Invitrogen, Karlsruhe) was used to prepare a stock solution of 1mg/ml of the dye in phosphate buffered saline (PBS) at pH 6.8. The working solution was prepared by diluting the stock to 1:5 (v/v). Samples were prepared for staining by growing cultures in K medium pH 6.8 for 24 h at 28 °C. Slides were smeared with the pre grown cultures and the bacteria fixed onto them by heat. Some drops of the dye were added to the smear and incubated in the dark at 5 °C for 1 h to allow diffusion. The cultures so stained were carefully mounted with a cover slip after washing with PBS to remove the excess of the stain before fluorescence analysis. The stained cultures were observed under an Olympus AHBT3 fluorescent microscope.

4.5.4 Preparation of Mn (II) stock solution

Stock solution having 100 mM, 150 mM, & 200 mM of $MnCl_2.4H_2O$ were prepared by dissolving the appropriate quantity of the salt in de ionized water. The stock solutions were stored at 4 °C and working solutions of required concentrations were obtained by appropriate dilution in de ionized water prior to each experiment.

4.5.5 Metal binding by cell wall containing polymers of SubDo-03

Prior to each metal-binding experiment, the concentration of the cell bound polymer was determined by the standard phenol-sulphuric acid test (4.5.2.1) and a final known concentration was used.This solution of final concentration of was taken in the 7 kDa molecular weight cut off dialysis bag and suspended in a wide mouth beaker having a concentration of 100 mM concentration of manganese (II) in deionised water. The metal solutions containing the dialysis bags were incubated at 28 ± 2 °C on a magnetic stirrer for 48 h. After incubation, the dialysis bags containing the cell bound polymers were removed and transferred to another container having de ionized water. Samples were dialyzed overnight to remove any loosely bound metal ions. These metal complexed polymers were then subjected to double beam atomic absorption

spectroscopy (Perkin-Elmer model 5500zl) to quantify the metal ions bound to them. For blanks, the bacterial polymer was replaced with equal volumes of deionized water and incubated under similar conditions, as also another control containing the bacterial cell bound polysaccharide without metal treatment was incubated in deionized water (Bhaskar et al, 2006).

Chapter 5 - Results

The bacterial strain of SubDo-03 which has been isolated from S. *domuncula* showed the following characteristics in microbial, and biochemical terms.

5.1 Isolation of Mn (II)-oxidizing sp SubDo-03

5.1.1 Growth characteristics of SubDo-03

Mn (II)-oxidizing microorganism had been isolated from tissue of *S. domuncula* after selection on Mn Agar plates and then grown in modified K medium, as described under "Materials and Methods". The isolated strain described in detail in the thesis, SubDo-03, oxidizes $MnCl_2$ (supplemented to the K-medium) or $MnCO_3$ (present in the agar) as can be deduced from the color change from whitish, absence of Mn (Fig.5), to brownish in Mn-containing liquid cultures (Fig.6). The morphology of the bacterial smears on agar changed with the development of the manganese precipitation. While in the absence of manganese in the agar the smear forms more a continuum (Fig.7), the colonies formed on Mn-containing agar show islands of a size around 50 µm, surrounded by manganese deposits (Fig.8).

Fig 5 Fig 6

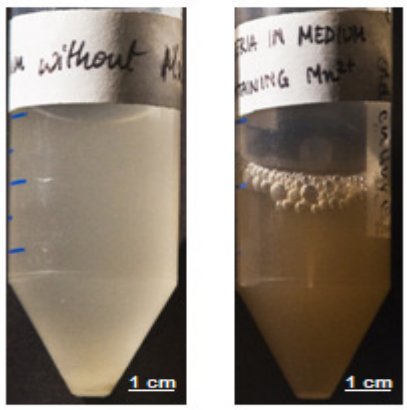


Figure 5: SubDo-03 cultures in medium without added $MnCl_2$.

Figure 6: Culture in medium containing 0.1mMMnCl $_2$ after incubation for 3 weeks at 28 °C shows the change of the medium colour to brown.

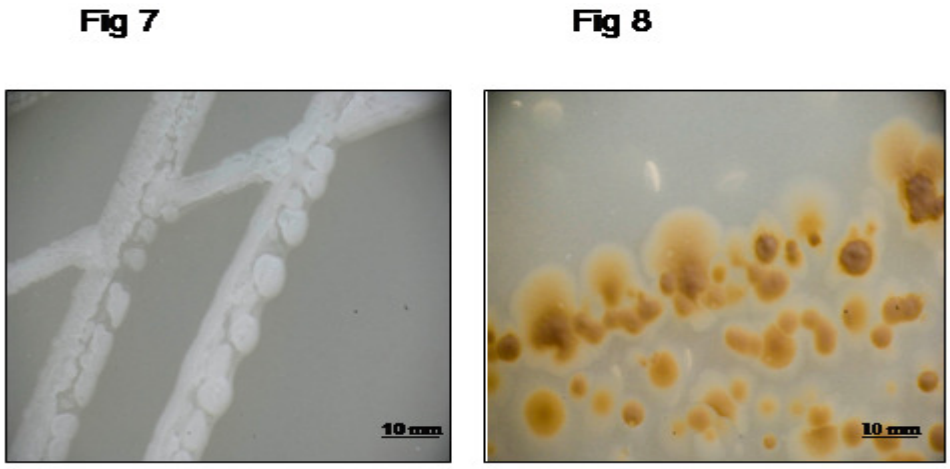


Figure 7: Close-up of SubDo-03 streak plates in medium lacking Mn.

Figure 8: and on Mn agar plates.

5.1.2 Growth curves and number of spore-forming bacteria produced by the cultures in presence or absence of added Mn (II)

The growth of the microorganisms is slow, at 28 °C in a modified K medium containing 0.1mM $MnCl_2.4H_2O$ as described under "Methods". The growth of strain SubDo-03 and its kinetics is dependent on the presence of manganese in the medium as is observed in all the experiments with inoculation volumes maintained at 1/10 to the K medium. In the absence of any Mn added a considerable lag phase of 20 to 24 hrs has been determined that is followed by a short increase in cell density prior to reaching the stationary phase after 32 h (Fig.10); Addition of 0.1 mM of $MnCl_2.4H_2O$ caused a reduction of the lag phase to 6 to 8 h; the stationary phase is reached after 16 to 20 h (Fig.9). Addition of Cu in the form of salt, at a concentration of 0.1 µM, does not change the growth kinetics of strain SubDo-03. The enumeration of spore-forming bacteria was carried out over the incubation periods by microscopic observations of the cultures after safranin/malachite green staining. The cultures showed the presence of the spore-forming bacteria only after 8 h of incubation in modified K medium, pH 6.8 at 28 °C. The number of spore forming bacteria showed an increase with the addition of 0.1 mM of $MnCl_2.4H_2O$, which signifies its dependence (Fig.9).

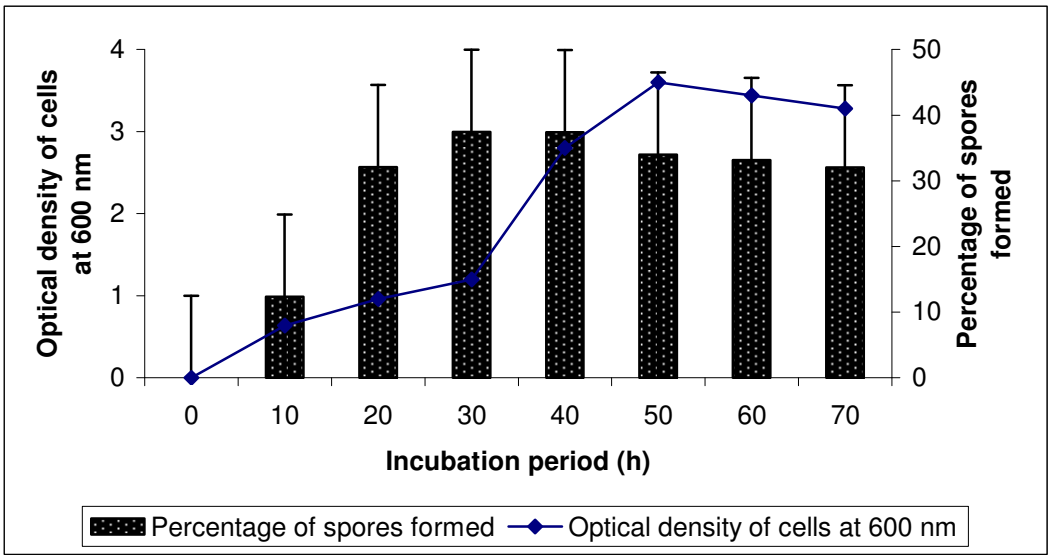


Figure 9: Measurement of cell growth given as the density of bacterial cells at 600 nm and enumeration of spore forming bacteria by SubDo-03 in presence of 0.1mM $MnCl_2$ added to K-medium (pH 6.8).

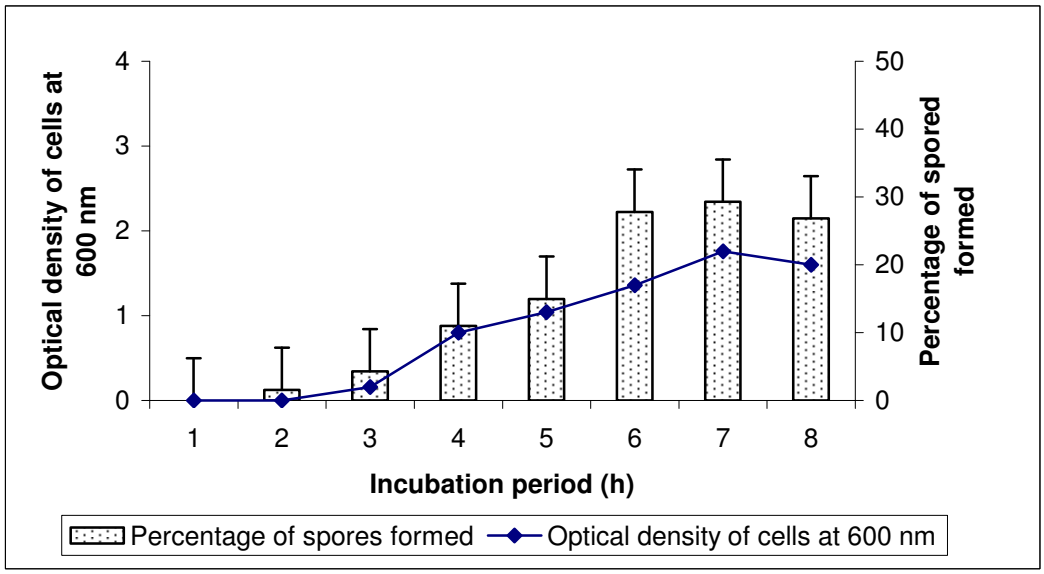


Figure 10: Measurement of cell growth given as the density of bacterial cells at 600 nm and enumeration of spore forming bacteria by SubDo-03 in the absence of added $MnCl_2$ to K-medium.

Growth kinetics and spore formation of strain SubDo-03 in K-medium (pH 6.8), in the absence or presence of 0.1 mM $MnCl_2.4H_2O$. The bacterial growth was measured spectrophotometrically as an increase of OD at 660nm while the concentration of spores forming bacteria at the same time intervals was microscopically examined after malachite green/safranin staining as described by Schaeffer & Fulton, (1933). Cultures are in triplicates.

5.1.3 Spore staining

Based on previous findings, indicating that the formation of spores is essential for the manganese oxidation activity by the bacteria (Rosson & Nealson, 1982; Francis & Tebo, 2002), experiments had been performed to determine if the presence of the spores in the cultures of SubDo-03 at various time intervals could be observed.

The spore staining procedure as described in the methods was able to demonstrate the presence of spore forming bacteria in the cultures. Spores are essentially refractile to most of the staining procedures and so appear as clear areas when stained with normal stains like gram stain (Knyasi, 1949). Just like poly-betahydroxybutyrate and other inclusions of the bacterial cells which are also not stained by normal staining procedures, they too appear as clear areas. Hence when the primary stain Malachite green is “driven” into the spore body by heat, the result is the formation of breaks in the spore wall such that the stain can penetrate the spore coat, cortex and the spore wall. After rinsing of the excess stain, the addition of safranin, acts as a counter stain, which will stain the vegetative cells pink. The spores can be observed as green bodies among them.

Spore staining can be observed in SubDo-03 culture after incubation of the cells in the K-medium for a minimum period of 8 h at the optimum temperature and pH conditions. The culture when stained shows the presence of spore like bacterial cells with a dark green central region among the negatively stained pink vegetative cells. The cultures grown in K-medium without any added $MnCl_2$ after 12 and 18 h of incubation showed the presence of the spore like bacteria as seen in (Fig.11) and (Fig.12). The SEM images of the same cultures grown in medium containing added $MnCl_2$ after 12 hours showed the presence of elongated spindle shaped structures as spore like bacteria which can be seen in (Fig.14) as compared to the cultures in the initial growth stages (Fig.13). After a prolonged incubation in the medium

containing added $MnCl_2$ up to 72 h, the SubDo-03 cells showed a brick like formation on their surface which is likely to be by the deposition of the Mn so oxidized (Fig.15).

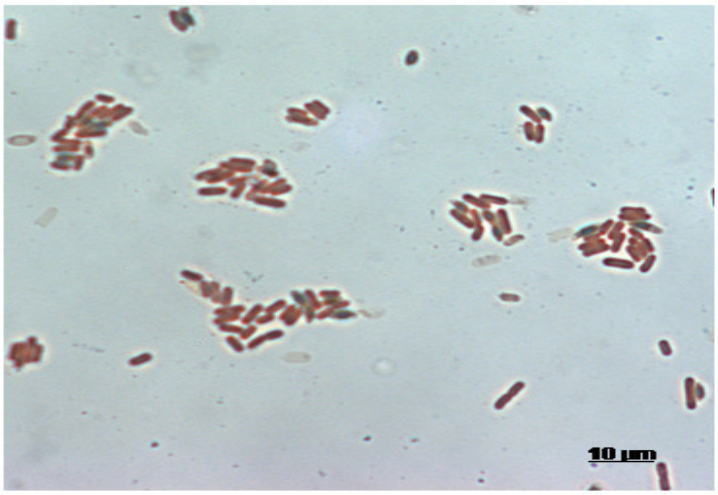


Figure 11: Spore staining of SubDo-03 cultures incubated in modified K-medium at 28 °C, pH 6.8 for 12 h, which is the time of onset of production of spores.

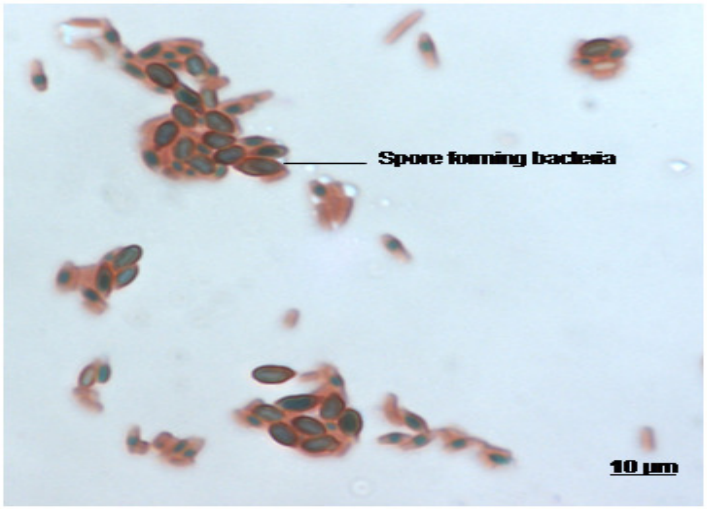


Figure 12: Incubation of the SubDo-03 cultures in modified K-medium for prolonged period of 72 hours at 28 °C, pH 6.8. Almost half of the normal cells have developed into spore forming bacterial cells which show increase in their size.

Spore formation of strain SubDo-03 as analyzed by light microscopy and SEM. Under conditions described in Fig 13 the onset of production of spore forming bacteria in the culture is between 16 h and 32 h in the standard assay. Hence samples were taken for observation between those times points.

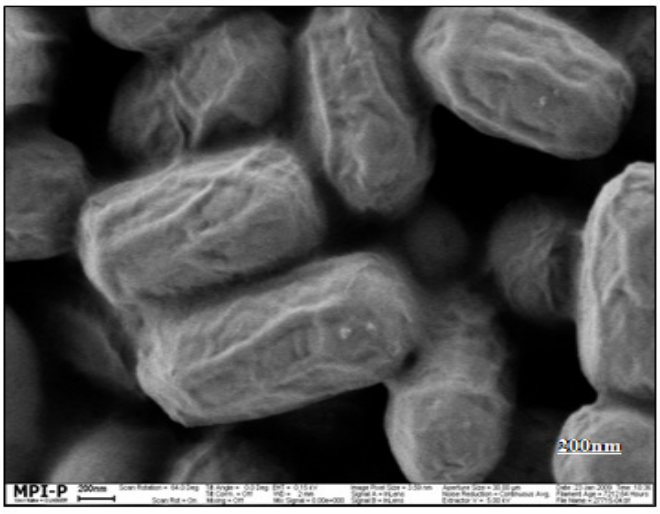


Figure 13: Culture incubated in medium without added $MnCl_2$ after 12 h as seen under SEM.

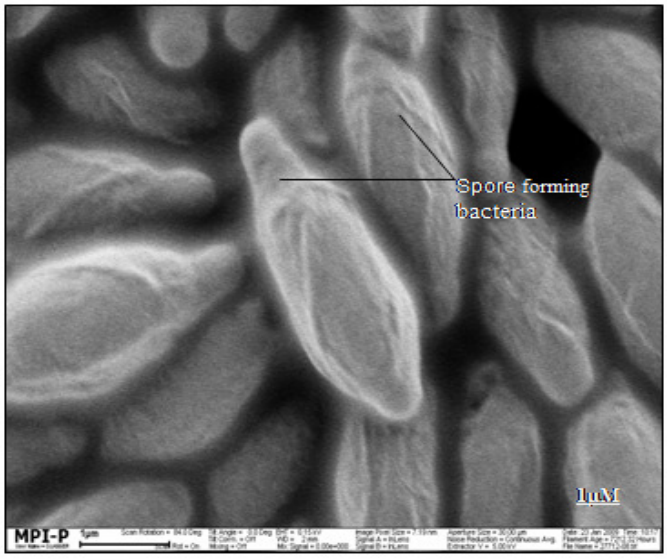


Figure 14: SEM image of the culture showing the spores as spindle shaped bodies after incubation in medium containing 0.1mM $MnCl_2$ for over 18 h.

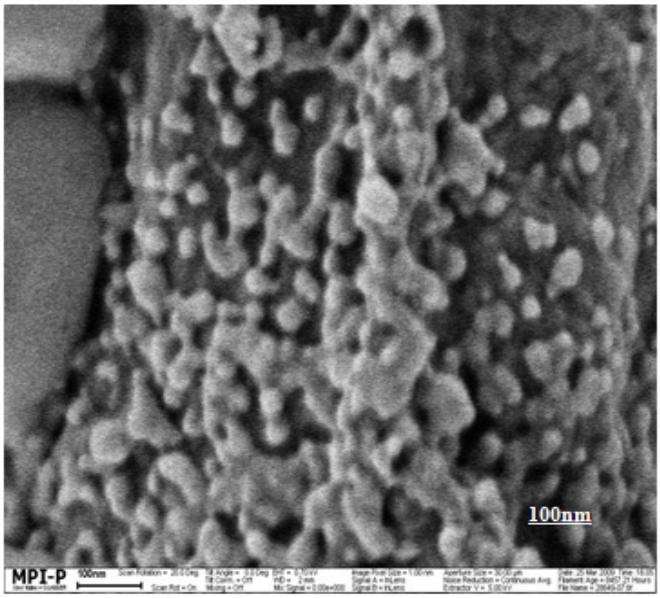


Figure 15: SEM image of the culture with a prolonged incubation of 72 h in medium containing 0.1mM $MnCl_2$, showing the presence of bricks formed by oxidized manganese on the surface of the cells.

5.2 Characterization of manganese oxidation activity by the spore forming bacteria of SubDo-03

5.2.1 In gel determination of manganese oxidizing activity

The Mn-oxidizing activity was determined in extracts from spore forming bacteria prepared from strain SubDo-03 that had been cultivated in modified K-medium for 24 h in the absence or presence of 100 µM of $MnCl_2$. Subsequently, the outermost layer from the spore forming bacteria has been obtained by physical treatment, as described under "Methods" where most of the Mn-oxidizing activity can be resolved (Francis & Tebo, 2002). Extracts of that layer was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE) to visualize (*i*) all proteins by Coomassie brilliant blue (Fig.16) and (*ii*) the Mn-oxidizing activity *in situ*, in the gel (Fig.17). The data revealed that after incubation of the gels in 100 µM $MnCl_2$ only in the extracts obtained from bacteria, grown in $MnCl_2$, a protein band could be visualized (Fig 17, + Mn^{2+}). The size of the band corresponds to 110 kDa. No band developed on the gel in extracts from those spores that had been developed in medium, lacking $MnCl_2$ (Fig 17, - Mn^{2+}). This implies that Mn causes an upregulation of the Mn-oxidation

enzyme in the outermost spore layer(s). In a previous study (De Vrind et al., 1986) had mentioned the existence of that activity in the layer(s) of spores formed from vegetative cells of Mn-oxidizing bacteria, grown in the presence of Mn. But until now no data existed about that activity in spores formed from cultures grown in medium not supplemented with Mn.
In controls, it has been clarified that the appearance of the band was inhibited by incubating the gels in the presence of the copper chelator, *o*-phenanthroline (data not shown). This finding is consistent with the experimental data that indicate the involvement of a metalloprotein (multicopper oxidase) in Mn-oxidation (Solomon et al., 1996).

In situ identification of Mn oxidizing activity in SubDo-03 cultures, from the outermost layer of spores. Samples from spores, developed in cultivating medium which had been Mn(II)-free, or had been supplemented with 100 µM of $MnCl_2$ [- Mn^{2+}; + Mn^{2+}], have been analyzed. The same extracts had been analyzed for the in-gel Mn-oxidizing activity.

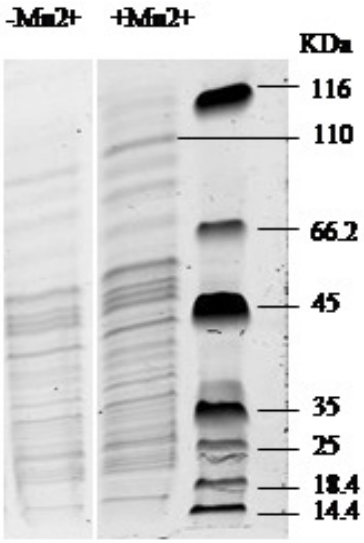


Figure 16: Total protein extract of SubDo-03 culture grown in medium without and with added 100 µM of $MnCl_2$ as seen after Coomassie staining.

After size-separation the gel has been treated in HEPES buffer containing 100 µM Mn(II) for 4 h to stain for Mn-oxidizing activity. It can be visualized that only in protein samples, obtained from spores that had been formed in the presence of 100 µM of $MnCl_2$ (+ Mn^{2+}) the 110 kDa band can be visualized. In contrast, no band was stained in a sample analyzed from spores, formed in the absence of Mn(II) (- Mn^{2+}).

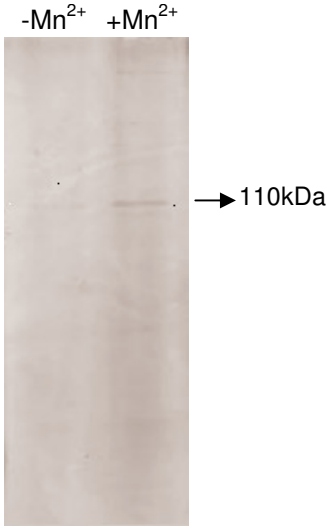


Figure 17: In situ Mn-oxidising activity of the SubDo-03 cultures.

The manganese oxidizing enzyme was observed as a protein of 110kDa band size as seen by Commassie staining on the SDS gel in Fig.16.

5.2.2 Effect of physical factors on the manganese oxidation activity of SubDo-03

To determine whether the Mn(II) oxidation by the cells of SubDo-03 was of enzymatic nature the concentration of oxidized manganese in the medium was determined from the cell free cultures using the colorimetric LBB reagent as described in "Materials and Methods".

5.2.2.1 Temperature

In order to substantiate an enzyme-driven oxidation of Mn(II) the microorganism samples had been incubated at the standard pH conditions over a range of different incubation temperatures (8 ºC to 37 ºC). The cell concentrations had been adjusted to a value of 3.26 x 10^8 cells/ml .At the standard temperature of 28 °C Mn-oxidizing activity was calculated from the standard curve to be 8.02µM/h over a period of 12 h of incubation (Fig.19).

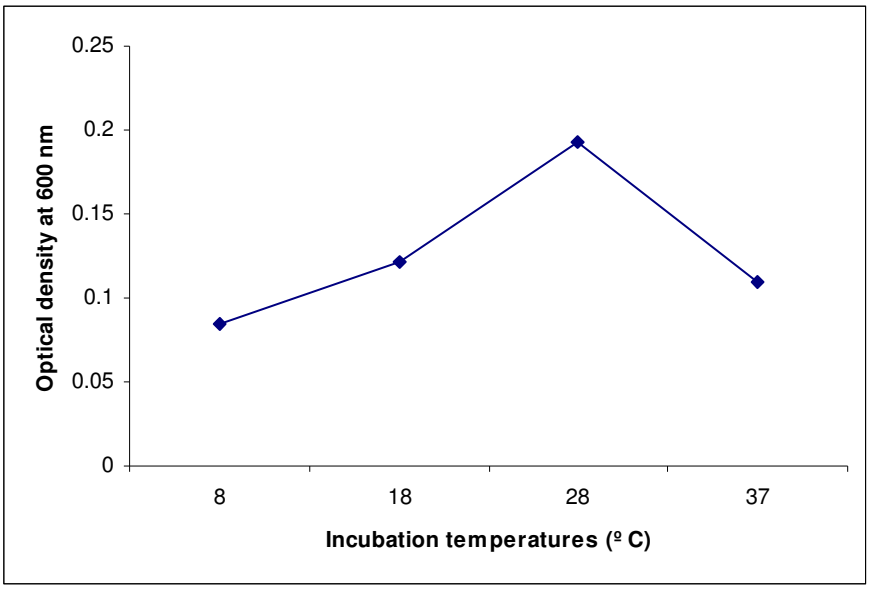


Figure 18: Growth of SubDo-03 cells at four different incubation temperatures, (8 º C, 18 º C, 28 º C and 37ºC) over a period of 12 h in the modified K medium at pH 6.8.

Mn(II) oxidation rates from the bacterial cultures were determined at four different temperatures, (8 ºC, 18 ºC, 28 ºC and 37 ºC) over a period of 12 h in the modified K medium at pH 6.8. The optimum growth of the cells was observed around 28 ºC, which was the temperature at which the bacteria were isolated (Fig.18). When the amount of Mn(II) oxide formed was studied over this range of temperature using the LBB test assay (as described in "Methods"), the maximum rate of oxidation was observed above the optimum temperature, which showed that the factor of temperature is important for the enzyme activity (Fig.19). The percentage of spores formed increased with increase in temperature and this in turn influenced the manganese oxidizing activity of the culture (Fig.19).

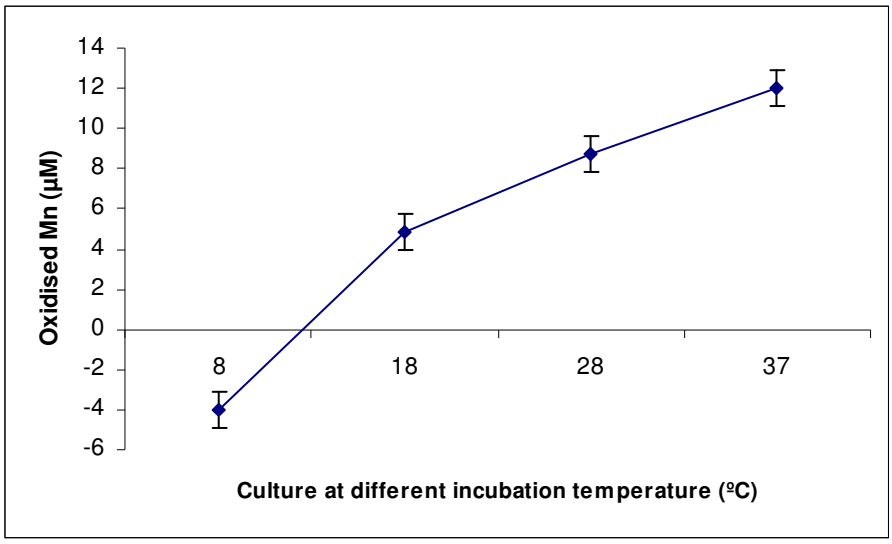


Figure 19: Manganese oxidation by the cultures at different incubation temperatures, values expressed as the observed colour shift of LBB dye at 620 nm. The enzyme activity was measured at a cell concentration of 3.26* x 10^8 *cells/ml over a period of 12 h.

5.2.2.2 pH

Similarly equal inoculation volumes of the culture samples were subjected to oxidation determination over a range of four different pH values (5.6, 6.1, 7.0 and 8.0) of the modified K medium with 0.1 mM Mn^{2+}added to it for 12 h. The cell concentrations were adjusted to a value of 1.38 x 10^8 cells/ml. At standard incubation temperature of 28 °C and at pH value of 7.0, a maximum oxidation was observed at 0.52 µM (Fig.20) as determined by the LBB test assay.

At pH values below and above the optimum range the cell growth was found to be slow (data not shown). This was comparable to all the studies that have so far found out that the rate of biologic Mn(II) oxidation is strongly influenced by pH, and are very slow at pH values less than 6 or greater than 8.5 (Fig.20).

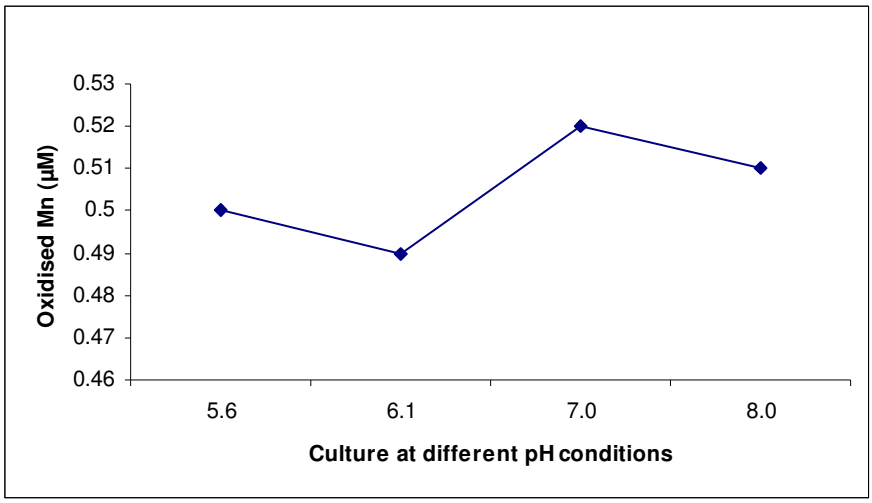


Figure 20: Manganese oxidation by the cultures in mediums with varying pH as measured by the LBB test assay using K-permanganate as a standard. The enzyme activity was measured at a cell concentration of* 1.38 x 10^8 cells/ml *over a period of 12 h.

5.3 Detection and quantification of manganese oxidation activity from SubDo-03

5.3.1 Colorimetric measurement of manganese oxidation activity, appearance and progress in the culture medium

The samples grown over a period of 24 h were used after which the rate of manganese oxidation was determined by the LBB assay as described above in "Methods". The culture with equal inoculation volumes was supplemented with different starting concentrations of $MnCl_2$ and their oxidation by the bacteria to the Mn(III) or Mn(IV) forms was measured by colorimetric ally measuring the colour shift at 620 nm at three different time intervals. In comparison, the medium with similar starting concentrations of $MnCl_2$ was measured at the similar conditions of incubation to determine the amount of auto oxidation by Mn. This was calculated to determine the reagent control.

Oxidation of manganese supplied in the medium by the culture of SubDo-03 as determined by the Leucoberberlin (LBB) test assay. Medium was supplemented with 50 µM to 200 µM of $MnCl_2$ as indicated and the concentration of oxidized Mn(II) by the bacteria against the auto oxidized Mn in the medium was determined colorimetrically at three different time periods between 6 to 24 h . Five independent experiments were performed and the mean values are indicated (Fig 21).

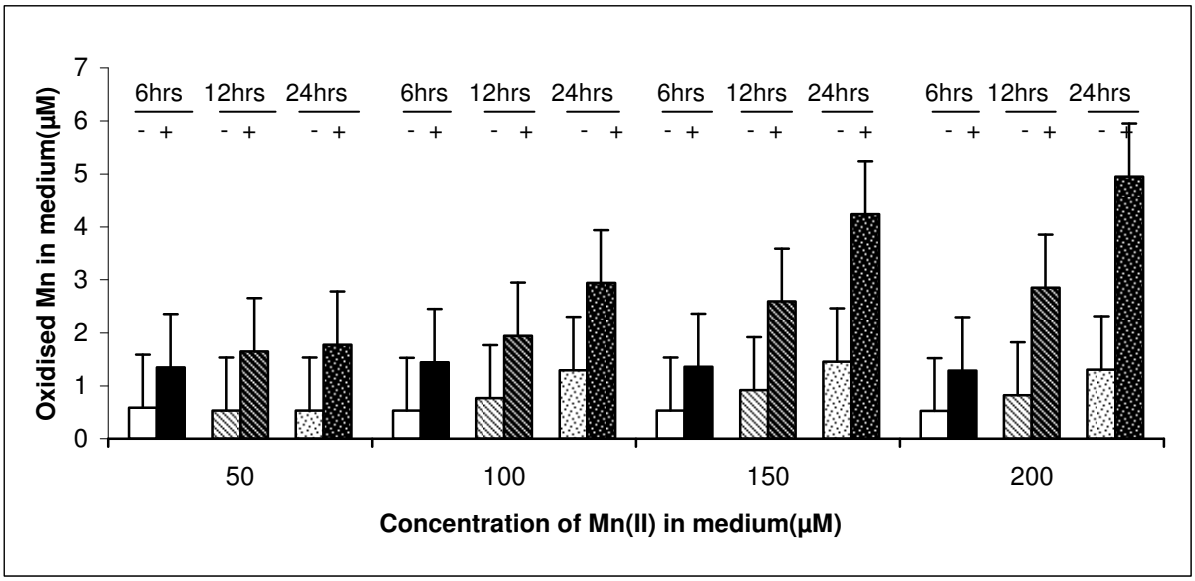


Figure 21: Quantification of oxidized manganese in the modified K medium pH 6.8, with different intial concentrations of added $MnCl_2$.The cell concentrations were maintained in all the samples to a value of 3.59* x 10^8 *cells/ml over three different incubation periods.(- , Sample without bacterial culture; + ,Sample with bacterial culture)

If the microorganisms were cultivated in the presence of 50 µM of $MnCl_2$ a time-dependent increase in oxidized manganese, Mn(III) or Mn(IV), was not determined in the medium. After 6 h the amount of oxidized manganese was determined to be 1.2 ± 0.253µM, a value which increases to 1.7 ± 1.414µM (12 h), or 1.8 ± 1.972µM (24 h), respectively. If the supply of $MnCl_2$ in the medium was raised to 200 µM a continuous increase of the concentration of oxidized manganese could be measured. At that concentration of $MnCl_2$ in the medium the level of oxidation increased to 1.2 ± 0.253µM (6 h), 2.82 ± 1.414µM (12 h) or 4.9 ± 1.972µM

(24 h), respectively (Fig.21).

5.3.2 Estimation and quantification of manganese oxidation in SubDo-03 cultures by spectrometric methods

The quantitative determination of the oxidation activity in the SubDo-03 cultures was carried out by measurement of the samples containing the spores for the manganese concentration by atomic absorption spectrometry. The determination of the enzymatic nature of the manganese oxidation prompted us to study the response of the enzyme to the change in the substrate concentration which is an important factor for any enzymatic reaction. Hence the SubDo-03 cultures were grown until they reached the initial exponential stage of their growth, thereafter supplemented with different initial concentration (10µM/l, 20µM/l, 30µM/l and 40µM/l) of Mn(II). To examine the effect of substrate concentration on the reaction velocity the increase in the manganese concentration was measured in the cultures at different initial substrate concentrations (Fig.22). Reaction velocity for different substrate concentrations was examined. And it was found out that the rate of Mn(II) oxidation was maximum of maximum initial Mn(II) concentration when the parameters of temperature, pH and copper concentration were kept constant (T=28 ºC, pH= 6.5 and zero Cu was added), as copper was found to be a cofactor affecting the enzymatic reaction.

To examine the effect of substrate concentration on the reaction velocity the increase in the manganese concentration was measured in the cultures at different initial substrate concentrations. The progressive curve of Mn^{2+} oxidation for different substrate concentrations was examined over a period of 12 h keeping the temperature constant at 28 ºC. The amount of Mn^{2+}oxidised increased with increasing substrate concentration The concentration of the manganese in the medium is observed to be higher than that in the cell free medium with the similar amounts of added $MnCl_2$ and after similar incubation times (Fig.22 and 21). This implies that the major portion of the oxidized Mn(IV) is associated with bacteria.

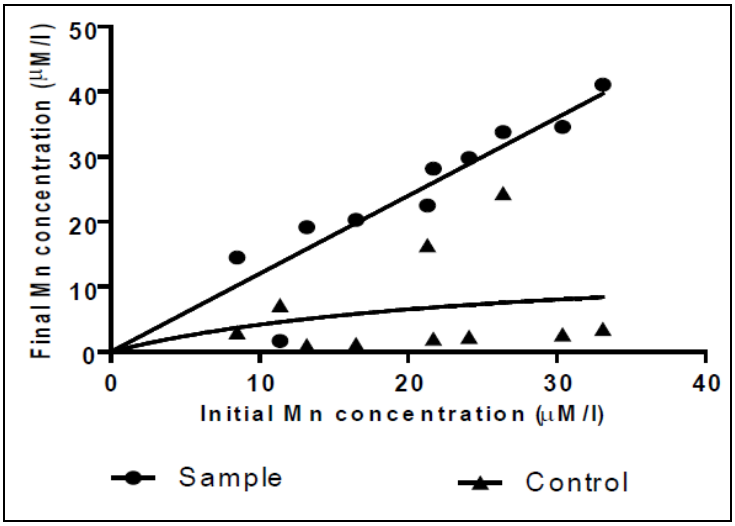


Figure 22: Manganese oxide formed by the cells of SubDo-03 on the surface over a period of 12 h, quantified using atomic absorption spectrometry. The cell concentration inoculated in each sample with different initial concentrations of added $MnCl_2$ was maintained at a value of 3.59 x 10^8 cells/ml.

In order to verify the assumption, that the bacteria from cultures, not supplemented with $MnCl_2$ or grown in the presence of $MnCl_2$ have a different morphology, the samples were processed for SEM analysis. As seen in Fig.23 it is apparent that the bacteria grown in the presence of $MnCl_2$ have, in comparison to those cultured in the absence of $MnCl_2$ (Fig.17), shell-like wrinkled coat formed by the deposition of the oxidized manganese around them.

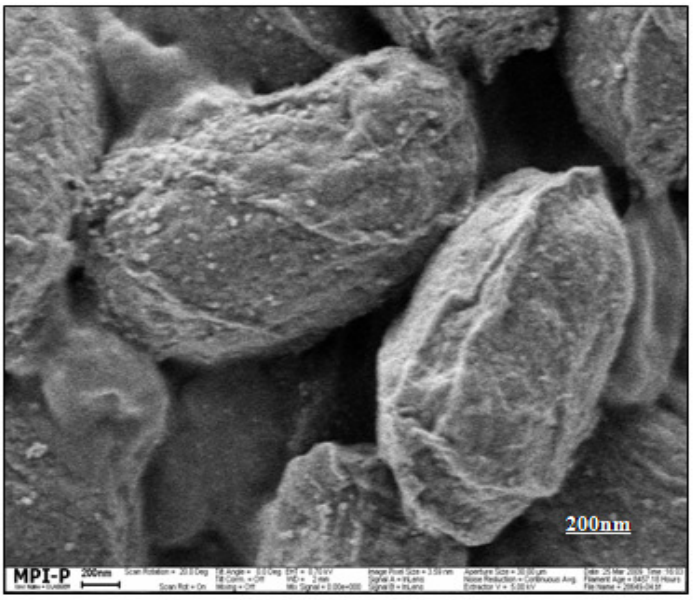


Figure 23: Incubation of the cells in the medium containing 0.1mM $MnCl_2$ for over 12 h resulted in the deposition of oxidized manganese around the cells.

5.3.3 Effect of copper on manganese oxidation by the spore forming SubDo-03 cultures

Evidence that the Mn(II) oxidizing enzyme contains a Cu binding domain, due to which Cu acts as a cofactor for the enzymatic reaction has been previously demonstrated in the in-gel analysis and also been confirmed by molecular investigations. Therefore in order to test the effect of Cu on the growth and the enzymatic Mn(II) oxidation activity of the spores (as described under 5.2.3), the reaction was carried out in presence of Cu concentration of 10 µM in the medium at the time of inoculation. Equal concentrations of culture volumes of 3.26 x 10^8 cells/ml were inoculated into modified K-medium pH 6.8 with and without Cu and the oxidation were carried out for 12 h at 28 °C. In which time the bacteria used the Cu as a cofactor for the manganese oxidation. The reaction was compared with the same volume of substrates in the absence of Cu and a significant difference was observed.

At the level of zero added Cu, which served as the reference condition for the experiment, a trace level of Cu (0.05 µM) may be still present in the medium as a reagent contaminant. The increase in the oxidation activity by the microorganisms was determined by spectroscopic ally measuring the amount of manganese in the medium and calculating the difference between the

initial and final concentrations.

A significant difference can be observed in the concentration of manganese oxidized between the cultures supplemented with and without Cu at the time of inoculation (Fig.24).

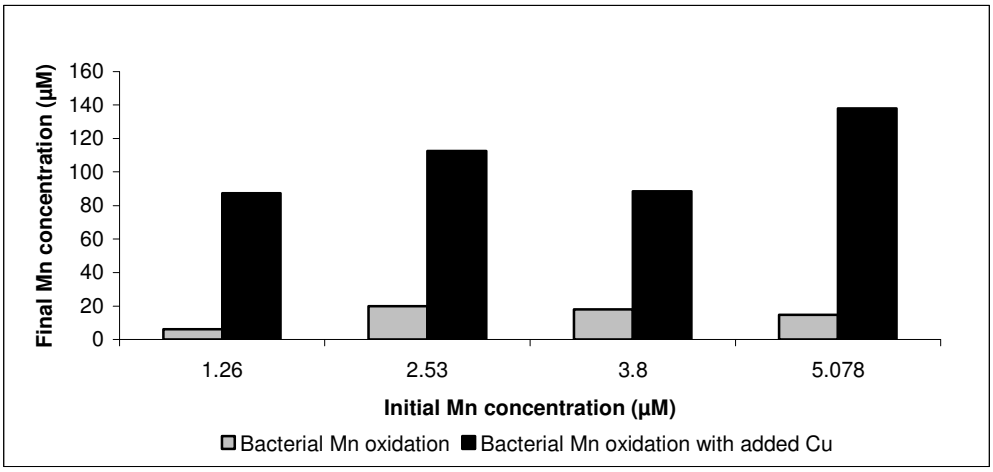


Figure 24: Effect of copper on the enzyme activity of SubDo-03 cultures. The enzyme activity was measured at a cell concentration of* 3.26 x 10^8 *cells/ml for a period of 12 h.

5.4 Characterization of cell wall containing polysaccharide from SubDo-03

5.4.1 Physico-chemical characterization of the cell bound polymer

Different parameters were used to characterize the cell bound polymer from the bacterial cultures. The highest concentration of cell bound polymer obtained after 7 days of culture was 210 mg/l.The freeze-dried exopolymer appeared as a yellow-brown solid material. This crude polymer was then analyzed for its protein, neutral sugars and uronic acid contents by colorimetric analysis and for its carbon, hydrogen, nitrogen and sulphur content by elemental analysis. The molar ratio of hydrogen-carbon in the sample was determined to be less than 2 (0.5), suggesting that the exopolymer produced by SubDo-03 cells are composed of heteropolysaccharides.

% C	% H	% N	% S
23	4.26	7.4	0.38

Table 1 Elemental analysis of the cell bound polysaccharide from SubDo-03 cultures showing the carbon, hydrogen, nitrogen and sulphur content.

5.4.2 Visualization of cell wall bound polysaccharide in SubDo-03 cells

The cell surface polysaccharides from the bacteria are thought to be involved in the attachment of cells to one another during the stationary phase to produce a precipitated clump of cell material. At the same time, it has been hypothesized in the thesis that, the bacteria form a cell capsule containing the factors which are involved in the oxidation of manganese. In order to demonstrate the presence of these polysaccharides on the surface of the cells, the SubDo-03 cultures, grown for a period of 24 h at 28 °C in modified K medium at pH 6.8 were observed for its presence or absence by the positive and negative staining of the liquid culture samples. The staining of the samples with India black ink (Fig.26) under phase contrast microscope provided a negative background to highlight the presence of capsule structures which remain unstained (McKinney, 1953).

The bacterial cultures from SubDo-03 grown for a period of 24 h at 28 °C in modified K medium at pH 6.8 was examined for the presence of cell wall containing polysaccharides by the fluorescent labeling with lectin. The bacteria when stained with WGA conjugated with Alexa Flour 488 demonstrated the presence of the cell wall bound polysaccharide as a layer coating the bacterial mass when observed under the fluorescent microscope (Fig.27, A & B). The cultures without any polysaccharide formation were stained negatively for a comparative study (Fig.27, C & D).

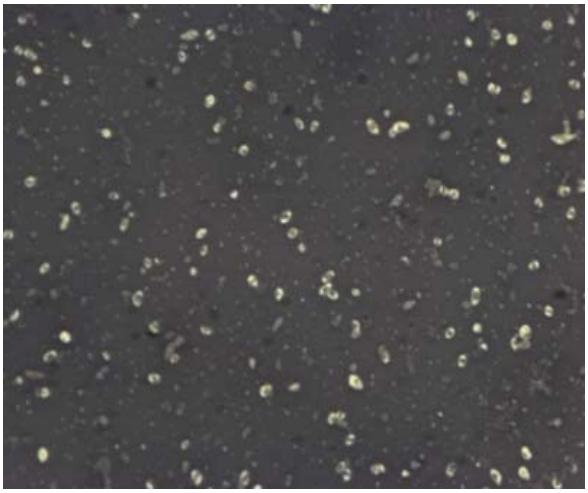


Figure 25 Negative staining with India ink showing the presence of the polysaccharide on the surface of the SubDo-03 cells.

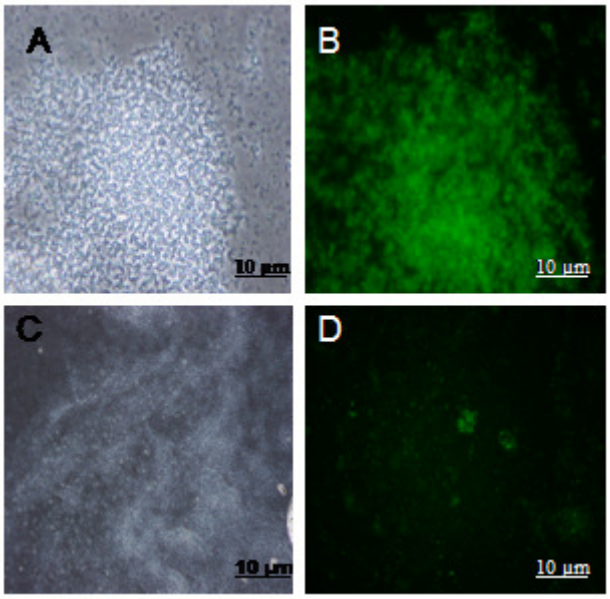


Figure 26 A; SubDo-03 cultures and B; visualisation of cell bound polysaccharide by fluorescent staining with lectin C and D; cultures without cell bound polysaccharide as a negative control.

5.4.3 Determination of manganese binding to the cell bound polysaccharide from SubDo-03 cultures

As a generalization, the binding of metal ions to biopolymers is likely to be via two major mechanisms, the first of these being simple ion-exchange and the second through the formation of complexes (co-ordination compounds) which may be chelates. Because of the complexity of the polysaccharide membrane , as observed on the surface of the SubDo-03, it can be said that more than one processes of binding is taking place at the same time.

The exopolymers from the bacterial cells showed the presence of heteropolysaccharides, which may be responsible for the manganese ion exchange by cation binding. In addition to this the presence of organic ligands such as neutral divalent oxygen or sulphur atoms or neutral trivalent nitrogen atoms which are known to donate one or more pairs of electrons to the metal atoms, thus forming a metal complex. This binding of the metal ions by the cell bound polymers of SubDo-03 cultures could thus be observed when the bacterial exopolymer sample was analyzed for the presence of manganese on its surface by atomic absorption spectroscopy (Fig.22).

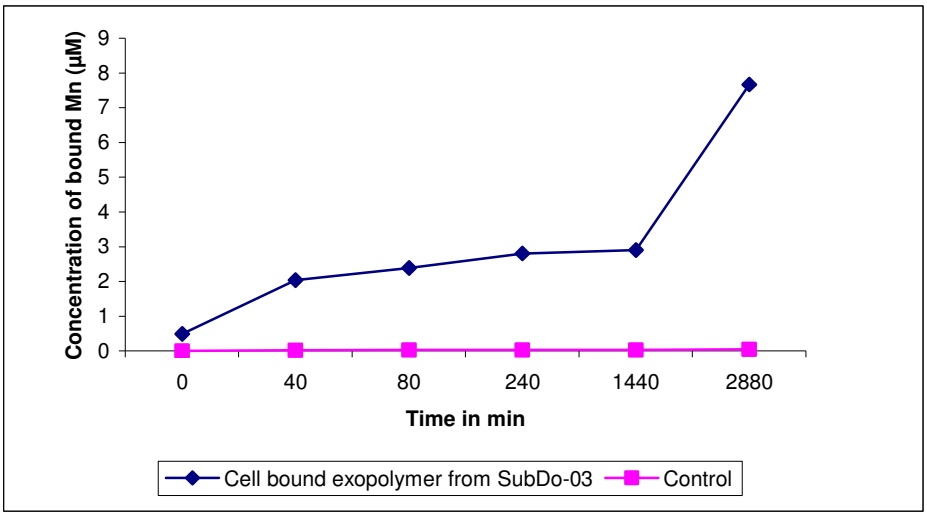


Figure 27 Measurement of Mn bound to the cell bound exopolymer of SubDo-03.

The binding of the metal ions to the bacterial cell wall bound polymers was observed when the purified exopolymer samples were incubated over a period of 48 h with the Mn(II) solution at 28 °C and could be seen to increase with time; by the end of 48 h the amount of Mn(II) present on the exopolymer surface was determined to be 8.67 µM; as measured by AAS. Control samples containing de ionized water incubated over the same period of time showed a very little concentration of Mn(II) binding (0.098 µM) which could be attributed to the sheer physical attraction of the metal ions. This proved the assumption that the attachment of the manganese to the cells of SubDo-03 is carried out by the cell wall bound polysaccharide.

Chapter 6 – Discussion & Conclusion

6.1 Discussion

6.1.1 Sponges, niches for microorganisms both symbiotic and non-symbiotic.

Sponges are filter feeders that take up microorganism from the seawater and digest them by phagocytosis. However major sponges are known to harbor massive consortia of symbiotic microorganisms which are phylogenetically distinct from those in the surrounding seawater, in their mesohyl matrix. The potentially unique nature of the sponge–microbe association, together with the importance of sponges from an ecological (Gili & Coma, 1998) and biotechnological (Osinga et al., 2001) perspective, makes it an ideal system for the examination of diversity and host specificity in marine prokaryote–eukaryote associations. As filter feeders, sponges are capable of processing large volumes of seawater equivalent to many thousands of liters $(kg\ sponge)^{-1}\ day^{-1}$ and rely on the uptake and digestion of microbial cells, through this so that the fate of most of the bacteria found within sponges is that of food particles. The role of bacteria as a food for the sponge was deducted among others from disintegration processes observed in electron microscopic observations (Weissenfels, 1976). It was supported by physiological experiments of bacterial uptake rates (Reiswig, 1971; 1975) and the utilisation of bacterial fatty acids by the sponge for phospholipid synthesis (Carballeira et al., 1986). This predator–prey relationship between bacteria and sponges obscures any possible symbiotic relationship.

Particle uptake occurs at least at three functional sites: large particles (>50 µm) are taken up at the sponge surface by epithelial pinacocytes, smaller particles (<50µm) capable of entering the ostia are taken up by the pinacocytes lining the progressively narrower canal walls and even smaller particles (<5 µm) are trapped in the choanocyte chamber. Although the sponges feed on microorganisms, large amounts of extracellular bacteria populate the mesohyl matrix of the so called bacteriosponges. In the literature, bacteria observed in and isolated from sponges often are commonly called symbionts, although only in a few studies have clear indications for such beneficial interrelationships been obtained. Often, the frequent and abundant presence of bacteria especially within the sponge mesohyl led authors to address

these bacteria as symbionts (Vacelet, 1975; DeVos et al., 1995; Burja et al., 1999), although their functions, and thereby the benefit for the sponge, remained unclear.

A benefit for the bacteria might be already the mere protection by the sponge and escape from the grazing pressure in the environment, although this holds only if they are not digested by the sponge or if at least a balance exists between bacterial growth and digestion. Benefit for the host, may result from the transfer of metabolites from the symbionts to their hosts (e.g. nutrients of nitrogen and carbon fixation, secondary metabolites). In fact, antibiotic activities found in sponges such as *Microciona prolifera* initiated discussions about the ecological role of these biologically active compounds in sponges and their possible production by bacteria (Jakowska & Nigrelli, 1960). The approval of antimicrobial activities by bacteria isolated from marine sponges further stimulated the idea that these bacteria may be involved in defence mechanisms of their host sponges and lives in a symbiotic interaction with them.

Another circumvential indication that supports the existence of symbiotic bacteria within sponges is the observation that mechanisms exist (*Spongia* sp. and *Hippospongia* sp.) to transfer bacteria from the parent to the embryo (Simpson, 1984; Kaye, 1991). Such transfer and re-establishment of a new bacterial population in daughter sponges, per se suggests some importance of a continued association and therefore does indicate a specific and possibly symbiotic association. Unfortunately, neither identity nor function of these bacteria has been established so far for this vertical transfer of bacteria in the sponge.

A very well explained model for the sponge microbe interactions is shown in the (Fig.29). The different types of bacterial associations are described; the bacteria which pass through the sponge unprossesed are depicted by the squares in the picture. Circles represent the bacteria that enter the mesohyl where their persistence depends on individual turnover times. Those bacteria which are transmitted via the reproductive stages and that are probably absent from the seawater are represented by triangles in the picture.

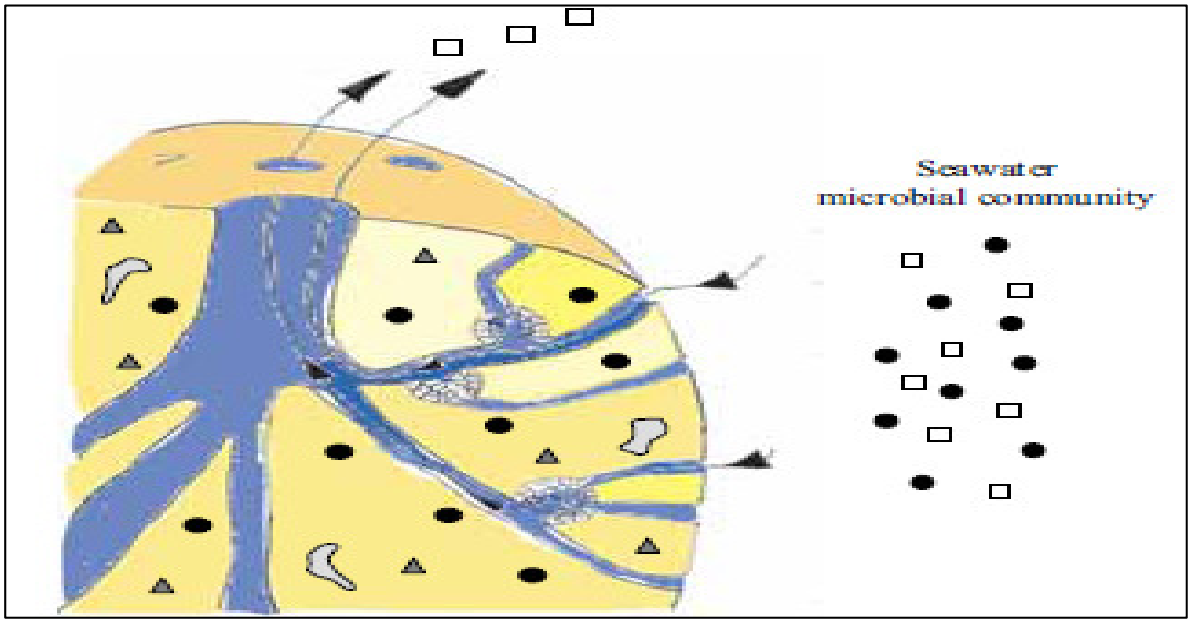


Figure 28: Sponge bacteria interaction model (Hentschel et al, 2002).

6.1.2 Bacteria associated with the mediterranean sponge *Suberites domuncula.*

So far the studies on bacteria associated with the sponge *S. domuncula* have shown the presence of gram negative bacteria belonging to the alpha subdivision of Proteobacteria and a possible novel *Pseudomonas* species (Thakur et al., 2005). As a result it was found that the demosponge *S. domuncula* is provided with a recognition system for gram negative bacteria (Weins et al., 2005). The lipopolysaccharide (LPS)-interacting protein was identified as a receptor on the sponge cell surface, which recognizes the bacterial endotoxin LPS. Many of the bioactive metabolites produced by the microorganisms which are associated symbiotically or commensally with the host have been reported on the surface of *S. domuncula* (Thakur et al., 2003), which protects the host against these bacterial invaders. Associated epibiotic and endobiotic micro-organisms may play a role in the antifouling chemistries of the sponge. This has been investigated by (Miki et al., 1996) and (Osinga, 2001) where preliminary screening results suggest that the epibionts do play a role against the fouling bacteria.

A logical question to ask is 'why do sponges tolerate micro-organisms inside their body'? The most obvious answer might be that the micro-organisms provide a source of food or other useful metabolic products to their host. Symbiosis with biochemically versatile microorganisms is an efficient strategy to accomplish chemical defense in a host. Several different morpho-types of bacteria are generally found in a given host. Accordingly, three types of associations of micro organisms with sponges are now recognized: (i) cosmo-politan bacteria similar in composition to those in the surrounding seawater which serve, most likely, as a food source; (ii) extra cellular bacteria which are probably specific to the sponge mesohyl and (iii) intracellular or even intra nuclear bacteria which permanently reside in host cells/nuclei (Vacelet, 1970). It has been suggested that growth of these useful micro-organisms may be under the control of the sponge host (Müller et al., 1981).
This growth of beneficial micro-organisms is termed 'gardening' or 'farming' and may occur frequently among sponges. A good example of gardening is the often described relationship between sponges and phototrophic microorganisms. Extra cellular cyanobacteria are frequently encountered free living in the mesohyl (Wilkinson, 1978a), zooxanthellae (dinoflagellate microalgae that usually live inside sponge cells) and zoochlorellae (green algae that have been found in cells of both marine and freshwater sponges) are also sometimes present (Simpson, 1984).

6.1.3 Occurrence and activity of manganese oxidizing bacteria in sponges.

6.1.3.1 Detection of a symbiotic manganese oxidizing bacteria in *S. domuncula.*

Mn is an essential trace element and is therefore actively assimilated and utilized by both plants and animals (Underwood, 1977; Woolhouse, 1983) as it is responsible for the accumulation of nitrate and carbondioxide in plants and acts as an activator of number of enzymatic reactions in the animals (Malmstron & Rosenberg, 1959). Mn in its reduced form Mn(II) is bioavailable and can be readily taken up by the benthic fauna. Concentrations of dissolved manganese in natural waters that are essentially free of anthropogenic sources can range from 10 to >10,000 µg/l (Reimer, 1999). However the manganese concentrations in

natural surface waters rarely exceed 1000 µg/L and are usually less than 200 µg/l (Reimer, 1999).

Mn exists in the aquatic environment in two main forms: Mn(II) and Mn(IV). Transition between these two forms occurs via oxidation and reduction reactions that may be abiotic or microbially mediated (Nealson, 1983; Thamdrup et al., 2000; Heal, 2001). The environmental chemistry of manganese is largely governed by pH and redox conditions; Mn(II) dominates at lower pH and EH, with an increasing proportion of colloidal Mn oxy hydroxides above pH 5.5 in non-dystrophic waters (LaZerte & Burling, 1990). The oxidation of soluble Mn(II) to insoluble Mn(III, IV) oxides and hydroxides is an environmentally important process because the solid-phase products; the Mn oxides, oxidizes a variety of organic and inorganic compounds (eg: humic substances, Cr(III) and Fe(III)), scavenge many metals (eg: Co, Cu, Cd, Zn, Ni and Pb), serve as electron acceptors for anaerobic respiration. Bacteria have long been recognized for their ability to deposit iron hydroxide or Mn oxide in structures outside their cells (Beger & Bringmann, 1953). And when involved in those reactions, they become very important for the environment by acting as the source for all the above stated reactions. Indeed, the classical Fe- and Mn-depositing bacteria, *Gallionella*, *Sphaerotilus*, *Leptothrix*, and *Clonothrix,* were all described during the nineteenth century (Buchanan et al., 1974 & Pringsheim, 1949). These bacteria are recognized in the natural environments by the metal encrusted structures surrounding them. Recent interest in the economic, environmental and geochemical importance of metal-transforming micro organisms has rekindled the interest in studying these bacteria. As they can be involved in the formation of ferromanganese deposits containing economically important trace metals. Also they can be used for recovering valuable and toxic metals from the dilute aqueous milieux. So far the presence of Mn oxidizing bacteria have been determined in only few of the environments such as hydrothermal vent deposits,(Cowen et al., 1986; Mandernack & Tebo, 1993) ferromanganese nodules and concretions in oceans, lakes, soils, oxic –anoxic interfaces (Emerson et al., 1982; Tebo, 1991). However the existence and symbiotic association of a Mn oxidizing bacteria with a marine sponge species has been demonstrated in this thesis which presents a novel opportunity to study the physiological implications of this bacterial metal oxidation in the sponge. This bacterial species, named as *SubDo-03* has been found to be a chemoheterotroph demonstrating its ability to oxidize manganese if

present in the surrounding environment. The reason behind which can be perhaps related to the shifts in the microbial communities due to climate change or environmental stress. As in today's climate of rapidly accelerating environmental change, a mechanistic understanding of the sensitivity of the sponge symbiosis to environmental change is urgently required.

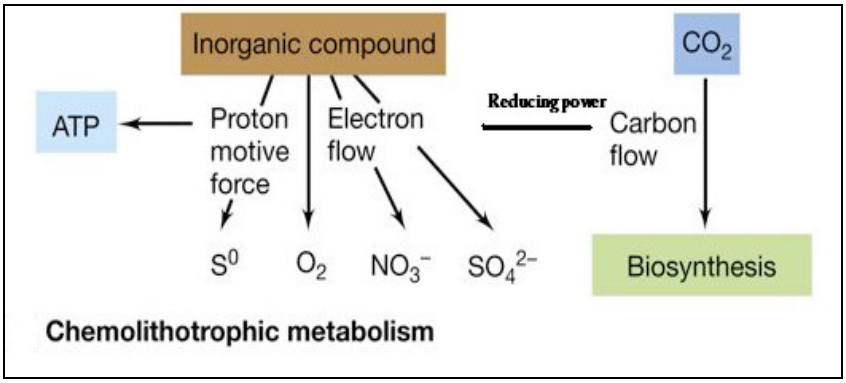


Figure 29: Schematic representation of chemolithotrophic metabolism carried out by bacterial cells.
Taken from Physiology and Diversity of Prokaryotes WS 2008/2009 (www.icbm.de/pmbio/)

Chemoheterotrophic bacteria can be described as those which assimilate organic compounds for their carbon needs by oxidizing inorganic compounds. They can be also termed as chemolithotrophs or literally "stone eaters", as they acquire all their energy by the oxidation of inorganic compounds and in the process, are able to fix inorganic carbon via the Calvin cycle (Balkwill, 1989). These types of bacteria participate intimately in the conversion and circulation of inorganic material on the Earth. Some of the commonly occurring type of chemolithotrophs can be the ammonia-oxidizing bacteria; *Nitrosomonas europaea* (Lee et al, 1999) nitrite oxidizing bacteria; *N.winogradskyi* (Aleem et al., 1965), denitrifying bacteria; *Thiobacillus denitrificans* (Aminuddin & Nicholas, 1973), sulfur oxidizing bacteria; *Acidothiobacillus thiooxidans* and *Acidothiobacillus ferrooxidans* (Kuenen, 1989), methanogens; *Methanobacterium thermoautotrophicum* (Zeikus, 1977).

6.1.3.2 Intra or extra cellular manganese oxidation by SubDo-03 in *S. domuncula.*

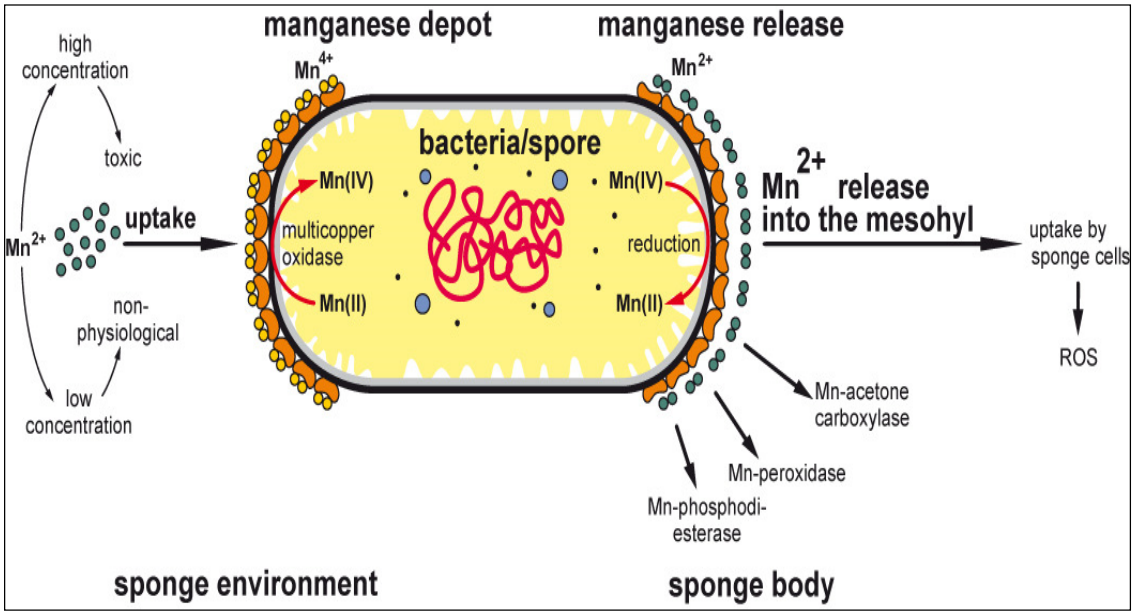


Figure 30: Schematic representation of uptake of manganese from the surroundings by SubDo-03 and its use by the sponge S.domuncula (unpublished observations).

Under typical ocean conditions, Mn in seawater can be found in three different oxidation states, as soluble Mn(II), Mn(III) and Mn(IV). Though it is believed that the dissolved fraction of Mn is as Mn(II), as the subsequent reoxidation to Mn(III) or Mn(IV) is relatively slow, the predominant form is as particulate MnO_X; as Mn is readily precipitated out under the alkaline ocean conditions (Strumm & Morgan, 1981; Chapin, 1990; von Langen, 1997). The concentrations of dissolved Mn in seawater have been documented to range from as low as 0.08nM to as high as 10nM, depending on whether the measurements were made in coastal or open ocean areas (Bruland et al., 1983). Hence there is a need for uptake and deposition of trace levels of Mn from the surroundings by non physiological means which, here is thought to be mediated by the SubDo-03 like species in the sponge body (unpublished observation).

As a trace nutrient, Mn is required for a large number of cellular functions in the sponge body. Therefore, it is worth considering whether Mn(II) oxidation by the bacterial spores of

SubDo-03 is related to the role of Mn in cellular functioning. For example, intracellular Mn(II) is known to act as an antioxidant, protecting some cells from reactive oxygen species (ROS), such as superoxide, even in cells that do not possess superoxide dismutase (Archibald & Fridovich, 1981). The reaction of Mn(II) with superoxide can produce Mn(III) or Mn(IV) species. These reactions of Mn(II) are likely to occur on the inner membrane, where ROS are produced as by-products of respiration (Ghosal et al., 2005). Thus, the question that arises for the Mn(II)-oxidizing bacteria which precipitate Mn oxides extracellularly, is whether Mn(II) oxidation is related to this intracellular ROS scavenging, or whether the bacteria are simply taking advantage of similar chemistry to protect themselves from oxidants in the environment?

The Mn oxidizing activity by the SubDo-03 species has been observed in the mid to late exponential phase of the growth of the bacteria. This can be explained by the rise in the number of spore-forming bacterial cells formed out of the normal cells. *Bacillus* species have been observed to produce spores in the normal stages of their growth cycle, and also require a higher concentration of manganese for this process (Nealson, 1982). Here the SubDo-03 bacterial cells have been found to utilize the Mn from the surrounding medium, when present and carry out its oxidation. It can be said that in seawater the effects of the spores of bacterial cells on Mn oxidation can be found to evident early on, before they become coated or lose their Mn (II) oxidizing capacity (Tebo, 1995), it has been observed that the Mn oxidation by the SubDo-03 cultures show similar results.

6.1.3.3 Manganese oxidation activity by SubDo-03 cultures.

The Mn oxidation carried out by the cells of SubDo-03 was also seen to be strongly affected by presence of Cu in the medium. This followed the analysis that the enzyme responsible for the Mn oxidation in this bacterium had a multicopper binding domain, and belonged to the family of multicopper oxidases (MCO). On the other hand the strongest support for this conclusion was provided by the fact that Mn oxidation activity is inhibited by *o*-phenanthroline.

Ortho-phenanthroline is a potent inhibitor of metallo-proteins, including multicopper oxidases and its inhibitory effect is likely due to interference with the metal centers of such

enzymes (Okazaki M et al., 1997). No bacterial Mn oxidase has been purified in quantities sufficient for detailed biochemical study, and to date, no multicopper oxidase gene thought to encode a Mn oxidase has been successfully expressed to yield active enzyme in a foreign host. Thus the direct role of multicopper oxidases in Mn (II) oxidation remains a hypothesis. Nevertheless, it seems reasonable that multicopper oxidases in bacteria could also directly oxidize Mn(II) because (*a*) genetic and biochemical studies point to the involvement of multicopper oxidases in Mn(II) oxidation in several unrelated bacteria, (*b*) some eukaryotic multicopper oxidases are known to directly oxidize Mn(II) (Hoofer & Schlosser, 1999; Schlosser & Hoofer, 2002), and (*c*) multicopper oxidases that oxidize Fe(II) occur in both eukaryotes (Solomon et al., 1996) and bacteria (Kim et al., 2001; Huston et al., 2002).

The question of bacterial multicopper oxidase specificity and function has broadened recently as genome sequencing has revealed that multicopper oxidases, identified by their copper binding site motifs, are prevalent in bacteria (Alexandre & Zhulin, 2000; Claus, 2003). Although most of these putative bacterial multicopper oxidases are uncharacterized, results so far indicate that they are involved in a wide range of functions, including copper resistance (Kim et al., 2001; Lee et al., 1994; Mellano & Cooksey, 1988; Yang et al., 1996), melanin production/UV protection (Hullo et al., 2001; Solano et al., 2000), iron oxidation and acquisition (Huston et al., 2002).One study showed that Yac K, a multicopper oxidase from *E. coli*, exhibits oxidase activity towards both iron and organic compounds, and suggested functional roles for both activities (Kim et al., 2001). It is interesting that this and another (Hullo et al., 2001) bacterial multicopper oxidase are unable to oxidize Mn (II). Genome sequencing of *Pseudomonasputida* KT2440, *P. fluorescens* strain PfO-1, and *Nitrosomonas europaea* has revealed that these organisms contain genes similar to *Bacillus* sp. *mnxG*, while KT2440 also has a putative *cumA* gene. The putative proteins encoded by these three *mnxG*-like genes are from distantly related bacteria and share a high degree of sequence similarity, suggesting perhaps that they represent a distinct class of multicopper oxidases.

The results of this study are consistent with what is currently understood about Mn oxidation by bacteria and fungi; all bacterial Mn-oxidation systems contain a multicopper oxidase system as a functional component (Brouwers et al., 2000; Tebo et al., 1997). Multicopper oxidase enzymes are associated with Mn oxidation in fungi as well, where laccase (Hofer &

Schlosser, 1999; Munoz et al., 1997; Schulze et al., 1995) and a laccase-like enzyme (Miyata et al., 2004) have been shown to oxidize Mn. Laccases fall under the broader classification of multicopper oxidases. They have broad substrate affinities, are involved in multiple biochemical processes (Mayer & Staples, 2002), and fungi typically harbor several laccase isozymes in their genomes (Hoegger et al., 2004; Kumar et al., 2003; Mayer & Staples, 2002).

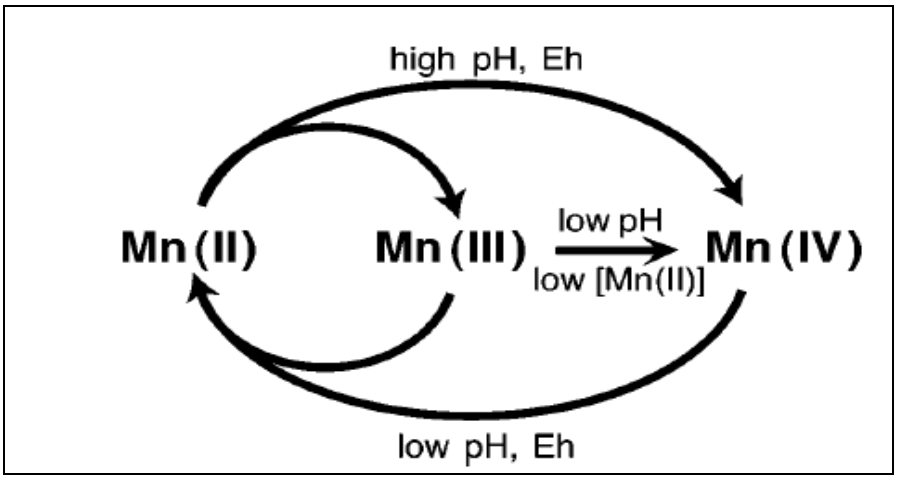


Figure 31: The Mn cycle of oxidation states in nature, (Tebo et al, 2004).

The further proof that the Mn oxidation by the cells of SubDo-03 followed an enzymatic nature was given by the determination of the oxidation over a range of incubation temperatures and at different pH of the culture medium. The bacterial cells showed oxidation at and above the isolation temperatures of 28 °C. While the biological Mn (II) oxidation was very slow at ranges below pH 6 and above pH 8.5 which followed an enzyme activity of biological nature. The manganese oxidation by the bacteria also showed a substrate dependant increase in its activity which followed a normal enzymatic reaction.
At low pH and low O_2 concentration the Mn(II) remains stable, but undergoes oxidation to Mn(III) and Mn(IV) forms under high pH conditions. Mn(IV) occurs as solid phase Mn oxides or hydroxide minerals and is the thermodynamically favored form under oxic conditions at circumneutral pH, while Mn(III) is thermodynamically unstable and disproportionates to Mn(II) and Mn(IV) in the absence of organic compounds that chelate and stabilize Mn(III).It has been found that enzymatic manganese oxidation in solution is not

favored at pH values much lower than neutrality. This is because the standard free energy change (ΔG°) when manganese is oxidized with oxygen as electron acceptor steadily decreases until it assumes a positive value near pH 1.0 (Ehrlich, 1978 a). Biological catalysis is more likely to be important in natural environments because Mn^{2+} does not auto oxidize rapidly below pH 9 (Stumm, 1981).

6.1.3.4 Metal binding by the bacterial exopolymer of SubDo-03.

In the marine environment, natural and artificial substrata are readily colonized by micro- and macroorganisms in a process known as "Biofouling" (Cooksey & Wigglesworth-Cooksey, 1995; Flemming, 2002; Wieczorek & Todd, 1998). Bacterial biofilm formation is believed to be influenced by environmental signals and regulatory pathway (Stanley & Lazazzera, 2004). Bacteria undergo profound changes in physiological features during biofilm formation, a dynamic process wherein, they transform from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community (Costerton J et al., 1995; Fletcher M**,** 1996; Heydorn A et al., 2000; O'Toole, Kaplan & Kolter, 2000; O'Toole & Kolter, 1998; Sauer, 2003; Stoodley et al., 2002). A five-stage process is generally accepted to describe the development of biofilm formation. This process includes initial reversible surface attachment, irreversible attachment by producing extracellular polymeric substance (EPS), early development of biofilm architecture, maturation of biofilm architecture, and dispersion of single cells from the biofilm (Stoodley et al., 2002). In plank tonic bacteria, EPS are often present as a capsule.EPS also plays an important role in bacterial biofilm formation (Danese, Pratt & Kolter, 2000; Stoodley et al, 2002, Sutherland, 2001). EPS consists not only of polysaccharides but also considerable amounts of proteins, nucleic acids, and lipids (Sutherland, 2001).The microbial EPS from the aquatic environments carry out the important function of assisting the bacterial colonization on to biological surfaces as a means of basic survival strategy (Costerton et al*.,* 1978). Colonization of a given surface is a sequential event leading to the formation of a biofilm. In the complex process of biofilm development the initial attachment of bacteria to a surface is followed by the irreversible adhesion of cells, facilitated by production of extracellular polymeric substances (EPS) often referred to as glycocalyx or slime (Lappin-Scott &

Costerton, 1989; Costerton et al., 1987; Characklis & Cooksey, 1983). The content of the macromolecules like polysaccharides, proteins and nucleic acids, in a given EPS, vary depending on bacterial species and growth conditions (Sutherland, 1985).

The concentration and speciation of metals, nutrients, and trace elements in rocks, soils, and aquatic environments can be dramatically influenced by sorption reactions at cell surfaces. Natural biofilms sequester several metallic and alkaline elements. For example, biofilms often contain K^{+},Ca^{2+}, Cr^{3+},Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} at concentrations 2 to 4 orders of magnitude greater than those measured in the surrounding bulk fluid (Brown et al., 1994; Ferris et al., 2000). The affinity of bacterial surfaces for aqueous cations is due in part to the low isoelectric points of the surfaces, resulting in a negative surface charge over a wide pH range. However, the structure and charge density of bacterial cell walls can vary greatly (Beveridge & Murray 1980). The carboxyl and phosphoryl sites are thought to dominate metal binding at low to neutral pH, and hydroxyl and amino groups have been invoked for metal complexation above pH 8. However, the surface complexation modeling can only tentatively infer the chemical identity of sorption sites from their acidity constants, and the modeling is also only sensitive to the dominant sites involved in metal binding.

Thus the metal binding by the bacterial polysaccharides of SubDo-03 could be associated to the presence of high amounts of carboxyl moieties in it. These neutral carbohydrates could be forming weak electrostatic bonds with the metal ions and thus helping in creating the cross links with the metal ions. In addition the SubDo-03 belongs to the gram positive group of bacteria, showing a very high percentage of peptidoglycan in the outer part of their cell walls. These peptidoglycan layers have shown to form a complexation followed by redox-type of reaction with the dissolved metal ions (Fortin D, 1997).Thus the formation of biogenic Mn oxides by the cells of SubDo-03 could be following the view that the Mn^{2+} may first bind to the polymers and then are oxidized biologically by the manganese oxidation enzymes present on the surface of the spore-forming bacterial cells.

6.2 Conclusions-Reasons for the possible symbiosis of SubDo-03 with *S. domuncula.*

The reasons for the presence of the Mn oxidizing bacteria in the marine Mediterranean sponge *S. domuncula* could be interesting. Because there appears to be a linkage between oxidative metabolism and Mn oxidizing ability, it is possible that both properties evolved together.

One advantage of possessing Mn oxidizing activity for the host would be that energy could be derived from the oxidation. Other possible selective advantages of metal deposition include nutrient scavenging and detoxification. Apparently, Mn-depositing activity is the property of many *Bacillus* spp, which form metal oxides in association with spores (Ghiorse, unpublished observations). These observations illuminate an important point concerning possible influences of bacteria on sedimentary metal deposition. They indicate the possibility for formation of bacterial microfossils-metal-encrusted bacterial corpses that could contribute to metal deposition in sedimentary environments.

The possible reasons which can be suggested for the presence of these bacteria in the marine environment are their role in natural detoxification of the metals (Hennebel et al., 2008). However how and to what degree do they detoxify the metals is still a topic of study. The probable hypothesis by which these bacteria function in the marine environment could be that they are able to resist the input of metal pollution and as survivors fulfill their role as decomposers. If they not only survive, but actually use the toxic metals to generate energy—as laboratory tests have suggested—the benefits to the bacteria may be considerable. If this is the case, then not only do the bacteria benefit from the energy they acquire by detoxifying metals, but their habitat benefits because the toxic metals are reduced.

Like initially proposed (Althoff et al., 1998) and soon accepted (Faulkner et al., 2000), bacteria live in a symbiotic/commensalic relationship with most of the marine sponge species living in the Mediterranean Sea , and contain, if kept in an aquarium for more then two weeks (Müller et al., 2004). And, focusing on *S. domuncula*, only a few different bacterial strains which are non abundant, are located in specific cells, the bacteriocytes (Böhm et al, 2001). This is in contrast to a few species like *Aplysina aerophoba* that abundantly contain microorganisms, which make up to 50% of the sponge body mass (Weiss et al., 1996).

From those specimens of *S. domuncula*, kept in quarantine, the Mn-precipitating bacteria described here have been isolated. Already this finding suggests that these bacteria display a crucial role in the physiology/metabolism of the sponge, perhaps supporting our assumption that the Mn-precipitating bacteria act as a reversible Mn store in the sponge. According to this view, the presence of SubDo-03 bacteria is required as a protection against higher, toxic concentrations of Mn in the surrounding environment. It is observed that after oxidation of Mn (II) to Mn (IV) in the nature the ion becomes insoluble (Bargar et al., 2000).Thus the bacteria are able to detoxify the higher concentrations of the metal, if present.

However, the bacteria appear to be also essential for the maintaining the physiological concentration of Mn in the sponge. Since only minute levels of Mn exist in the surrounding seawater a substantial accumulation of Mn has to arise, which is implicated by a release of bacterial-precipitated Mn(IV). According to the reaction mechanisms described recently for the MCO from *Escherichia coli*, and basing on the experimental proven four-electron reduction of dioxygen by the MCO (Kataoka et al., 2009), a reduction and in turn a dissolution of Mn(IV) to Mn(II) might be postulated.

Yet another function of the presence of the Mn oxidizing bacteria in the sponges could be postulated to be intracellular response against the oxygen toxicity, where in mM concentrations of intracellular Mn^{2+} could replace µM concentrations of super oxide dismutase (Archibald & Fridovich, 1981). Its role as an antioxidant in low molecular weight complexes is likely to involve redox reactions between the Mn(II) and Mn(III) states.

An oxygen destroying mechanism could be also proposed in which Mn(II) can act as a catalytic scavenger of superoxide (O_2^-) and get oxidized to Mn(IV) by oxygen; the Mn(lII) so formed in the process could then be reduced by NADPH, reforming Mn^{2+} for further reaction with oxygen. The equation shown below represents the scavenging of superoxide by Mn(II). This equation is not balaced for charge.

$$Mn(II)+O_2^-+2H^+ \rightarrow Mn(III)+H_2O_2 \rightarrow Mn(II)+O_2+2H^+$$

Equation 3: Mn(II) scavenging of superoxide (Horsburgh et al., 2002)

As an alternative to the above mechanism, the H_2O_2 formed during the O_2^- scavenging by Mn(II) can be dismutated by catalse with subsequent reductant of Mn(III) by a cellular reductant such as cysteine or glutathione.

6.3 Future directions of the study.

Despite new information on the diversity of Mn(II)-oxidizing bacteria and the molecular mechanisms likely to be involved in Mn(II) oxidation, factors controlling the distribution, activity and biochemical function of Mn(II)-oxidizing bacteria remain unknown. Several major questions that are currently under investigation include: what are the direct links between Mn oxide biomineralization and the transformation and sequestration of Fe, C, N, S, nutrients and trace metals; are there chemolithoautotrophic Mn-oxidizing bacteria that can use oxygen or nitrate as an electron acceptor during Mn(II) oxidation and conserve energy for growth – this is particularly interesting when there is a supply of Mn(II) derived from geological processes; and are there biogenic Mn oxide minerals that can be used to identify microbial Mn(II) oxidation in diverse geochemical environments? More importantly, are these oxides preserved in the geological record as recognizable microfossils, particularly when the minerals coat unique morphological forms such as sheaths and capsules?
The presence of a plasmid controlling the manganese oxidation by SubDo-03 could be a topic to be studied for any interesting piece of information, regarding the presence of the bacterium in the sponge, if it is perinneal.
Future work is also needed to further characterize the substrate specificity of bacterial Mn oxidases. Do Mn oxidases feature a substrate-binding pocket that ensures greater specificity as in ascorbate oxidase and ceruloplasmin (Solomon et al., 1996), or are they nonspecific, such as the plant and fungal laccases? Perhaps the answers to such questions will shed light on the functional role of bacterial Mn(II) oxidation.

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Appendix

AAS	Atomic absorption spectroscopy
µl	Microliter
µg	Microgram
BSA	Bovine Serum Albumin
EDTA	Ethylendiamin-N,N,N´,N´-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FTIR	Fourier transform infrared spectroscopy
g	gram
x g	times gravity (units of gravity)
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kD	kilo-Dalton
L	Liter
M	molar (mol/l)
Mn	Manganese
min	Minute
ml	Milliliter
mm	Millimeter
mM	millimolar
NaCl	Sodiumchlorid
NaDodSO4	Sodium Dodecyl Sulfate
OD	Optical density
PAGE	Poly-Acrylamid-Gelelectrophoresis
PMSF	Phenylmethane sulfonyl fluoride
PBS	phosphate buffered saline
pH	potentia hydrogenii
PVDF	Polyvinylidendifluorid
rpm	rounds per minute

RT	Room temperature
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
Tris	Tris-(hydroxmethyl)-aminomethan
Tween 20	Poly(oxyethylen)20-sorbitan-monolaurat
U	Unit
UV	Ultraviolett
v/v	volume/volume (Vol.%)

Name: **Mugdha Divekar**

Date of Birth: 11 Jan 1982

Nationality: Indian

Email: divekar@uni-mainz.de

Education:

2006-present: Ph.D. student, Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Johannes Gutenberg Universität, Mainz, Germany

2003-2005: Master of Science in Biotechnology from University of Bangalore, India

2000-2003: Bachelor of Science in Zoology and Biotechnology from University of Mumbai, India

Research Experience:

2005-2006:. Identification and screening of culturable and unculturable bacteria and actinomyces from marine sponges and algae for the development of drugs from marine sources. Phylogenetic identification of actinomyces strains from marine sources by 16S RNA typing. Nicholas Piramal Research Centre, Nirlon complex, Mumbai in the department of Marine Molecular Biology

2003-2005: Chromatographic detection and estimation of Cardiac glycosides from the leaf extracts of Calotropis gigantea, Department of Biotechnology, V.G.Vaze College, Mumbai.

2002: Detection of Microbial contamination from drinking water sources, Department of Biotechnology, V.G.Vaze College, Mumbai.

2001: Bacteriological and serological testing from animal sources, Bombay Veterenary college, Parel, Mumbai.

2000: Human karyotyping and animal tissue culture from blood, Moghe's Cytogenetics Lab,Pune.

Publications:

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W.Tremel.

Bioencapsulation of living bacteria (Escherichia coli) with poly(silicate) after transformation with silicatein-alpha gene

Werner E G Müller, Sylvia Engel, Xiaohong Wang, Stephan E Wolf, Wolfgang Tremel, Narsinh L Thakur, Anatoli Krasko, Mugdha Divekar, Heinz C Schröder

Poster Presentation:

Diversity assessment and biotechnological exploration of sponge-associated bacteria: Molecular biological approach

Narsinh L. Thakur, Roopesh Jain, Mugdha Divekar, Sushma Parab, Ashwini Vartak

Silicatein expression on bacterial cultures
Sylvia Engel, Mugdha Divekar, S.Pervoric-Ostadd, Anatoli Krasko, Dr. W.E.G. Mueller

Bacterial assisted biomineralisation in marine sponges
Mugdha Divekar, Dr.Vladislav Grebenyuk, Prof. Dr.W.E.G Mueller