Chapter 2
Halophiles in Nanotechnology

Abstract Halophiles are salt loving organisms that flourish in saline environments such as marine and estuarine environments, solar salterns, salt lakes, brines and saline soils. They offer potential applications in various fields of biotechnology. They can be used as a source of metabolites, compatible solutes and other compounds of industrial value. The biodegradation of organic pollutants in hypersaline environments and treatment of saline effluents contaminated with organic by halophiles have been investigated. Some halophiles are a potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions and harsh conditions of pH or/and ionic strength. Recently, they are being explored as potential sources of metal tolerant microorganisms with the ability to synthesize metallic nanoparticles. This chapter covers the various halophilic organisms and their by-products that have been exploited for nanomaterial synthesis, the mechanisms that may be involved in the nanomaterial fabrication and the possible applications of the fabricated nanoparticles.

2.1 Introduction

Halophiles are salt loving organisms that flourish in saline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Currently, over 182 species distributed in 48 validly described genera in *Halobacteriaceae* cover aerobic halophiles and a few anaerobic and halophilic methanogens are known in *Archaea* domain. The number of halophiles in *Bacteria* domain is higher and they are distributed in many groups (phyla). Representatives of halophilic bacteria are included in Phylum *Bacteroidetes*, *Cyanobacteria*, *Proteobacteria*, *Firmicutes* and *Sulphur-Green* bacteria. Furthermore, halophilic Fungi, Plants, Ciliates and Flagellates have also been known in *Eucarya* domain. These organisms cope with harsh environmental conditions by two strategies, namely salt-in and compatible solutes (Oren 1999a, b, 2002a, b). The salts are

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accumulated inside of cells to osmolarity equivalent with external environment in first strategy, and synthesis of organic molecules known as compatible solutes occurs or they are accumulated from environments if they are present in the second strategy (Oren 1999a, b). Special adaptations are requested for proteins and enzymes in case of fi rst strategy, and the most well-known fact is the increasing acidic amino acids residues on their surface (Lanyi 1974; Graziano and Merlino 2014). Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes (DasSarma 2009; DasSarma and DasSarma 2012). Halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement for NaCl. The extremely halophilic archaea, in particular, are well adapted to saturating NaCl concentrations and have a number of novel molecular characteristics, such as enzymes that function in saturated salts, purple membrane that allows phototrophic growth, sensory rhodopsins that mediate the phototactic response, and gas vesicles that promote cell flotation.

The saline environments that halophiles inhabit include the marine and estuarine environments, solar salterns, salt lakes, brines and saline soils (Oren 1999a, b, 2002a, b; Tiquia et al. 2007; DasSarma and DasSarma 2012). In the latter, the matrix potential of the soil adds to the water stress caused by high salt concentrations. High saline waters originate either by seawater condensation (thalassohaline) or by evaporation of inland surface water (athalassohaline). The salt composition of thalassohaline waters resembles that of seawater with NaCl as the main constituent. Athalassohaline lakes can differ in their ion composition from seawater derived lakes. Some athalassohaline waters have a very high concentration of divalent cations (for example, the Dead Sea with the main cation Mg^{2+} instead of Na^{+}), while others are free of magnesium and calcium due to the presence of high levels of carbonate. Increased carbonate concentrations lead to the formation of soda lakes, which have pH-values well above 10 (for example, the Wadi Natrun in Egypt). Microflora have been found in all of the above types of saline waters, indicating that halophilic microorganisms tolerate high salinity and can adapt to different stressors like high pH or extreme temperatures (Kunte et al. 2002). The salinity of saline environments is generally understood by biologists in terms of sodium chloride content. This compound represents over 90 % of the total salt content in many cases and the presence of other compounds in terms of influence to physical or chemical parameters of the saline area could be considered as low. On the other hand, these compounds could have a signifi cant infl uence on the diversity of halophilic microorganisms if we considered the high concentrations of magnesium in Dead Sea or carbonates in soda lakes such as Magadi, in Kenya, Wadi Natrum in Egypt, Sambhar, India (Enache et al. 2015). The predominant microorganisms in the saline environments are represented by both halophilic archaea and bacteria (DasSarma and DasSarma 2012).

Halophilic bacteria grow over an extended range of salt concentrations (3–15 % NaCl, w/v and above), unlike the truly halophilic archaea whose growth is restricted to high saline environments (Litchfield 2002). According to Kushner (1978), many

marine organisms are slight halophiles (with 3 % w/v NaCl in seawater). Moderate halophiles optimally grow at 3–15 % w/v NaCl; extreme halophiles at 25 % w/v NaCl (halobacteria and halococci) and borderline extreme halophiles require at least 12 % w/v salt. Halophilic microorganisms usually adopt either of the two strategies of survival in saline environments: 'compatible solute' strategy and 'salt-in' strategy (Ventosa et al. 1998). Compatible solute strategy is employed by the majority of moderately halophilic and halotolerant bacteria, some yeasts, algae and fungi. In this strategy cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential through the synthesis or uptake of organic compatible solutes. Hence these microorganisms are able to adapt to a wide range of salt concentrations. The compatible solutes include polyols such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine and ectoines. The salt-in strategy is employed by true halophiles, including halophilic archaea and extremely halophilic bacteria. These microorganisms are adapted to high salt concentrations and cannot survive when the salinity of the medium is lowered. They generally do not synthesize organic solutes to maintain the osmotic equilibrium. This adaptation involves the selective influx of K^+ ions into the cytoplasm. All enzymes and structural cell components must be adapted to high salt concentrations for proper cell function (Shivanand and Mugeraya 2011).

Halophilic bacteria offer potential applications in various fields of biotechnology (Tiquia and Mormile 2010; Margesin and Schinner 2001). These microorganisms can be used as a source of metabolites, compatible solutes and other compounds of industrial value. Novel halophilic biomolecules may also be used for specialized applications, e.g. bacteriorhodopsin for biocomputing, pigments for food colouring and compatible solutes as stress protectants (DasSarma and DasSarma 2012). Biodegradation of organic pollutants by halophilic bacteria and archaea has been reviewed (Borgne et al. 2008; Tiquia 2010). These microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. Understanding the degradation process would also shed light on the enzymes involved and on the regulation of metabolism. Halophilic bacteria are a potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions (Shivanand and Jayaraman 2009).

Halophiles organisms represent a valuable resource of enzymes (extremozymes) with stability in harsh conditions of pH or/and ionic strength. Thus, their investigations as biocatalysts in the presence of novel nanomaterials are attractive. Several biomolecules produced by these halophilic organisms, i.e. enzymes, halocins (halobacterial proteins with antibiotic activities), exopolysaccharides etc. show biological activity in harsh conditions. Combining of these bio-molecules with various nanomaterials like thin-layers, nanotubes, nanospheres results in novel compounds harboring both biological properties of biomolecules and physico-chemical characteristics of nanomaterials. Recently, they are being explored as potential sources of metal tolerant microorganisms with the ability to synthesize metallic nanoparticles (Agnihotri et al. 2009; Kathiresan et al. 2009; Venkatpurwar

and Pokharkar 2011; Ali et al. 2011). This chapter covers the various halophilic organisms and their by-products that have been exploited for nanomaterial synthesis, the mechanisms that may be involved in the nanomaterial fabrication and the possible applications of the fabricated nanoparticles.

2.2 Nanoparticle Synthesis by Halophiles

Marine environments could be good source of metal tolerant microbes as most of these organisms exist at the bottom of the sea, and contribute towards biogeochemical cycling of inorganic elements. Besides, the marine econiche is continuously exposed to metallic pollution due to volcanic eruptions, natural weathering of the rocks, anthropogenic activities such as mining, combustion of fuels and industrial and urban sewage. Estuaries and solar salterns may also contain high concentrations of metals as they serve as effective traps for river borne metals (Chapman and Wang 2001). Thus, halophiles are continuously exposed to metals and could be exploited for nanoparticle synthesis. Nanoparticles synthesis by halophiles has been reported in few organisms like bacteria, archaea, fungi, and algae (Table 2.1).

2.2.1 Nanoparticle Synthesis by Halophilic Bacteria

Reports on nanoparticles synthesis by halophilic bacteria and their metabolites are mostly confined to metallic nanoparticles. These halophilic bacteria include, *Halomonas salina*, *H. maura*, *Pseudomonas* sp., and *Idiomarina* sp. PR-58-8 (Table 2.1). A highly silver tolerant halophilic marine bacterium *Idiomarina* sp. PR 58-8 synthesizes intracellular crystalline silver nanoparticles (SNPs) with an average particle size of 26 nm. Non-protein thiols that are known to be expressed in response to metal stress are involved in metal tolerance (Seshadri et al. 2012). This bacterium synthesizes SNPs when silver is added at the time of inoculation unlike the terrestrial bacteria such as *Lactobacillus* sp. and *Escherichia coli*, wherein silver is added in the mid-log phase of growth. This is attributed to the high silver-tolerance of *Idiomarina* sp. PR58-8 and eliminates the requirement of growth phase monitoring during synthesis of SNPs. UV-visible absorbance scan of the 48 h culture from 300–800 nm revealed a broad peak at 450 nm, a characteristic of SNPs. XRD of lyophilized cell pellets obtained from 48 h cultures corresponded to silver (3C-syn) in the ICDD. TEM showed presence of SNPs in the 26 nm size range which upon purification could be applied in bio-labelling, antimicrobial coatings etc. The bacterium was found to respond to silver stress by inducing the expression of NP–SHs at extremely high levels (261 % on average) over the control, peaking at 42 h. Thus *Idiomarina* sp. PR58-8 is a promising microorganism for metal accumulation and metal nanoparticle synthesis.

Table 2.1 Halophiles in biosynthesis of nanoparticles

Halophile	NPs	Reference
Bacteria		
Idiomarina sp. PR 58-8	Ag	Seshadri et al. (2012)
Pseudomonas sp. *591786*	Ag	Muthukannan and Karuppiah (2011)
Halomonas salina	Au	Shah et al. (2012)
Bacillus megaterium BSB6	Se	Mishra et al. (2011)
Bacillus megaterium BSB12	Se	Mishra et al. (2011)
Archaea		
Halococcus salifodinae BK3	Ag	Srivastava et al. (2013)
Halococcus salifodinae BK18	Ag	Srivastava et al. (2014)
Fungi		
Penicillium fellutatum	Ag	Kathiresan et al. (2009)
Aspergillus niger	Ag	Kathiresan et al. (2010)
Pichia capsulata	Ag	Manivannan et al. (2010)
Yarrowia lipolytica	Ag	Bankar et al. (2009)
Yarrowia lipolytica	CdO	Pawar et al. (2012)
Yarrowia lipolytica	CdS	Pawar et al. (2012)
Rhodosporidium diobovatum	PbS	Seshadri et al. (2012)
Thraustochytrium sp	Ag	Gomathi (2009)
Algae		
Sargassum wightii	Ag	Singaravelu et al. (2007)
Sargassum wightii	Au	Oza et al. (2012)
Sargassum ongifolium	Au	Rajeshkumar et al. (2014)
Pterocladia capillacae	Ag	El-Rafie et al. (2013)
Jania rubins	Ag	El-Rafie et al. (2013)
Ulva faciata	Ag	El-Rafie et al. (2013)
Colpmenia sinusa	Ag	El-Rafie et al. (2013)
Navicula atomus	Au	Schrofel et al. (2011)
Diadesmis gallica	Au	Schrofel et al. (2011)

Similarly, a novel halophilic strain of *Pseudomonas* sp. 591786 can also synthesize polydisperse intracellular silver nanoparticles (Muthukannan and Karuppiah 2011). The silver nanoparticles formed were polydisperse, predominantly spherical with some nanotriangles in the range of 20–100 nm. Some small particles in the regime of 10–20 nm are also present. On treatment of aqueous solution of 1 mM silver nitrate with bacterial strain, SNPs can be rapidly fabricated at intracellular level. The formation of intracellular SNPs occurred only after 6 h of incubation.

The halophilic proteobacteria *Halomomas salina* can synthesize anisotropic gold nanoparticles under acidic conditions and spherical nanoparticles under alkaline conditions. The nanoparticle synthesis is extracellular and the NADH-dependent nitrate reductase is involved in the silver reduction and nanoparticle synthesis (Shah et al. 2012). *Halomonas salina* is a halophilic *Proteobacteria*, rich in reductases.

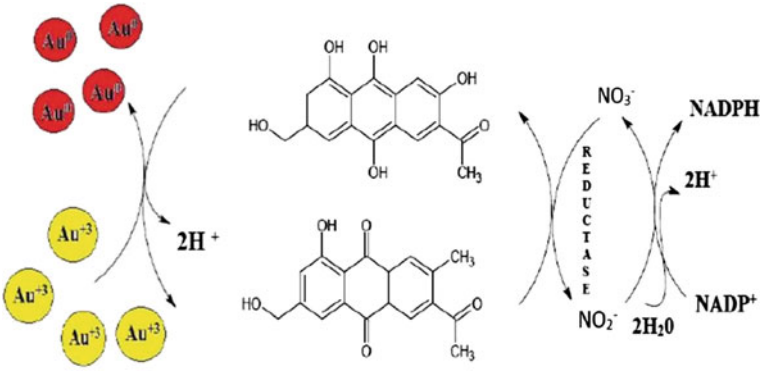

Fig. 2.1 Possible mechanism of reduction of gold salt and formation of gold nanoparticles. *Source* Shah et al. (2012). Copyright © 2012, Scholar Research Library

Studies have indicated that NADH and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles. *Halomonas salina* is known to secrete the cofactor NADH and NADH-dependent enzymes, especially nitrate reductase, which may be acting as a scaffold or nucleating agent and might be responsible for the bioreduction of Au^{+3} to Au^0 and the subsequent formation of gold nanoparticles (Fig. 2.1). The same enzyme later then acts as a capping agent, thus ensuring complete formation of thermodynamically stable nanostructures.

Similarly, two halophilic strains of *Bacillus megaterium* BSB6 and *Bacillus megaterium* BSB12 isolated from Bhitarkanika mangrove soils, synthesize spherical selenium nanoparticles (SeNPs) both intra- and extra-cellularly with an average size of 200 nm). The mechanism involved for the reduction of selenite to selenium, however remains unexplored (Mishra et al. 2011). Both the strains were found capable of reducing Se(IV) to elemental selenium even in the presence of high salt concentrations. Under optimized set of conditions (at 37 C, initial pH 7.5) almost complete reduction of Se(IV) up to 0.25 mM was achieved within 40 h incubation. Microbial reduction of selenium oxyanions generates red elemental selenium particles with either crystalline or amorphous structure. Often Se^0 formed by bacterial reduction is structurally unique compared to elemental selenium formed by chemical synthesis (Oremland et al. 2004). The formation of selenium nanoparticles was also evident from the UV–Vis spectra (Fig. 2.2) of the centrifuged and washed culture with selenium particles. The absorption bands with maxima at 226 and 285 nm, located between 200 and 300 nm, was due to the formation of selenium nanoparticles from Se(IV).

The intracellular selenite reduction is usually driven by reduced thiols, e.g., glutathione, in microorganisms (Kessi and Hanselmann 2004; Kessi 2006). Selenite reacts with glutathione to form selenodiglutathione (GS-Se-SG), which can be further reduced by NADPH to unstable selenopersulfide (GS-Se–) in the presence of glutathione reductase. Then, dismutation of GS-Se– will produce GSH and Se^0. In addition to the thiol groups, terminal reductases for anaerobic respiration in some

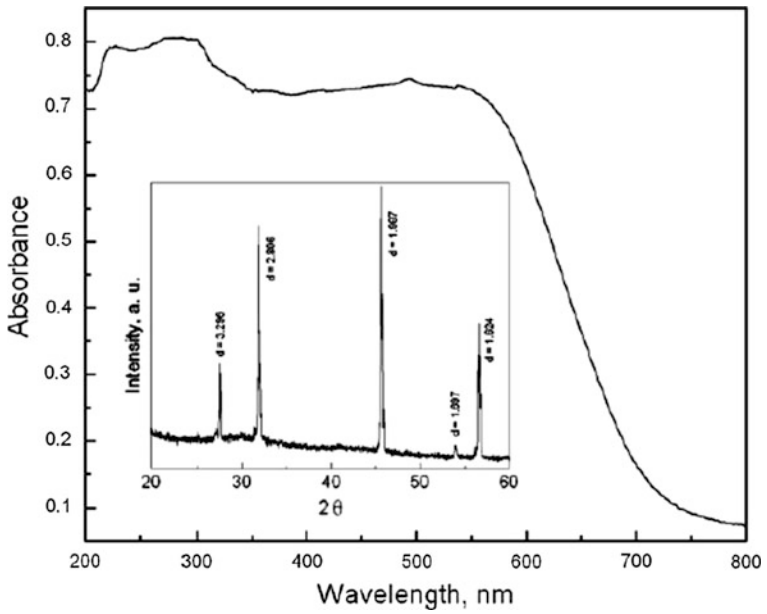

Fig. 2.2 UV–Visible diffuse reflectance spectra of Se^0 particles associated with *B. megaterium* (BSB12). (*Inset*) X-ray diffraction patterns of Se^0 particles associated with *B. megaterium* (BSB12). *Source* Mishra et al. (2011). Copyright © 2011, Elsevier. Reproduced with permission

microorganisms may also reduce selenite as they are redox-reactive in cells. It is reported that two nitrite reductases and an inducible sulfite reductase are able to conduct selenite reduction in cells (DeMoll-Decker and Macy 1993; Basaglia et al. 2007; Harrison et al. 1984). However, the possible involvement of other various respiration reductases in selenite reduction, as well as the physiological and ecological influence of this process to cells (Fig. 2.3). For instance in *Shewanella oneidensis* MR-1, selenite reduction is dependent on central respiration c-type cytochrome CymA. In contrast, nitrate reductase, nitrite reductase, and the Mtr electron transfer pathway do not work as selenite reductases (Li et al. 2014).

2.2.2 *Nanoparticle Synthesis by Halophilic Archaea*

Haloarchaea are the predominant population of thalassohaline and athalassohaline environments where salinity reaches up to 300 g/L (Zafrilla et al. 2010) and contribute to the red coloration of solar salt crystallizer. These organisms maintain osmotic balance with hypersaline surroundings by building up potassium ion concentration within their cells (Oren 2008). Haloarchaea are also known to encounter metals in their environment, but their metal tolerance has not been well documented (Srivastava and Kowshik 2013). Metal resistance genes have been

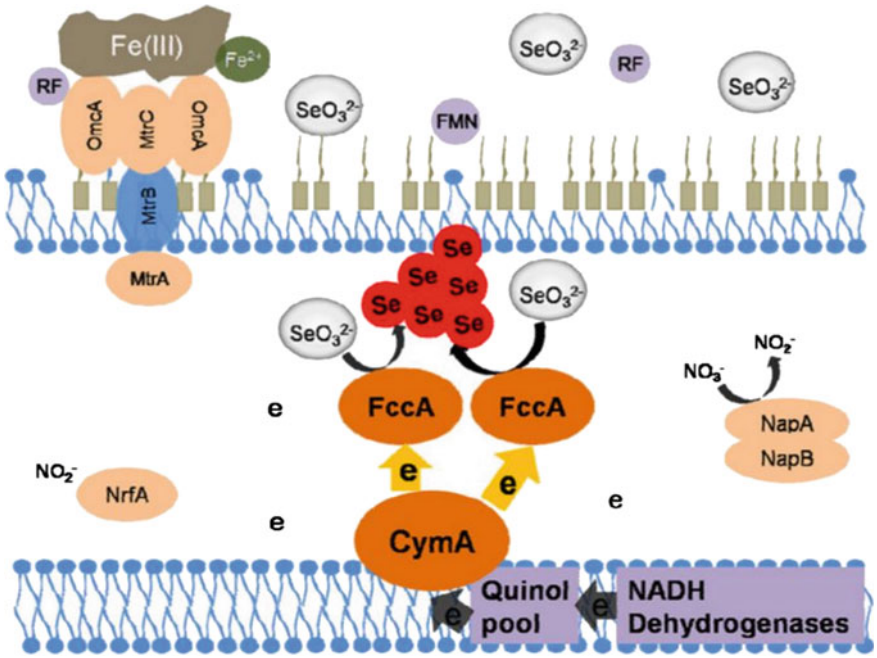

Fig. 2.3 Schematic diagram of the proposed pathways of selenite reduction and anaerobic respiration in *Shewanella oneidensis* MR-1. The oxidation of lactate provides electrons in the form of NADH, which further deliver the electrons to CymA through NADH dehydrogenases and the quinol pool. Shunt of electrons from CymA to various reductases enables execution of usual anaerobic respiration and selenite reduction. *Source* Li et al. (2014). Copyright © Scientific Reports (Springer Nature). Reproduced with permission

annotated in model organism *Halobacterium* sp. strain NRC-1 (Ng et al. 2000), but only arsenic resistance has been demonstrated experimentally (Wang et al. 2004). A system-level analysis of this organism has shown various strategies such as enhanced efflux and reduced influx that result in metal tolerance (Kaur et al. 2006). Haloarchaea are known to encounter metals in their natural habitat, yet reports on metal tolerance and nanoparticles synthesis by haloarchaea are few.

With the exception of two organisms, *Halococcus salifodinae* BK3 and H. *salifodinae* BK6, there are no other reports on metallic nanoparticles synthesis by haloarchaea. The intracellular synthesis of silver nanoparticles by *H. salifodinae* BK3 and BK6 involves the enzyme NADH-dependent nitrate reductase that helps in silver ion reduction. These organisms adapt to the metal stress and thus their growth kinetics parameters in presence of silver nitrate are similar to that of organism grown without silver nitrate. The reduction of silver ions to its metallic form in bacterial and fungal systems has also been shown to involve enzymatic detoxification by enzymes like nitrate reductase, where toxic metal form is converted to non-toxic nanoparticulate form (Srivastava et al. 2013, 2014).

Nitrate reductase is normally involved in the reduction of nitrate to nitrite using NADH as the electron donor, however, in the presence of silver the electron is shuttled from NADH to reduction of silver ions. The haloarchaeal isolate *H. salifodinae* BK3 exhibited nitrate reductase activity as indicated by the pink color development on addition of Griess Ilosvays reagent (Srivastava et al. 2013). This mechanism has been excellently described in the organism *B. licheniformis* (Kalimuthu et al. 2008). *B. licheniformis* is known to secrete the cofactor NADH and NADH-dependent enzymes, especially nitrate reductase, that might be responsible for the bioreduction of Ag^+ to Ag^0 and the subsequent formation of silver nanoparticles. Figure 2.4 shows that the nitrate reductase present in the bacteria may aid the synthesis of silver nanoparticles (Kalimuthu et al. 2008). The silver nanoparticles synthesized by *H. salifodinae* BK3 and BK6 exhibit good antibacterial activity against both Gram-positive and Gram-negative bacteria (Srivastava et al. 2013, 2014). The following are the effects by which silver ions exhibit their antimicrobial functions. (1) binding of silver ions to the negatively charged DNA thereby making the DNA to lose its structure and also inhibiting the replication of DNA; (2) binding of silver ions with the thiol-containing proteins,

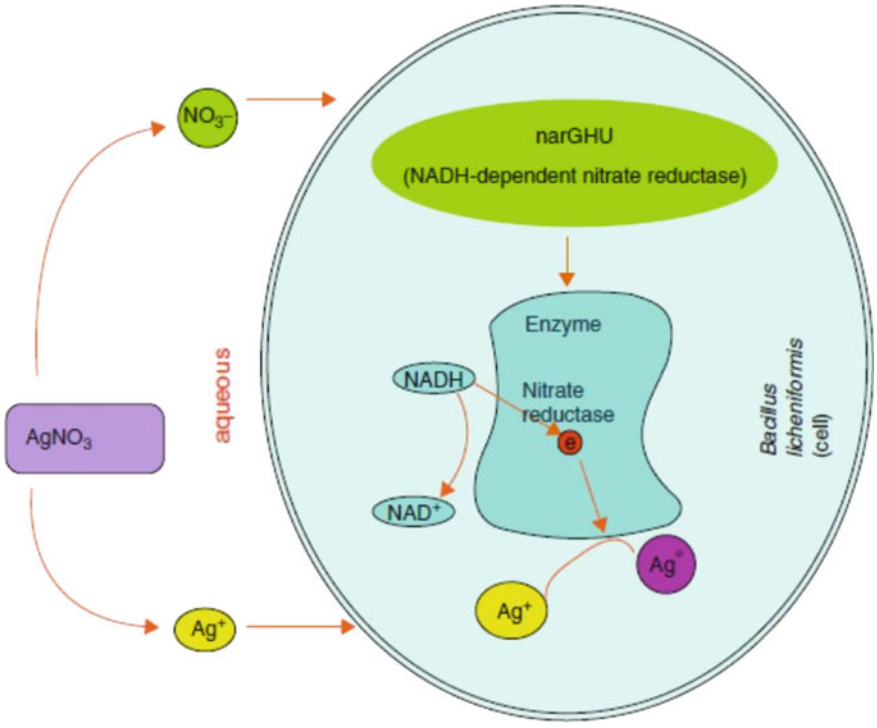

Fig. 2.4 Reduction of silver ions to silver atom by the enzyme nitrate reductase. *Source* Kalimuthu et al. (2008). Copyright © 2008, Elsevier. Reproduced with permission

thereby inhibiting the function of proteins; and (3) induction of reactive oxygen species synthesis leading to the formation of highly reactive radicals that destroy the cells (Deepak et al. 2011).

2.2.3 Nanoparticle Synthesis by Halophilic Fungi

A few halophilic yeasts and fungi are known to synthesize nanomaterials. *Pichia capsulata*, a mangrove derived halophilic yeast, is capable of synthesizing silver nanoparticles extracellularly (Manivannan et al. 2010). Yeasts are unicellular fungi that predominantly reproduce by budding. A rapid method for the synthesis of silver nanoparticles from *P. capsulata* was demonstrated. The optimum conditions were pH 6:0, 0:3 % NaCl concentration, and a temperature of 5 °C. The nanoparticles were mostly spherical with a size of 525 nm. An NADH-dependent (NADH: nicotinamide adenine dinucleotide) protein similar to nitrate reductase was partially purified and was suggested to mediate the reduction process.

Yarrowia lipolytica is biotechnologically important in the bioremediation of hydrophobic substrate contaminated environments, in the treatment or up-gradation of wastes, biotransformation of organic compounds, production of novel enzymes, and in the cloning and expression of heterologous proteins. This yeast has been isolated from polluted areas containing toxic and hazardous metals. Metal tolerance in this yeast is attributed to the presence of superoxide dismutase (a copper tolerating protein), reductases, CRF1, metallothioneins, efflux mechanisms, and melanin (Bankar et al. 2009). A tropical marine isolate of *Y. lipolytica* (NCIM 3589), obtained from oil-polluted seawater near Mumbai, India, mediated the synthesis of gold nanoparticles. The synthesis took place at 30 °C within 72 h. The cell wall-associated synthesis of nanoparticles was confirmed by TEM analysis. The yeast, as well as the mycelial forms of this dimorphic fungus, synthesized the gold nanostructures. The size of the nanoparticles was found to be pH dependent. At pH 2:0, gold nucleation was observed within 15 min. Over a period of time, these developed into large triangular and hexagonal plates. The size of the nanostructures at pH 7:0 and at 9:0 was 15 nm (Agnihotri et al. 2009). In a further study, this system was used in the custom designing of gold nanoparticles with specific sizes. With increasing cell numbers and the same concentration of gold salt, the particle size was found to decrease. On the other hand, with increasing concentration of the gold salt and the same cell numbers, there was an increase in the size of the particles. The cell-associated gold nanoparticles could be released into the medium by incubation at 20 °C (Pimprikar et al. 2009). Melanin, a dark-colored pigment from this yeast was found to be one of the factors responsible for nanoparticle synthesis. Cell-extracted and induced melanin (obtained by incubating resting cells with L-3,4-dihydroxyphenylalanine (L-DOPA) mediated the synthesis of gold nanostructures. Another cold-adapted marine strain of *Y. lipolytica* (NCIM 3590) also synthesized gold nanostructures (Fig. 2.5a). The synthetic ability in this case was also associated with the dark-colored pigment, melanin. These nanoparticles

displayed antibiofilm activity against pathogenic bacteria. Since the inherent content of melanin in this organism was low, the yeast was induced to overproduce melanin by incubation with a precursor, L-DOPA. This melanin also mediated the rapid formation of silver and gold nanoparticles. The former displayed effective antifungal properties against a wall-disfigurement causing fungus (Apte et al. 2013). In addition, the tropical marine strain (*Y. lipolytica* NCIM 3589) described above was able to synthesize CdO and CdS nanostructures in a cell-associated and extracellular manner. The SEM images of cell-associated nanostructures in this yeast are depicted in Fig. 2.5b. Certain functional groups on the cell surface were thought to play a role in the reductive and stabilization processes (Pawar et al. 2012).

Another fungus, *Penicilium fellutanum* associated with the rhizosphere of an Indian endemic mangrove plant (*Rhizophora annamala*) was isolated and evaluated

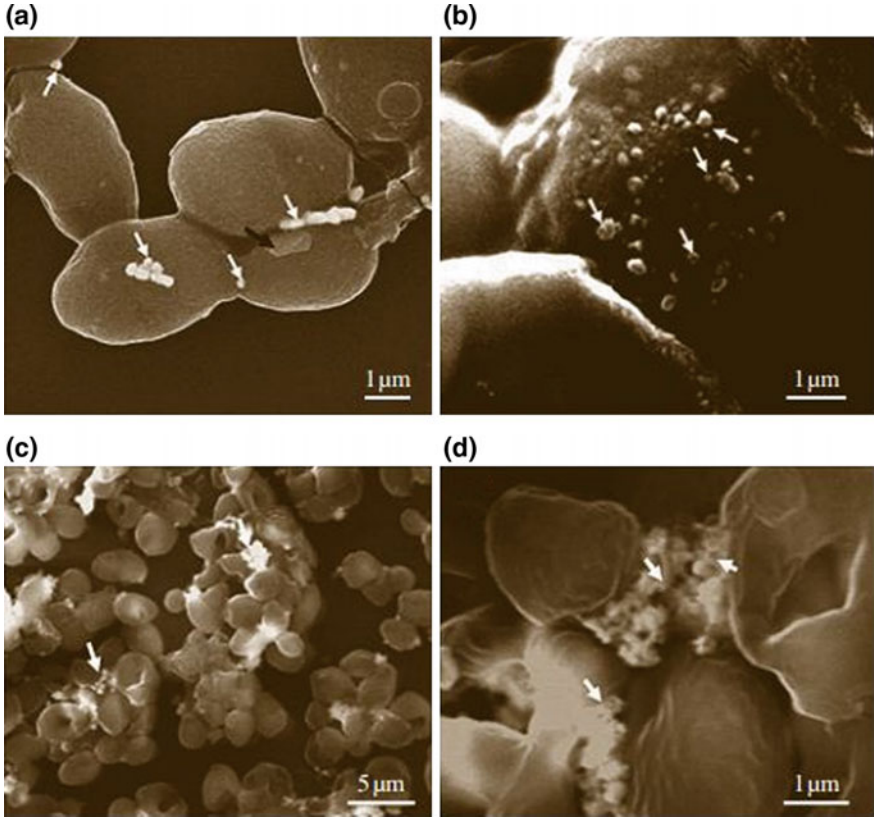

Fig. 2.5 Nanostructures associated with marine yeasts. **a** Gold nanoparticles and microplates synthesized by Yarrowia lipolytica NCIM 3590; **b** CdO nanoparticles mediated by *Y. lipolytica* NCIM 3589. *White arrows* point to nanostructures and the *black arrow* to a microplate. *Source* Pawar et al. (2012). Copyright © 2012, American Scientific Publishers

for the synthesis of silver nanoparticles. The biosynthesis of nanoparticles was the maximum when the culture filtrate was treated with 1.0 mM $AgNO_3$, maintained at 0.3 % NaCl and pH 6.0, incubated at 5 °C for 24 h. The extracellularly synthesized nanoparticles are spherical in shape with size ranging from 5–25 nm. A protein of about 70 kDa from the cell-free supernatant was proposed to be responsible for converting the metal ions to their zero valence state (Kathiresan et al. 2009). Presence of silver nanoparticles in the culture filtrate was confirmed by absorption peak at 430 nm, as well under transmission electron microscope.

Silver nanoparticles are also synthesized by the halophilic fungi *Aspergillus niger*. *Aspergillus species* are found in almost all climatic conditions worldwide. This fungus is often associated with environments that are rich in starchy material. Since mangrove ecosystems offer such conditions (due to the accumulation of debris from leaves, inflorescence, and stems), *Aspergillus* sp. have been isolated from these habitats (Seelan et al. 2009). A strain of *A. niger* (AUCAS 237) isolated from a mangrove sediment (Vellar estuary, India) synthesized silver nanoparticles. The nanoparticles were 5–35 nm in diameter and spherical in shape. They displayed effective antimicrobial activity towards clinical pathogens (especially Gram negative bacteria and some fungi). The activity was enhanced when the nanoparticles were stabilized with polyvinyl alcohol. FTIR analysis revealed the possibility of proteins as possible capping and stabilizing agents (Kathiresan et al. 2010). Silver nanoparticles were also synthesized by using another strain of *A. niger* isolated from the Gulf of Cambay, India. The nanoparticles were spherical, 5–26 nm in size, and displayed laser optical speckles, which could have several applications in the future (Vala et al. 2012). Microorganisms are known to be symbiotically associated with different marine forms such as sponges. A strain of *A. terreus* (MP1) was isolated from a marine sponge. The mycelial extract of this fungus synthesized silver nanoparticles. SEM and TEM analysis showed the presence of spherical nanoparticles 1520 nm in size. These particles effectively inhibited the growth of pathogenic bacterial strains of *Staphylococcus aureus, Klebsiella pneumoniae*, and *Salmonella typhi* (Meenupriya et al. 2011).

Rhodospiridium diobovatum, a marine yeast, synthesizes lead sulphide (PbS) nanoparticles intracellulary with the help of non-protein thiols (Seshadri et al. 2011). The PbS nanoparticles were characterized by UV-visible absorption spectroscopy, X-ray diffraction (XRD) and energy dispersive atomic spectroscopy (EDAX). UV-visible absorption scan revealed a peak at 320 nm, a characteristic of the nanosize range. XRD confirmed the presence of PbS nanoparticles of cubic structure. Crystallite size as determined from transmission electron microscopy was found to be in the range of 2–5 nm. Elemental analysis by EDAX revealed the presence of particles composed of lead and sulfur in a 1:2 ratio indicating that PbS nanoparticles were capped by a sulfur-rich peptide. A quantitative study of lead uptake through atomic absorption spectrometry revealed that 55 % of lead in the medium was accumulated in the exponential phase, whereas a further 35 % was accumulated in the stationary phase; thus, the overall recovery of PbS nanoparticles was 90 %. The lead-exposed yeast displayed a marked increase (280 % over the control) in nonprotein thiols in the stationary phase. A sulfur-rich peptide was

suggested to be the capping agent. In the presence of lead, this yeast produced increasing contents of non-protein thiols during the stationary phase. These were possibly involved in forming the nanoparticles (Seshadri et al. 2011).

2.2.4 Nanoparticle Synthesis by Halophilic Algae

The reports on nanoparticles synthesis by halophilic algae are few and mostly recent. All studies so far are on extracellular synthesis of inorganic (metallic) nanoparticles. Algae inhabit natural as well as metal-contaminated freshwater and marine environments. Various species of algae are also known to interact with heavy metal ions. Some of these species are involved in the detoxification and bioremediation of metal wastes from water (Scarano and Morelli 2003; Mehta and Gaur 2005). The first alga reported to synthesize gold nanoparticles was the brown alga Sargassum wightii (Singaravelu et al. 2007). The marine brown algae *Sargassum wightii* synthesizes stable gold (30–100 nm) and silver nanoparticles (8–12 nm) when its extract is exposed to gold chloride and silver nitrate, respectively (Singaravelu et al. 2007; Oza et al. 2012). The first report on the synthesis of highly stable gold nanoparticles using marine alga, *S. wightii* was carried out by Singaravelu et al. (2007). The reduction of the metal ions resulted in the formation of high density, extremely stable gold nanoparticles in the size ranging from 8 to 12 nm with an average size ca. 11 nm. Figure 2.6a shows the powder of marine alga with gold ions at the beginning of the reaction and Fig. 2.6b shows the color change of the medium to ruby red after 15 h of incubation. The change in color of the medium was noted by visual observation. An important potential benefit of the described method of synthesis of nanoparticles using marine algae is that they are quite stable in solution and this is a very important advantage over other biological methods currently in use (Singaravelu et al. 2007).

Similarly, extracts of *S. longifolium* can reduce silver nitrate to spherical silver nanoparticles that exhibit excellent antifungal activity. The extracts contain various active molecules rich in hydroxyl groups or carboxyl groups that are responsible for the reduction of the metallic ion (Rajeshkumar et al. 2014). The synthesized silver nanoparticles exhibited excellent antibacterial activity against pathogenic fungi such as *Aspergillus fumigatus*, *Candida albicans*, and *Fusarium* sp. (Fig. 2.7). The antifungal activity of *S. longifolium* mediated synthesized silver nanoparticles against harmful pathogenic fungi at different concentrations (50, 100, and 150 μL) was carried out. As the concentration of silver nanoparticles increased, the zone of inhibition increased.

Diatoms are unicellular photosynthesizing microorganisms belonging into the group of brown algae (division *Chromophyta*, class *Bacillariophyceae*) encased in siliceous cell walls—frustules (Fig. 2.8). The biosynthesis of gold nanoparticles has been successfully conducted using two strains of diatoms (*Navicula atomus* and *Diadesmis gallica*) mixed with aqueous $HAuCl_4$ ($\sim$500 mg/L Au) at laboratory conditions. The interaction of diatoms with aqueous salt promoted the precipitation

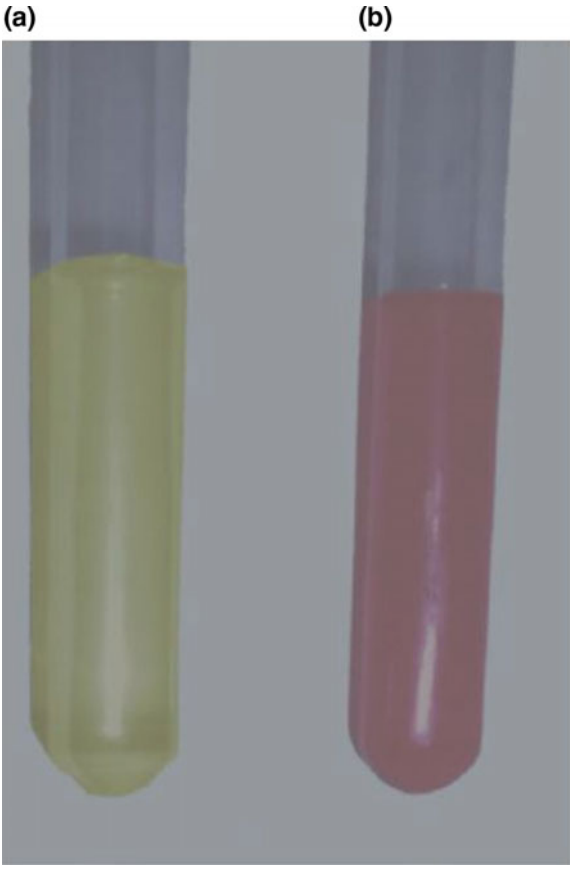

Fig. 2.6 *Yellow color* due to aqueous auric chloride (**a**) and *ruby red color* indicating the formation of gold nanoparticles (**b**). *Source* Singaravelu et al. (2007). Copyright © 2007, Elsevier. Reproduced with permission

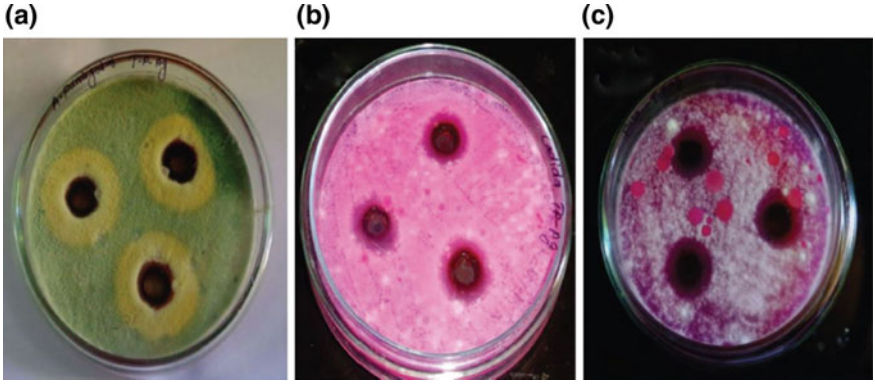

Fig. 2.7 Antifungal activity of AgNPs synthesized by using marine algae *S. longifolium*. *Source* Rajeshkumar et al. (2014). Copyright © 2014, Hindawi Publishing Corporation. Reproduced with permission

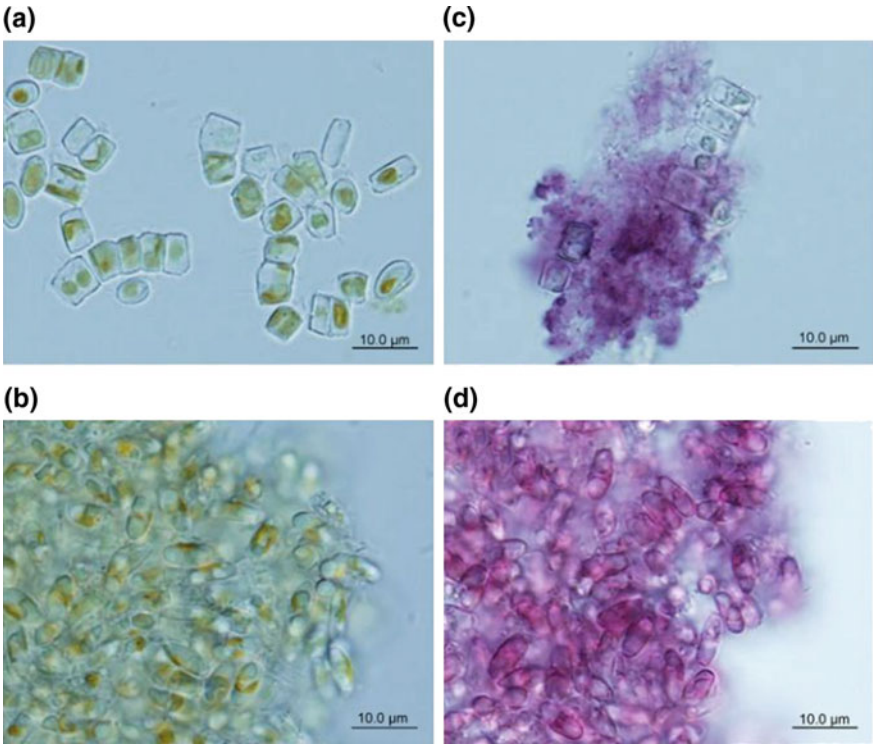

Fig. 2.8 Light microscope photographs of the diatom cells before (*left*) and 12 h after (*right*) tetrachloroaureate addition for **a**, **c** *Diadesmis gallica*, and **b**, **d** *Navicula atomus*. *Source* Schrofel et al. (2011). Copyright © 2011, Springer. Reproduced with permission

of gold nanoparticles. When the diatoms are grown in the presence of tetrachloroaurate, the diatoms reduces it to gold nanoparticles that are associated with the diatom frustules and exopolysaccharides (EPS) excreted by the diatoms. The size of the biosynthesized nanoparticles differed in each strain. Whereas *Diadesmis gallica* showed larger mean particle size (around 22 nm) and wider range of the size distribution, AuNPs synthesized by NA strain had smaller mean particle size (9 nm) and higher homogeneity in size (Schrofel et al. 2011).

2.3 Biomolecules Produced by Halophiles with Implications in Nanotechnologies

The synthesis of silver nanoparticles is attributed to the presence of peptides, amino acids, reducing sugars, and enzymes such as reductases and proteases. Thus, many biomolecules including proteins/enzymes/oligopeptides (Crespilho et al. 2009),

antibody/antigens (Haes et al. 2004; Pengo et al. 2007), biotin/streptavidin (Haes et al. 2004), and DNA/oligonucleotides/aptamers (Nykypanchuk et al. 2008) have been immobilized on the surface of nanoparticles to form noble metal nanoparticle–biomolecule conjugates. The similarity in size of nanoparticles and biomolecules makes them relatively easy to integrate.

The synthesis and applications of biomolecule-nanoparticle hybrid systems have been reviewed (Katz and Willner 2004). There have also been a number of articles that review different specific applications of biomolecule-nanoparticle hybrids, such as biosensing (Willner et al. 2007), probing cells (Roca and Haes 2008), delivery (Ghosh et al. 2008) and diagnosis (Baptista et al. 2008). For example, Katz and Willner (2004) reviewed advances in the synthesis of biomolecule (proteins or DNA)-nanoparticle (metal or semiconductor nanoparticles) conjugates as well as their application. Aubin-Tam and Hamad-Schifferli (2008) reviewed studies of the conjugation of protein and nanoparticles using a linker species. In addition, many specific applications have also been reviewed: biomolecule nanoparticle conjugates for the assembly of electrochemical biosensors (Willner et al. 2007), noble metal nanoparticle aggregates as tags for probing cells (Roca and Haes 2008), gold nanoparticles as non-toxic carriers for drug and gene delivery (Ghosh et al. 2008), and gold nanoparticles for application in clinical diagnosis (Baptista et al. 2008).

The development of biomolecule-nanoparticle systems has been very rapid, and many new materials have been reported. As a result, it is very imperative to review of recent research results including new synthesis methods, optical properties and applications. Conjugation of noble metal nanoparticles with biomolecules has mainly been achieved by one of four major mechanisms: electrostatic adsorption, direct chemisorption of thiol derivatives, covalent binding through bifunctional linkers, and specific affinity interactions (Zhang et al. 2012). Among these mechanisms, specific affinity interactions can be further classified into sub-categories including biotin-streptavidin binding, antibody-antigen conjugation, and complementary DNA association. These mechanisms are schematically described in Fig. 2.9.

Several biomolecules currently produced by these halophilic organisms, such as exopolysaccharides (EPS) and enzymes showed biological activity in harsh conditions. Combination of these biomolecules with various nanomaterials like thin-layers, nanotubes, nanospheres results in novel compounds possessing both biological properties of biomolecules and physico-chemical characteristics of nanomaterials. The present chapter deals with the main biomolecules produced by both halophilic microorganisms and bacteria revealing their potential implications in some nanotechnologies.

2.3.1 Exopolysaccharides from Halophiles

Exopolysaccharides (EPS) are often found surrounding the outermost structures of both prokaryotic and eukaryotic microbial cells. They may be closely associated

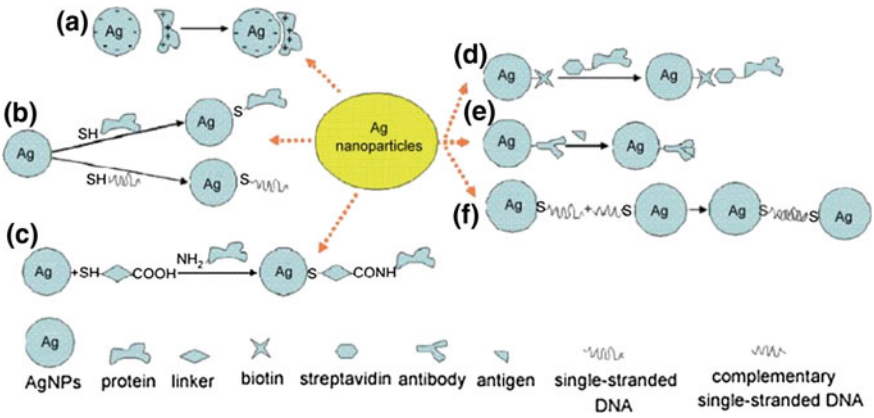

Fig. 2.9 Synthesis of biomolecule-nanoparticle systems by different routes: **a** assembly of nanoparticle (NP)–protein conjugates by electrostatic adsorption; **b** conjugation of biomolecules (proteins/DNA) on NPs through direct chemisorption of thiol derivation; **c** covalent binding through bifunctional linkers to form biomolecule-NP hybrids. *Source* Zhang et al. (2012). Copyright © 2012, Springer. Reproduced with permission

with the cell in the form of discrete capsules or else excreted as slime, unattached to the cell surface as such. EPSs exist in a wide variety of unique and often complex chemical structures and they are believed to provide self-protection against antimicrobial substances (Kumon et al. 1994), antibodies and bacteriophages (De Vuyst and Degeest 1999; Wingender et al. 1999) and/or afford adherence to other bacteria, animal and plant tissues or inert surfaces (Sutherland 2001), thus forming biofilms. Increasing interest is being generated in the study of these molecules because of their wide applications in food, pharmaceutical, petroleum and other industries (Dawes 1990; Sutherland 1990; Vandamme et al. 2002). Nevertheless, the strains used for the industrial production of EPS belong to a small number of taxa, such as *Xanthomonas campestris* (Evans et al. 1979), *Pseudomonas* (Jarman 1979), *Azotobacter* (Jarman et al. 1978), *Sphingomonas* (Lobas et al. 1992), *Alcaligenes* (Sutherland 1990).

Several halophilic microorganisms produce such exopolysaccharides in copious amounts, and therefore their commercial exploitation has been considered. The archaeon *Haloferax mediterranei*, excretes large amounts of anionic exopolysaccharides. The sulfated acidic heteropolysaccharide of *Haloferax* species has a high viscosity at low concentrations, its rheological properties are excellent and it is resistant to extremes of pH and temperature (Oren 2012). *Aphanothece halophytica*, a halophilic unicellular cyanobacterium found in the benthic cyanobacterial mats of solar salterns and in many other hypersaline lakes, is also known for its massive synthesis of polysaccharides. In solar salterns excessive polysaccharide production can have a negative impact on the salt production process, as explained above. However, a recent report on the immunomodulating properties of the sulfated polysaccharide of *A. halophytica* is of special interest: when administered orally in

mice, it significantly inhibited pneumonia induced by influenza virus H1N1 (Zheng et al. 2006). Among the halophilic representatives of the *Bacteria*, the *Halomonas* species (*H. maura*, *H. eurihalina*) shows considerable promise as a producer of large amounts of an extracellular polyanionic polysaccharide, a potent emulsifying agent that exhibits a pseudoplastic behaviour (Quillaguamán et al. 2007). The *H. maura* exopolysaccharide ('mauran') has also been shown to be an immunomodulator (Béjar et al. 1998; Arias et al. 2003; Llmas et al. 2006).

Polysaccharides are emerging as stabilizing and reducing agents for nanoparticles synthesis, however the commercial polysaccharides are not economically viable. Therefore, the exopolysaccharide from microbial origin such as bioflocculants are promising alternate for the synthesis and stabilization of nanoparticles. Polysaccharides have hydroxyl groups, a hemiacetal reducing end, and other functionalities that can play important roles in both the reduction (Mata et al. 2009) and the stabilization of metallic nanoparticles that creates vast opportunities for their utilization and potential mass production. Polysaccharide bioflocculants can be used for high-performance nanomaterials production, since they easily form a variety of liquid crystals in aqueous solutions and bioflocculant-mediated processes are highly profitable. Bacterial metabolites or products such as polysaccharides/bio-flocculants are now being used as reducing agents to synthesize inorganic nanoparticles. In most cases the synthesized nanoparticles are capped by reducing agents. AgNPs have were synthesized by using polysaccharide bioflocculant as reducing and stabilizing agent by Sathiyanarayanan et al. (2013). In their study, the polysaccharide bioflocculant produced by a halophilic bacterium *Bacillus subtilis* MSBN17 reduced silver nitrate to spherical AgNPs in reverse micelles. The electrostatic forces between the amino groups of the polysaccharide MSBF17 and the silver ions in the solution are proposed to be the driving force for the formation and stabilization of the silver nanoparticles. The carboxyl, hydroxyl and methoxyl groups of MSBF17 form a coating on the silver nanoparticles thereby stabilizing them. These nanoparticles exhibit anti-microbial activity against a host of pathogenic organisms. The AgNPs were spherical shaped (60 nm) and stable for 5 months (Sathiyanarayanan et al. 2013).

Besides the various inorganic and organic nanoparticles, the exopolysaccharides of the halophilic bacteria have also been utilized for fabrication of polymer hybrid nanomaterials. Raveendran et al. (2013b) reported the synthesis of an extremophilic bacterial sulfated polysaccharide based nanoparticle as a stable biocompatible material for drug delivery, evaluation of anticancer efficacy and bioimaging. Sulfated polysaccharides (SPSs) are gaining attention since last few decades because of their exceptionally best physico-chemical properties and bioactivities. Owing to their unique properties like stable structure, composition, fluid dynamics, extreme stability, biodegradability and biocompatibility, they are widely exploited in modern biotechnology and material science (Raveendran et al. 2013b). *Halomonas maura* is a moderately halophilic bacterium, which is capable of producing highly sulfated EPS residues into the external milieu (Fig. 2.10). As reported by Arias et al. (2003), *Halomonas* polysaccharides are rich in sulfate residues and hence posses various biological properties. Biologically active sulfated

Fig. 2.10 SEM micrograph of *Halomonas maura* showing the exopolysaccharide, MR, covering the cell wall. *Source* Raveendran et al. (2013c). Copyright © 2013, Elsevier. Reproduced with permission

polysaccharide produced by *H. maura* is called MR and it has exceptionally high sulfate content and uronic acid content. MR is a high molecular weight acidic polysaccharide with repeating units of mannose, galactose, glucose and glucuronic acid. It is highly anionic in nature due to the presence of sulfate and uronic acid moieties. Viscoelasticity, pseudoplasticity and thixotropic behavior of MR make it an ideal molecule for material science applications. Similarly rheological properties of MR are not easily affected by the presence of any salts, sugars, surfactants, lactic acid, and changes in pH and freeze thawing (Llamas et al. 2006). Another important striking property of MR is the ability to withstand various harsh conditions like temperature, freeze thawing, extreme pH values and salt conditions. High temperature over 55 °C has detrimental effect on viscosity, although it can regain its 70 % of its property on cooling to 25 °C (Arias et al. 2003). MR can form stable gels on binding to various metal ions that helps in efficient removal of toxic ions from the polluted environments and water. The unusually high sulfate content of MR contributes to immunomodulating and antiproliferative effects on human cancer cells (Llamas et al. 2006). The highly sulfated anionic exopolysaccharide, Mauran (MR) secreted by the halophilic bacterium *H. maura*, is well characterized and has been successfully used for generation of such hybrid nanomaterials. The high sulphate content imparts immunomodulating and anti-cancer properties to MR. Thus, MR can be used for various biomedical applications due to their biological and the physicochemical properties. MR-Chitosan (MR/CH) hybrid nanoparticles (Fig. 2.11) fabricated via the ionic-gelation technique when used for encapsulation of drugs exhibits controlled and sustained drug release and biocompatibility (Raveendran et al. 2013b).

Similarly, electrospun MR-polyvinyl alcohol (MR-PVA) nanofibre membranes (Fig. 2.12) boost the cellular adhesion, migration, proliferation and differentiation, properties desirable for tissue engineering applications (Raveendran et al. 2013c). Nanofibers synthesized from biocompatible and bioactive polymers are of great

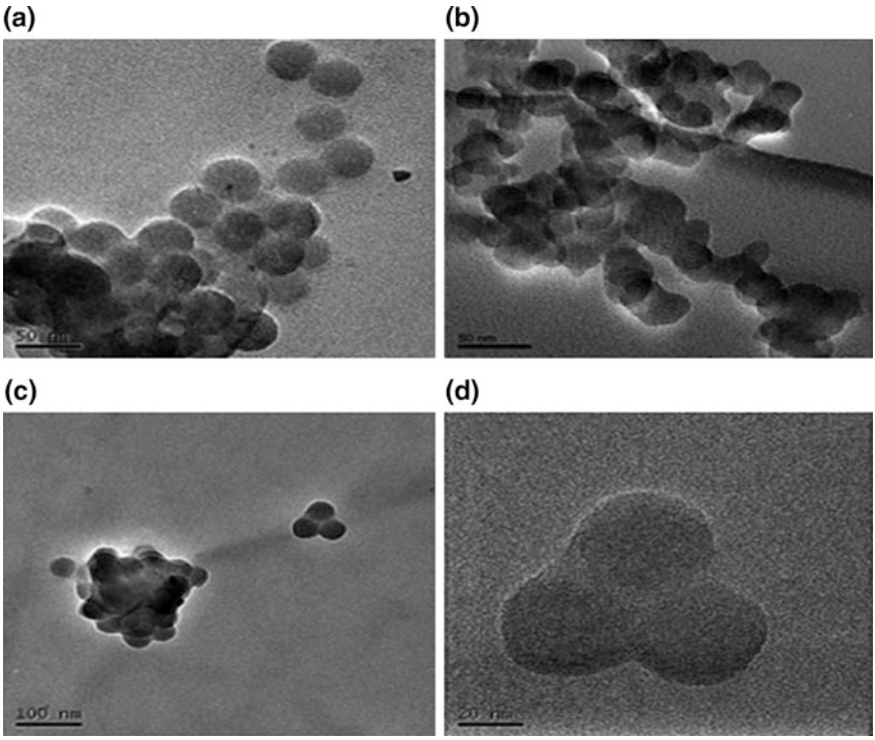

Fig. 2.11 TEM micrographs (**a**–**d**) depicting the morphology of the Mauran-Chitosan nanoparticles fabricated using the exopolysaccharide mauran secreted by the halophilic bacteria *Halomonas maura. Source* Raveendran et al. (2013b). Copyright © 2013, Elsevier. Reproduced with permission

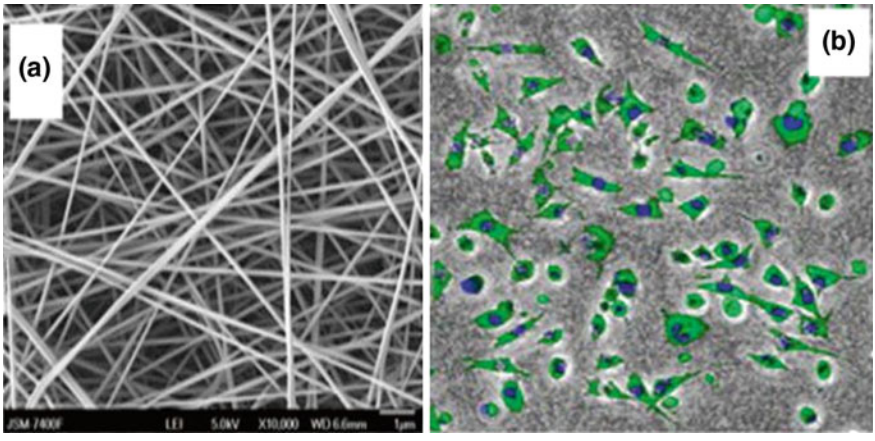

Fig. 2.12 SEM micrographs of the MR/PVA nanofi bres fabricated using the exopolysaccharide mauran (×10,000) (**a**). Confocal microscopy images of L292 cells attached and proliferating on MR/PVA nanofi bres (merged microtracker green and DAPI stained images) (**b**). *Source* Raveendran et al. (2013c). Copyright © 2013, Elsevier. Reproduced with permission

importance in the new generation biomedicine and nanotechnology. They are well established in the biomedical field for various applications like tissue engineering, wound dressing, drug delivery and enhanced cell adhesion (Vlierberghe et al. 2011; Dhandayuthapani et al. 2012). Recently optically transparent cellulose nanofibers have opened a wide scope of developing nanofiber matrices for enumerable applications even in the field of microelectronics (Nogi et al. 2009). Natural and synthetic polysaccharides are proven to be a good matrix material for generation of excellent tissue engineering scaffolds (Vlierberghe et al. 2011). Bacterial polysaccharide from moderately halophilic *Halomonas maura* based biocompatible nanofibers were produced for the first time via electrospinning technique by Raveendran et al. (2013c). In their study, mauran (MR), an extremophilic sulfated exopolysaccharide was extracted from *H. maura* and characterized for the application of nanofiber synthesis. Thin-uniform MR nanofibers were produced using homogenous solutions of poly (vinyl alcohol) (PVA) blended with different concentrations of MR. An average of 120 nm sized nanofibers were produced and tested for an enhanced cell growth under in vitro conditions in comparison with control. MR and MR/PVA nanofibers were found to be an excellent biomaterial for the migration, proliferation and differentiation of mammalian cells (Raveendran et al. 2013c).

Mauran may be used in augmenting the biocompatibility of quantum dots that are usually cytotoxic (Fig. 2.13). The nanocrystals, so-called quantum dots (QDs), are undisputedly excellent fluorescent markers for imaging and clinical diagnostics. However, their toxicity is always a perturbing issue and remains as the major hindrance for biocompatible imaging and other biomedical applications. Raveendran et al. (2014) demonstrated the extraction and application of an extremophilic bacterial polysaccharide, mauran (MR), from a moderately halophilic

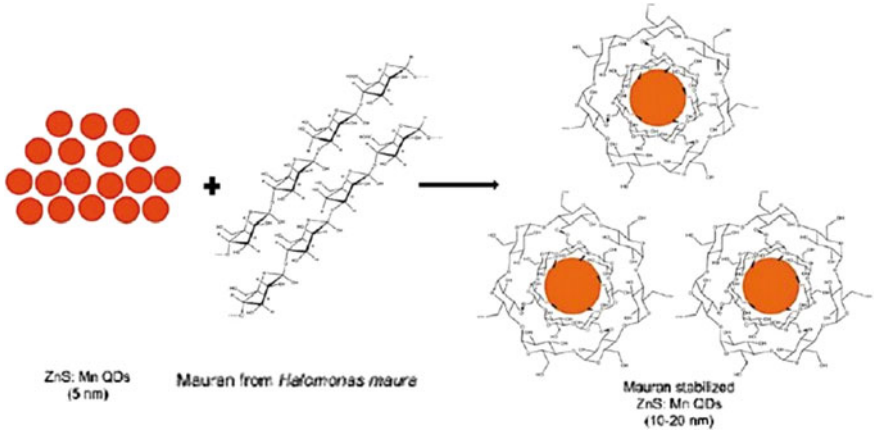

Fig. 2.13 Schematic representation of stabilization of QDs by Mauran, an exopolysaccharide secreted by *H. maura*. *Source* Raveendran et al. (2013b). Copyright © 2013, Elsevier. Reproduced with permission

bacterium called *Halomonas maura* in the stabilization of ZnS:Mn2+ QDs for the first time. Mauran was used as a natural polymer for bioconjugation to enhance the cellular acceptance and decrease the cytotoxicity of QDs while being used as a fluorescent marker for imaging purposes. Five nanometer-sized QDs were stabilized using an aqueous MR solution under ambient conditions to yield 10−20 nm-sized nanoparticles. MR conjugated with ZnS:Mn^{2+} QDs at a concentration of 0.05 mg resulted in a drastic increase in cell viability as compared to cell viability of bare QDs (Raveendran et al. 2014). Therefore, in addition to fabrication of polymer based nanomaterials, MR can also be used in conjugation with inorganic nanoparticles for enhancing their biological applications.

The water soluble exopolysaccharides extracted from the marine algae *Pterocladia capillacae, Jania rubins, Ulva faciata* and *Colpmenia sinusa* reduce silver ions to silver nanoparticles. In this work, a well-stabilized Ag-NPs solution with a concentration of 108 ppm was prepared using 30 mg of the prepared polysaccharides from *C. sinusa*, *J. rubins*, *U. faciata* and *P. capillacae* algae as reducing agents for silver ions as well as stabilizing agents for the formed AgNPs. The biosynthesized Ag nanoparticles are stable for six months and have the ability to be immobilized on the cotton fibers bringing a good antimicrobial property to the final textile product and rendering it to be used as an antiseptic dressing or bandage, which is in high demand for biomedical applications (El-Rafie et al. 2013).

EPS-gold and silica-gold bio-nanocomposites can be generated using the diatoms *Navicula atomus* and *Diadesmis gallica*. The diatoms when grown in presence of tetrachloroaureate, reduce it to gold nanoparticles that are associated with the diatom frustules and exopolysaccharides (EPS) excreted by the diatom cells. Scanning electron microscopy (SEM) and TEM showed that the nanoparticles were associated with the diatom frustules and extracellular polysaccharides (EPS) excreted by the diatom cells. Due to its accessibility, simplicity, and effectiveness, this method of nanocomposites preparation has great importance for possible future applications. The gold bio-nanocomposites may find applications in the field of catalysis (Schrofel et al. 2011).

2.3.2 Gas Vesicles

Buoyant gas vesicles are prokaryotic organelles that are widely distributed among bacterial and archaeal microorganisms and constitute protein nanoparticles (GVNPs) that may be engineered for biotechnological applications (DasSarma et al. 1994, 2010a, b; Shively et al. 2011; Cai et al. 2012). These organelles naturally promote flotation and increase the availability of light and oxygen to many aquatic microorganisms, especially those with photosynthetic or phototrophic capabilities. Water is excluded from the interior, a property that is thought to be a consequence of the hydrophobicity of the interior surface of the proteinaceous membrane. While the exact protein composition of the membrane has been difficult to ascertain due to its extreme stability against solubilization, production of these structures is easily

scaled-up and they are simple to purify by hypotonic lysis of the host and concentrate by flotation, enhancing their intrinsic value for biotechnological applications (Stuart et al. 2001, 2004). Gas vesicle nanoparticles (GVNPs), that may be engineered for various biotechnological applications are the buoyant gas vesicles widely distributed among bacteria and archaea. These organelles that naturally promote floatation are present in abundance in haloarchaea (Srivastava and Kowshik 2015). These vesicles are plasmid encoded with the genetic cluster gvp MLKJIHGFEDACNO involved in gas vesicle formation (DasSarma 1989; DasSarma and Arora 1997; DasSarma et al. 1987, 1994; Halladay et al. 1993). The proteins encoded by the gene clusters include the GvpA, J and M of Pfam 741 family, involved in gas vesicle membrane formation, and GvpF and L, coiled-coil protein (Pfam 6386) involved in the nucleation process of nanoparticles due to their self-associative properties (Jones et al. 1991; Shukla and DasSarma 2004). Genes corresponding to these proteins have been found in other organisms as well, with the exception of gvpC gene, which is found only in haloarchaea and cyanobacteria (van Keulen et al. 2005). In the haloarchaeon *Halobacterium* sp. NRC-1, the GvpC protein is hydrophilic and insertion mutations within this gene results in gas vesicles with altered shape and size (Fig. 2.14) (DasSarma et al. 2013). Thus, by genetic manipulation of gvpC gene, the gas vesicles may be made to express different proteins or display antigens, thereby increasing their applications in the field of biotechnology. A new *Halobacterium* sp. NRC-1 derived host strain and a series of smaller, more versatile plasmid expression vectors have been constructed. These represent a significantly improved genetic system for expression of GvpC-fusion proteins. For example, active *Gaussia princeps* luciferase enzyme can be fused to GvpC that would result in the expression of the luciferase enzyme on the surface of the GVNPs (DasSarma et al. 2013). Such GVNPs may be used for antigen display and vaccine development. These vesicle nanoparticles are stable biological structures resistant to degradation, devoid of nucleic acids, easy to harvest by lysis and flotation and are nontoxic. In preliminary studies, mice immunized with recombinant gas vesicles expressing an simian immunodeficiency virus (SIV) peptide elicited a strong antibody response and immune memory (Stuart et al. 2001). The potential for rapid, low-cost vaccine production, and increased safety make *Halobacterium* an excellent candidate for production of a vaccine vector.

2.3.3 Graphene Sheet

Graphene, the reduced form of graphene oxide (GO) is now being actively investigated for applications in areas including drug delivery and cellular imaging (Gilje et al. 2007; Sun et al. 2008) as well as nanoelectronics (Lee et al. 2011) molecular sensors, composite materials, and energy storage (Rao et al. 2009; Allen et al. 2010; Eda and Chhowalla 2010; Loh et al. 2010). In spite of many methods reported for producing graphene sheets, such as mechanical exfoliation of reduced GO (RGO), thermal expansion of graphitic oxide, (Eda and Chhowalla 2010) and

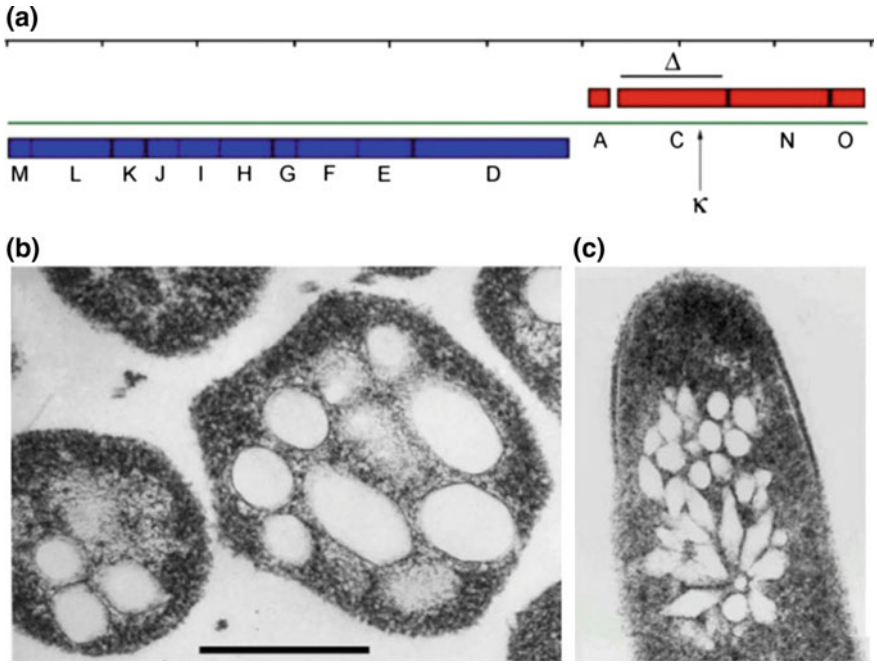

Fig. 2.14 *Halobacterium* sp. gas vesicle gene cluster and thin-sections. **a** Genetic map of the gas vesicle gene cluster from *Halobacterium*sp. NRC-1 pNRC100 is shown with genes transcribed rightward colored *red* and genes transcribed leftward colored *blue* (DasSarma et al. 2012). The scale is noted above (divided into kilobase pairs) and the positions of the *gvp*C deletion (Δ) and kappa insertion (κ) are indicated. **b** and **c** Thin sections of *Halobacterium* sp. NRC-1 (B) and SD109 (pFM104*gvp*C::κ1) mutant (**c**) viewed by transmission electron microscopy (*bar*, which is 325 nm long, applies to both **b** and **c**). Gas vesicles are visible as empty *oval* or *spindle-shaped* regions. Shapes observed reflect different planes of sectioning. *Source* DasSarma et al. (2013). Copyright © 2013, BioMed Central. Reproduced with permission

deoxygenation of graphene oxide via chemical reduction (Stankovich et al. 2007), a stable, cost-effective, and ecofriendly process for producing highly conductive graphene is proving to be elusive. Conventional methods for the preparation of graphene from dispersions from graphite oxide (Park and Ruoff 2009), involve either high temperatures or toxic and unstable gases. Recently, there have been several reports on the microbial reduction of GO to produce graphene. Many heterotrophic metal-reducing bacteria both facultative anaerobic and aerobic strains are capable of utilizing various organic compounds as terminal electron acceptors (Wu et al. 2005; Cho et al. 2012). Graphene sheets have been used for nanoparticle encapsulation (Myung et al. 2011).

Raveendran et al. (2013a) have successfully employed two strains of halophilic bacteria *H. eurihalina* ATCC 49336 and *H. maura* ATCC 700995 to convert GO to biocompatible graphene in a growth medium (Fig. 2.15). Microbial reduction experiment was performed under both aerobic and anaerobic conditions. At the end of

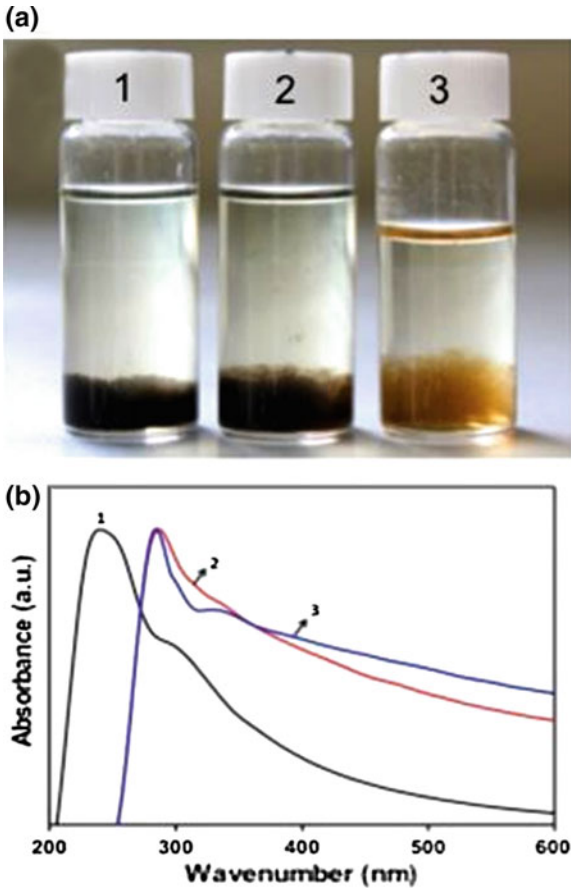

Fig. 2.15 **a** Extremophilic reduction of 10 times diluted concentration of GO—*a1*, *Halomonas eurihalina* reduced GO; *a2*, *Halomonas maura* reduced GO and *a3*, GO control; **b** UV–Vis spectrum—*b1*, GO spectrum; *b2*, ERGO spectrum; *b3*, MRGO spectrum. *Source* Raveendran et al. (2013a). Copyright © 2013, Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission

incubation, GO reduction was clearly seen from the change in the color of the GO in the medium, which changed from brown to a black precipitate settled at the bottom of the bottles (Fig. 2.15). It shows that the anaerobic reduction by *H. eurihalina* is much greater than that by the *H. maura*. Reports on biological synthesis of graphene by microorganisms are scarce. The microbially reduced GO sheet exhibits an increased conductivity as compared to chemically reduced GO and is bio-compatible. Such bio-compatible Graphene sheets may be used for green electronics and biological applications such as detection of cancer biomarkers, encapsulation of enzymes and nanoparticles (Myung et al. 2011; Raveendran et al. 2013a).

2.3.4 Halophilic Enzymes

Halophiles have been perceived as potential source of novel enzymes in recent years. Halophiles have been perceived as potential source of novel enzymes in

recent years. The interest emanates from their ability to catalyze efficiently under high salt and organic solvents The unique properties of halophilic enzymes such as the requirement of salt for stability and activity, the high resistance to several denaturation methods (Karan et al. 2012) and the ability to perform catalytic activity at low water activity or in organic solvents attracted the interest for research in this area (Tokunaga et al. 2008; Enache and Kamekura 2010; Oren 2010; DasSarma and DasSarma 2012; Karan et al. 2012). Halophilic enzymes are thought to remain active by having a predominance of negatively charged residues on the solvent-exposed surfaces of the protein. These negative charges attract water molecules and thereby keep the proteins hydrated so that they do not precipitate. It has also been shown that the hydrogen bonds formed between the negative side chains and the water molecules lead to the formation of a stable hydration shell. The increase in negative charges also results in an increase in ion-pair networks in halophilic enzymes (Bell et al. 1997; Britton et al. 2006; DasSarma and DasSarma 2012). The most well investigated haloezymes are hydrolases such as amylases (Amoozegar et al. 2003), lipases and esterases, xylanases, chitinases, proteases, cellulases, nucleases, etc. (Mellado and Ventosa 2003; Oren 2010; Moreno et al. 2013).

2.3.4.1 Proteases

Proteases are important hydrolytic enzymes which find significant applications in the biotechnology. Proteases have been immobilized on various supports like gold colloids [8], carbohydrate polymers (Zanphorlin et al. 2010; Zhao et al. 2010; Singh et al. 2011), polyvinyl alcohol beads (Hayashi et al. 1993) and microbial polysaccharides (Davidenko 1999). Recently attempts to immobilize protease on TiO_2, magnetic, bimetallic Ag-Au, silica nanoparticles have also been successful (Sadjadi et al. 2009; Jin et al. 2010; Soleimani et al. 2012). Halophilic proteases, apart from being highly salt stable, have also been recognized for their polyextremophilicity, as evident from their increased resistance to denaturation by higher temperatures, chemical reagents, detergents, chaotropic agents, organic solvents and extreme pH values (Karan and Khare 2011; Sinha and Khare 2014). Sinha and Khare (2015) explored the feasibility of using functionalized silica nanoparticles as an effective enzyme support for crude halophilic *Bacillus* sp. EMB9 protease to fabricate an active, stable, reusable enzyme preparation. The immobilization efficiency under optimized conditions was 60 %. Characterization of the immobilized preparation revealed marked increase in pH and thermal stability. It retained 80 % of its original activity at 70 °C while $t_{1/2}$ at 50 °C showed a five-fold enhancement over that for the free protease. The immobilized enzyme showed improved enzymatic and kinetic properties as well as reusability in comparison to the free enzyme. The thermally stable immobilized preparation was able to successfully hydrolyse whey proteins at high temperature with a high degree of hydrolysis.

2.3.4.2 Amylases

Amylases are important class of industrial enzyme finding wide scale applications in food, textile, paper, detergent, analytical chemistry, beverage, and pharmaceutical industry. Amylases have been characterized from many halophilic strains including *Natronococcus amylolyticus* (Kanai et al. 1995); *Haloferax mediterranei* (Perez-Pomares et al. 2003); *Haloarcula hispanica* (Hutcheon et al. 2005). *Halomonas meridiana* (Coronado et al. 2000); *Chromohalobacter* sp. TVSP 101 (Prakash et al. 2009); and *Nesterenkonia* sp. strain F (Shafiei et al. 2010). The production of amylases in halophiles is very low. However, conditions have been optimized in case of *Halomonas meridiana* (Coronado et al. 2000), *Halobacillus* sp. strain MA-2 (Amoozegar et al. 2003), and *Bacillus* sp. strain TSCVKK (Kiran and Chandra 2008) for enhancing the yield, yet maximum 3.2 U/mL could be attained.

The hydrolysis of starch to low molecular-weight products using α-amylase is one of the most important enzyme processes (Tanyolaç et al. 1998). In this regard, conversion of starch into sugars, syrups, and dextrins forms the major part of the starch processing industry. Immobilization of α-amylase on water-insoluble carriers seems to be the most promising way to obtain more stable and reuse forms of the enzyme (Cong et al. 1995). The immobilization of enzymes on solid support offers several advantages over the free enzyme, including easy recovery from the reaction medium, reusability, possibility of operation in continuous reactors, enhanced stability, and catalytic efficiency (Sheldon and Van Pelt 2013). There have been many reports on immobilization of α-amylase. Some examples involve reactive polymer films (Cordeiro et al. 2011), magnetic nanoparticles (Chen et al. 2012), mesoporous silica thin films (Bellino et al. 2010), and adsorption on zirconia (Reshmi et al. 2007). In recent years, nanostructured materials such as nanoporous media, nanofibers, nanotubes, and nanoparticles have emerged as amazingly effective enzyme support/matrix (Kim et al. 2006, 2008; Khan et al. 2013; Cipolatti et al. 2014). They provide the highest possible surface area for immobilization, enabling very high loading of enzyme on the support. This results in surprisingly high enzyme activities per unit volume (Ansari and Husain 2012).

Kumar and Khare (2015) utilize the α-amylase from moderately halophilic *Marinobacter* sp. EMB8 for nanoimmobilization and efficient starch hydrolysis. Amylase producer halophilic bacteria *Marinobacter* sp. EMB8 was isolated during screening of Indian saline habitats (Kumar et al. 2012). The α-amylase was purified and found to be salt and solvent stable. It was used for synthesis of industrially useful maltooligosaccharides (Kumar and Khare 2012). Kumar and Khare (2015) attempted to optimize production and immobilization on functionalized silica NPs for competitive yield and efficient application of α-amylase from *Marinobacter* sp. EMB8. Bacterial growth and enzyme production are greatly influenced by the nutritional factors (carbon and nitrogen sources, metal ions, etc.) and physical factors (pH, temperature, inoculation volume, and incubation time). Optimization of various culture parameters resulted in 48.0 U/mL amylase production, a 12-fold increase over that of unoptimized condition (4.0 U/mL). α-Amylase was

immobilized on 3-aminopropyl functionalized silica nanoparticles using glutaraldehyde as cross-linking agent. Optimization of various parameters resulted in 96 % immobilization efficiency. Starch hydrolyzing efficiency of immobilized enzyme was comparatively better. Immobilized α-amylase retained 75 % of its activity after 5th cycle of repeated use (Kumar and Khare 2015).

2.4 Conclusions and Future Perspectives

A variety of prokaryotic and eukaryotic halophilic microorganisms have been investigated with respect to their nanoparticle synthetic abilities. The vast biodiversity encountered in the prokaryotic world has been relatively less explored. Furthermore, most of the studies are related to the synthesis of silver nanoparticles, followed by those of gold. One reason for this could be the relative ease with which the noble metal ions of gold and silver are reduced. Microbial synthesis of nanoparticles of platinum, bismuth, cadmium (CdO, CdS, CdTe), antimony sulfide, copper oxide, zinc oxide, and titanium oxide described in Chap. 1, have been reported on fewer occasions on halophilic microorganisms.

The majority of the reports deal with application of novel biological systems in mediating the synthesis of nanoparticles, their characterization, and applications in the biomedical field, particularly as antimicrobial agents. However, there is a need to understand the mechanisms involved in the synthetic process. Another limitation of the studies is that the experiments have been conducted at laboratory scale and there are hardly any efforts for the scale-up of these processes. In the future, these shortcomings need to be addressed in an effective manner to harness the actual nanoparticle synthetic potential of the halophiles to their full extent.

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