Chapter 3
Thermophiles and Psychrophiles in Nanotechnology

Abstract Some thermophiles and psychrophiles have developed the ability to resort to specific defense mechanisms to quell stresses like toxicity of heavy metal ions or metals. Some of them could survive and grow even at high metal ion concentrations and are capable of binding large quantities of metallic cations. Moreover, some of these microorganisms are able to synthesize nanoparticles. The remarkable ability of these group of microbes to reduce heavy metal ions make them one of the best candidates for nanoparticle synthesis. In this chapter, thermophilic and psychrophilic microorganisms used in nanoparticle biosynthesis are presented. The aim of chapter is to make a reflection on the current state and future prospects and especially the possibilities and limitations of the use of extremophiles in bio-based technique for industries.

3.1 Introduction

Thermophiles are heat-loving organisms, which not only tolerate high temperatures but also usually require these for their growth and survival. Unlike other types of bacteria, thermophiles can survive at much hotter temperatures, whereas other bacteria would be damaged and sometimes killed if exposed to the same temperatures. Temperatures for their growth range from 50 °C to as high as 121 °C. Thermophiles are, for the most part, prokaryotic, and the most hyperthermophilic are archaea, which possess a large number of high-temperature adaptations. Only few eukaryotes are known to grow above 50 °C temperature, but some fungi grow in the temperature range 50–55 °C (Maheshwari et al. 2000). Several years ago Kristjansson and Stetter (1992), suggested a further division of the thermophiles and a hyperthermophile boundary (growth at and above 80 °C) that has today reached general acceptance. Thus, thermophiles are now classified into moderate thermophiles, extreme thermophiles, and hyperthermophiles. Most thermophilic bacteria characterised today grow below the hyperthermophilic boundary with some exceptions, such as *Thermotoga* and *Aquifex* (Stetter 1996; Takahata et al. 2001) while hyperthermophilic species are dominated by the Archaea.

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Habitats for the occurrence of thermophiles may be natural or man-made. Natural habitats that harbor a considerable variety of thermophilic microorganisms include terrestrial geothermal and volcanic areas and deep-sea hydrothermal vents (submarine hydrothermal vents) (Mehta and Satyanarayana 2013). Most of the currently known extreme thermophiles and hyperthermophiles have been recovered from these regions by culture-dependent as well culture-independent approaches. Geothermal and volcanic areas include terrestrial fumaroles (e.g., solfataras), terrestrial hot springs, and geysers. Other natural habitats include geothermally heated oil and petroleum reservoirs and sun-heated soils/sediments (Greene et al. 1997; Engle et al. 1995; Völkl et al. 1993; Ward et al. 1987; Zillig et al. 1980; Mehta and Satyanarayana 2013). Man-made thermophilic habitats include acid mine drainage and acidic effluents (Kelly and Wood 2000), self-heated compost piles (Fujio and Kume 1991; Tiquia 2005; Tiquia et al. 1996, 2002, 2005), biological wastes (Mehta and Satyanarayana 2013), and waste treatment plants (Mehta and Satyanarayana 2013). These are comparatively lower temperature habitats, as compared to the natural habitats, and are ideal for the isolation of moderate and extreme thermophiles.

As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperatures. Thermophiles have been found to improve protein thermostability by expressing heat-shock chaperonins to assist protein folding, prevent protein denaturation, and, in the case of small heat-shock protein "holdases," disaggregate amyloids (Trent 1996; Luo and Robb 2011). Protein folding is also aided by moving charged residues to the interior of the protein, effectively neutralizing the surface charge (Fukuchi et al. 2003). At high temperatures, neutral proteins have lower dielectric properties and desolvation penalties (Thomas and Elcock 2004). In addition, more densely packed protein folding and exposed hydrophobic shells minimize regions where high-temperature water can destabilize the protein (Vetriani et al. 1998). Interior salt and disul fi de bridges further improve thermostability (Robb and Maeder 1998). High temperatures promote the degradation of nucleic acids, so many thermophiles upregulate DNA repair pathways in order to preserve genetic stability (Toueille and Sommer 2011).

In industrial applications with thermophiles and thermostable enzymes, isolated enzymes are today dominating over microorganisms (Tiquia-Arashiro 2014; Tiquia and Mormile 2010; Satyanarayana et al. 2013). An enzyme or protein is called thermostable when a high defined unfolding (transition) temperature (Tm), or a long half-life at a selected high temperature, is observed. A high temperature should be a temperature above the thermophile boundary for growth (>55 °C). Most, but not all proteins from thermophiles are thermostable. Extracellular enzymes generally show high thermostability, as they cannot be stabilised by cell-specific factors like compatible solutes (Santos and da Costa 2002). The discovery and use of thermostable enzymes in combination with recombinant production and development using site-directed and enzyme evolution technologies, have erased some of the first identified hinders (e.g. limited access and substrate specificity) for use in

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industrial biocatalysis. Recently, thermophilic microorganisms have been explored to synthesize metallic nanoparticles with well-defined chemical composition, size, and morphology.

Psychrophiles are a class of extremophiles that have the ability to live in extremely low temperature conditions. About 75 % of the Earth's biosphere, including polar, deep ocean, and atmospheric habitats, is permanently cold, and 70 % of the surface of the planet has a temperature between 1 and 5 °C (Feller and Gerday 2003; Cavicchioli 2006). Thus, the existence of psychrophiles, or organisms that live in these cold environments, is intuitive. Psychrophiles are commonly divided into two categories: stenopsychrophiles and eurypsychrophiles. Stenopsychrophiles, or obligate psychrophiles, can only survive at temperatures below 15 °C, while eurypsychrophilic or mesotolerant, organisms grow optimally below 15 °C but can also survive at higher temperatures (Feller and Gerday 2003; Cavicchioli 2006). Psychrophiles are those which have an optimum growth temperature below 15 °C and cannot grow at temperature beyond 20 °C. Psychrotolerant microorganisms, also known as psychrotrophs are those which can live in cold conditions, but have a higher optimum growing temperature, much greater than 20 °C. Many organisms remain metabolically active at temperatures well below freezing (Koshima 1984). Psychrophilic microorganisms, including the bacterium *Psychromonas ingrahamii*, the archaeon *Methanosarcina baltica*, and the fungus *Humicola marvinii*, metabolize and reproduce optimally at temperatures below 15 °C and, in some cases, have been reported to survive with greatly reduced metabolism down to −20 °C (Von Klein et al. 2002; Auman et al. 2006; Kumar et al. 2007; Weinstein et al. 1997).

These cold-loving microorganisms are commonly found in Polar region, deep sea, mountains, glaciers, fresh and marine waters, polar and high alpine soils. Psychrophiles face many challenges in this environments, including membrane rigidity, protein misfolding, and slower reaction rates. Many psychrophilic microorganisms, such as the alga *Chlamydomonas* sp. ICE-L, have been found to increase membrane fluidity by incorporating more unsaturated fatty acids into the phospholipid bilayer (Zhang et al. 2011). Methanogens also express a variety of unique protein adaptations designed to assist both anabolic and catabolic metabolism (Von Klein et al. 2002; Kumar et al. 2007). For example, a psychrophilic α-amylase was found to have a higher turnover rate (kcat), lower activation energy ($\Delta G\ddagger$), and lower change in enthalpy ($\Delta H\ddagger$) than mesophilic and thermophilic amylases (Feller and Gerday 2003). This reduction in activation energy is partially achieved through increased flexibility in the psychrophilic enzyme's active site, which reduces the substrate-enzyme complex binding energy and activation energy (Roulling et al. 2011). Eurypsychrophilic methanogens have also been found to possess temperature-dependent transcription factors that respond to variations in temperature by changing proteome composition (Goodchild et al. 2004). Finally, extracellular polysaccharide substances (EPS) and teichoic acid are cryoprotectants, and intracellular fumarate and glycerol are chaotropes commonly produced upon cold shock (Rice et al. 2008; Marx et al. 2009; Chin et al. 2010).

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Psychrophiles became an important resource for bioprospecting because of its unique cold adaptations, which helped them successfully live in such frigid living conditions. Psychrophiles are successful in surviving such extreme conditions by optimizing various basic cell processes like enzyme function, nutrient transport and cell membrane function. The most important of these adaptations, which has immense potential to be exploited, are the production of polyunsaturated fatty acids and cold adaptive enzymes. The membranes and proteins of these microbes have a special property of increased structural flexibility that enhance the catalytic function and the presence of unsaturated fatty acids help in easy nutrient cell transportation, due to better fluidity. When temperature drops, psychrophiles produce cold shock or antifreeze proteins that enhance the activity of enzymes by improving enzyme kinetics and stabilizing microtubules. Psychrophilic enzymes have the advantage of having a low temperature optimum for activity with enhanced specific activity at low temperatures and rather high thermostability. The useful applications of cold active enzymes are widespread to a large number of industries such as the textile industry, the brewing and wine industry and the food and dairy industry. Psychrophiles are also a good source of polyunsaturated fatty acids, which can be extensively used in pharmaceutical industry for developing new therapeutic agents.

Microorganisms possess remarkable ability to reduce heavy metal ions and are one of the best candidates for nanoparticle synthesis. For instance, some thermophiles and psychrophiles have developed the ability to resort to specific defense mechanisms to quell stresses like toxicity of heavy metal ions or metals. Some of them could survive and grow even at high metal ion concentrations and are capable of binding large quantities of metallic cations. Moreover, some of these microorganisms are able to synthesize nanoparticles. The remarkable ability of some psychrophiles to reduce heavy metal ions make them one of the best candidates for nanoparticle synthesis. In this chapter, thermophilic and psychrophilic microorganisms used in nanoparticle biosynthesis (Table 3.1) are presented. The aim of chapter is to make a reflection on the current state and future prospects and especially the possibilities and limitations of the use of extremophiles in bio-based technique for industries.

3.2 Synthesis of Nanoparticles by Thermophiles

3.2.1 Thermophilic Bacteria

3.2.1.1 *Geobacillus* spp.

The genus *Geobacillus* comprises a group of Gram-positive thermophilic bacteria, including obligate aerobes, denitrifiers, and facultative anaerobes that can grow over a range of 45–75 °C. Originally classified as group five *Bacillus* spp., strains of *Bacillus stearothermophilus* came to prominence as contaminants of canned food

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Table 3.1 Thermophiles in biosynthesis of nanoparticles

Thermophile	Nanoparticle	References
Bacteria		
Geobacillus stearothermophilus	Ag, Au	Fayaz et al. (2010)
Geobacillus sp.strain ID17	Au	Correa-Llantén et al. (2013)
Geobacillus wiegelii Strain GWE1	Se	Correa-Llantén et al. (2014)
Thermomonospora sp.*	Au	Ahmad et al. (2003a)
Thermomonospora sp. 67 Th*	Au	Kalabegishvili et al. (2013)
Ureibacillus thermosphaericus	Ag	Juibari et al. (2011)
Thermoactinomycete sp. 44 Th	Au	Kalabegishvili et al. (2013)
Thermus scotoductus SA-01	Au	Erasmus et al. (2014)
Thermincola ferriacetica strain Z-0001	Fe_2O_3	Koksharov et al. (2009)
Caldicellulosiruptor saccharolyticus	Pd	Shen et al. (2015)
Thermoanaerobacter sp.BKH1	SiO_2	Show et al. (2015)
Thermoanaerobacter sp.	CdS	Moon et al. (2013)
Thermoanaerobacter TOR-39	Zinc ferrite	Yeary et al. (2011)
Thermoanaerobacter sp. TOR-39	L-substituted magnetites	Moon et al. (2007)
Thermoanaerobacter ethanolicus TOR-39	Metal-substituted magnetite nano-crystals	Roh et al. (2006, 2007)
Thermoanaerobacter sp. X513	Cu	Jang et al. (2015)
Archaea		
*Sulfolobus islandicus***	Ag	Kalabegishvili et al. (2015)
Fungi		
Humicola sp.	Ag	Syed et al. (2013)
Humicola sp.	CeO_2	Khan and Ahmad (2013)
Humicola sp.	Gd_2O_3	Khan et al. (2013)

*Thermoalkaliphilic
**Thermoacidophilic

and soon became the organism of choice for comparative studies of metabolism and enzymology between mesophiles and thermophiles. More recently, their catabolic versatility, particularly in the degradation of hemicellulose and starch, and rapid growth rates have raised their profile as organisms with potential for second-generation (lignocellulosic) biorefineries for biofuel or chemical production (Hussein et al. 2015).

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Geobacillus stearothermophilus

Biogenic synthesis of silver and gold nanoparticles by *Geobacillus stearothermophilus* was explored by Fayaz et al. (2010). The exposure of *G. stearothermophilus* cell free extract to the metal salts leads to the formation of stable silver and gold nanoparticles in the solution. The silver and gold nanoparticles have absorption maxima at 423 and 522 nm respectively. The TEM micrograph revealed the formation of polydispersed particles in the case of silver nanoparticles and monodispersed particles with respect to the gold nanoparticles. High stability of the nanoparticle solution could be attributed to the secretion of certain capping proteins by the bacterium in the reaction mixture (Fayaz et al. 2010).

Geobacillus sp. strain ID17

The production of gold nanoparticles by the thermophilic bacterium *Geobacillus* sp. strain ID17 is reported by Correa-Llantén et al. (2013). The strain was isolated from the Deception Island, Antarctica. The Deception Island is a complex strato-volcano with a "horseshoe" shape whose central part has a caldera structure. This volcanic island has been very active during the last century: fumarolic emissions, thermal springs and hot soils are evidence of Deception Island's continuing activity (Caselli et al. 2004), making it an interesting site for isolating new thermophilic bacteria. In order to study the biocatalytic reaction that takes place in the reduction of Au^{3+}, crude extracts of ID17 were assayed for their ability to reduce $HAuCl_4 \times 3H_2O$. Extracts from ID17 were able to catalyze the NADH-dependent reduction of Au^{3+} (Fig. 3.1a, b). NADH-dependent Au^{3+} reductase activity was dropped approximately in 98 % with SDS or proteinase K treatment, reflecting the enzymatic nature of this reaction. The activity was associated to at least four bands present in non denaturing gel PAGE corresponding to proteins in free cells extracts. One of these bands is present in high amount, based on the intensity of the band in a non denaturing gel PAGE (Fig. 3.1c). These results strongly suggest that the biosynthesis of gold nanoparticles by *Geobacillus* sp.strain ID17 is mediated by enzymes and NADH as a cofactor for this biological transformation. The intracellular localization and particles size were verified by TEM showing two different types of particles of predominant quasi-hexagonal shape with size ranging from 5 to 50 nm. The majority of them are between 10-20 nm in size (Correa-Llantén et al. 2013).

Geobacillus wiegelii Strain GWE1

Geobacillus wiegelii, strain GWE1, an aerobic thermophile belonging to genus *Geobacillus*, isolated from a drying oven (Correa-Llantén et al. 2014). This thermophile has the ability to reduce selenite evidenced by the change of color from colorless to red in the culture (Fig. 3.2). The SeNPs have a defined spherical shape

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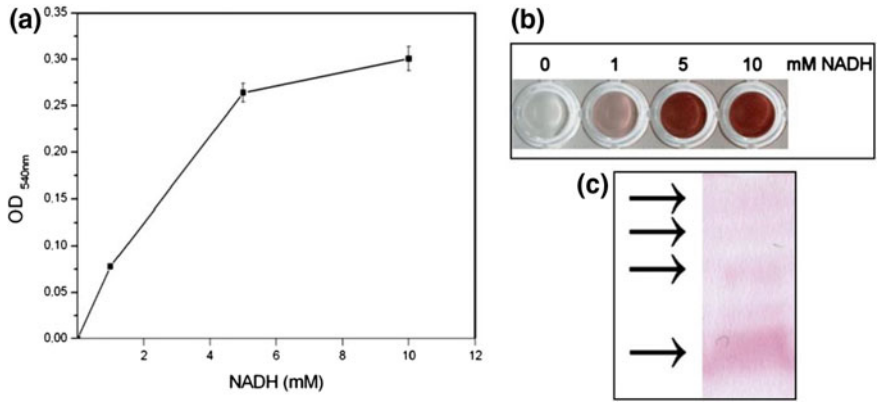

Fig. 3.1 Evidence of enzymatic Au^{+3} reduction in ID17. **a** OD at 540 nm using different concentrations of NADH (1, 5 and 10 mM); **b** NADH dependence for the $HAuCl_4$ reduction concentration; colour intensity indicates gold nanoparticles formation; **c** Zymogram analysis of enzymes (*indicated with arrows*) involved in Au^{+3} reductase activity. *Source* Correa-Llantén et al. (2013). Copyright © 2013, BioMed Central. Reproduced with permission

and a selenium elemental state. Size and shape of SeNPs can be modulated by pH and temperature. Size distribution ranged from 40 to 160 nm, where 70 % of nanoparticles have less than 100 nm in size. The size of all nanoparticles was less than 100 nm at pH 4.0; over 50 % of nanoparticles have less than 100 nm at pH 5.0; at pH 6.0 and 8.0 over 90 % of nanoparticles have less than 100 nm in size (Correa-Llantén et al. 2014). At neutral pH (7.0) nanoparticles reach a size around 120 nm and only 20 % of them were less than 100 nm. Nanoparticles did not show a significant difference in size when they were incubated between 0 and 3 h at 60 °C (Correa-Llantén et al. 2014). Meanwhile at 80 °C the nanoparticles suspension lost its homogeneity. A change in size was observed from 0 h of incubation at 80 °C,

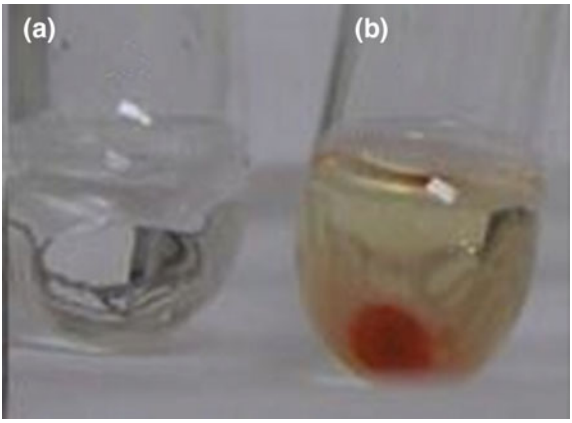

Fig. 3.2 Enzymatic selenite reduction by crude extract of *Geobacillus wiegelii* GWE1 and NADH. *Reddish color* in tube (**b**) indicates SeNPs formation. Tube (**a**) is a control without NADH. *Source* Correa-Llantén et al. (2014). Copyright © 2014, World Academy of Science, Engineering and Technology

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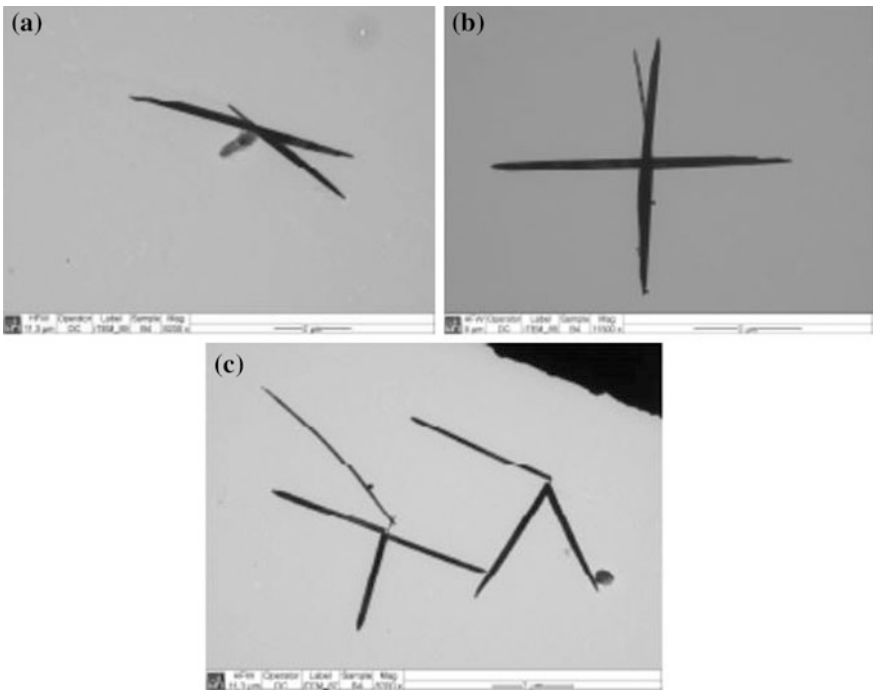

Fig. 3.3 Nanoparticles incubated for 3 h at 100 °C. **a**, **b** and **c** larger structures formed called nanorods. *Source* Correa-Llantén et al. (2014). Copyright © 2014, World Academy of Science, Engineering and Technology

observing a size range between 40 and 160 nm, with 20 % of them over 100 nm. Meanwhile after 3 h of incubation at size range changed to 60–180 nm with 50 % of them over 100 nm. At 100 °C the nanoparticles aggregate forming nanorod structures (Fig. 3.3). These results indicate that is possible to modulate size and shape of biologically synthesized nanoparticles by modulating pH and temperature (Correa-Llantén et al. 2014).

3.2.1.2 *Thermomonospora* sp.

Ahmad and group have performed a series of studies on bacterial synthesis of gold nanoparticles. In one such study, they used the extremophilic actinomycete *Thermomonospora* sp. to efficiently synthesize monodisperse gold nanoparticles (Ahmad et al. 2003a). By comparing this with their earlier work on gold nanoparticle synthesis from a fungus, *Fusarium oxysporum* (Ahmad et al. 2003b) they postulated that reduction of metal ions stabilization of the gold nanoparticles occurs by an enzymatic process. Furthermore, they attributed the synthesis of monodisperse gold

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nanoparticles by *Thermomonospora* sp. to extreme biological conditions (i.e., alkaline and slightly elevated temperature conditions) (Ahmad et al. 2003a).

3.2.1.3 *Ureibacillus Thermosphaericus*

Ureibacillus thermosphaericus showed high potential for silver nanoparticle biosynthesis with extracellular mechanism and selected for the biosynthesis optimization (Juibari et al. 2011). Biosynthesis reactions were conducted using the culture supernatant at different temperatures (60–80 °C) and silver ion concentrations (0.001–0.1 M). Figure 3.4. shows the results of visual and spectral analysis of biosynthesis optimization reactions conducted at different silver nitrate concentrations (0.001 and 0.01 M) and temperatures (60, 70, and 80 °C). Visual analysis revealed that the intensity of color change enhanced with the increasing in temperature and ion concentration The maximum nanoparticle biosynthesis was achieved at 0.01 M $AgNO_3$ and 80 °C. However, ion concentration seemed to have a more significant effect on particle size, since silver particles were the main products at the higher ion concentrations such as 0.1 M $AgNO_3$. The average size of AgNPs at the silver ion concentration of 0.001 M and different temperatures of 60, 70 and 80 °C was 57, 29 and 13 nm, respectively (Fig. 3.4b). Whereas, these values

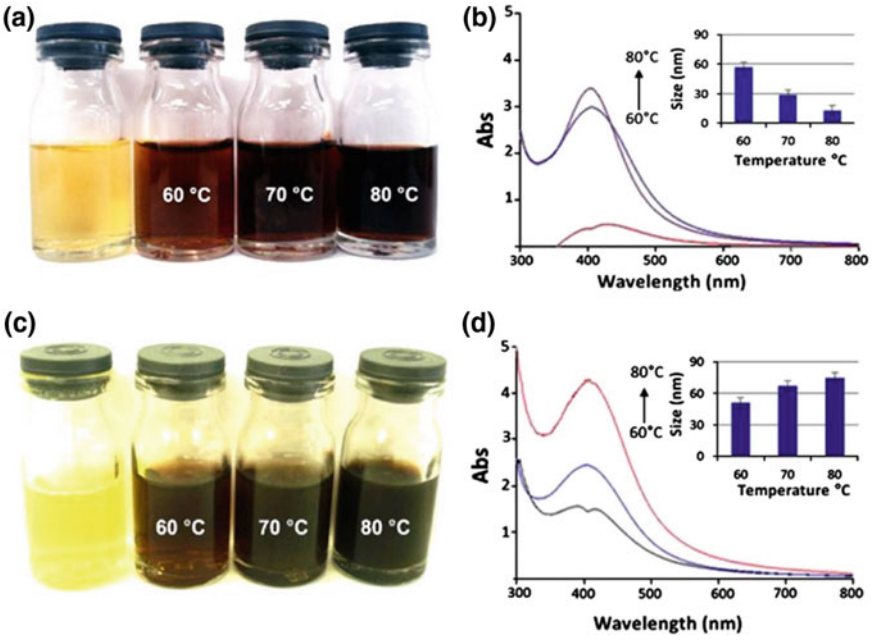

Fig. 3.4 Comparison of change in color intensity and UV–Vis spectrum of biosynthesis reaction conducted at different silver nitrate concentrations and temperatures, 0.001 M of $AgNO_3$ (**a**, **b**) and 0.01 M of $AgNO_3$ (**c**, **d**) Juibari et al. (2011). Copyright © 2011, Elsevier. Reproduced with permission

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showed an increasing trend; 51, 67, and 75 nm, respectively, when the silver ion concentration 0.01 M was used (Fig. 3.4d). The increasing trend observed in the average particle size at the silver ion concentration of 0.01 M $AgNO_3$ by increasing the temperature could be the result of increasing in the secondary reduction process which is a common phenomenon in high ion concentrations (Juibari et al. 2011). The results obtained showed that pure spherical nanoparticles in the range of 10–100 nm were produced, and the maximum nanoparticle production was achieved using 0.01 M $AgNO_3$ at 80 °C. The findings of this study confirmed the great biocatalyzing potential of the extremophilic *U. thermosphaericus* supernatant for intensified biosynthesis of silver nanoparticle at elevated temperatures and high silver ion concentrations. These results demonstrated the great advantage of thermophilic bacteria as biocatalysts such as *U. thermosphaericus* for enhanced nanoparticle biosynthesis over currently developed biocatalysts which obtained from mesophilic strains and plant cells (Juibari et al. 2011).

3.2.1.4 *Thermoactinomycete* sp. 44 Th

The cultivation of *Thermoactinomycete* sp. 44 Th for preparation of samples containing Au nanoparticles was carried out using $HAuCl_4$ 10^{-3} M. AuNPs were formed after 3–4 days of reaction. The nanoparticles size distributions range from 5 to 60 nm (Kalabegishvili et al. 2013).

3.2.1.5 *Thermus scotoductus* SA-01

Thermus scotoductus SA-01 has the ability to reduce Au(III) and produce nanoparticles, making it a suitable candidate for the production of AuNPs (Erasmus et al. 2014). Physico-chemical parameters have influence on particle size with lower pH and higher temperatures resulting in larger particles and in contrast, higher pH and lower temperatures produce smaller particles. Gold reduction primarily occurred in the cell envelope which is strong evidence for gold-specific reduction process (Erasmus et al. 2014). An ABC transporter, peptide-binding protein of *T. scotoductus* SA-01, able to reduce and synthesize gold nanoparticles was purified to homogeneity. Even though this type of protein is not a classical oxidoreductase, a cysteine–disulphide bridge electron shuttle mechanism is likely involved in reducing Au(III). Moreover, the protein also acts as nucleation seed sites that initiate and direct nanoparticle synthesis. Through manipulation of physico-chemical parameters, it is clear that particle formation can be influenced in terms of size, shape and number of particles formed. However, since biological Au(III) reduction and nanoparticle synthesis is a complex process, manipulations of single parameters are unlikely to result in the best conclusive results. This is witnessed by a lack of particle monodispersity (with the exception of small spherical particles) when evaluating any of the parameters. Varying and investigating multiple parameters simultaneously will

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likely shed light on the way forward to controlling and directing *T. scotoductus* SA-01 AuNP synthesis (Erasmus et al. 2014).

3.2.1.6 *Thermincola ferriacetica* Z-0001

Thermincola ferriacetica Z-0001 is an anaerobic iron-reducing bacterium that uses Fe^{3+}-hydroxide as an electron acceptor, and acetate as an electron donor for anaerobic growth (Koksharov et al. 2009). Koksharov et al. (2009) studied the Fe_2O_3 synthesis and the electro magnetic resonance spectra of Fe_2O_3 nanoparticles related to the reduction metabolism of the dissimilatory bacterium *Thermincola ferriacetica* Z-0001. The magnetization of the biologically-induced nanoparticles increases with time. However, the Fe_2O_3 nanoparticles obtained were not stable during later storage under open air conditions (Koksharov et al. 2009).

3.2.1.7 *Caldicellulosiruptor saccharolyticus*

Shen et al. (2015) examined the of coupling palladium (Pd) nanoparticle synthesis and H_2 production by *Caldicellulosiruptor saccharolyticus* for wastewater treatment under extreme thermophilic conditions. Na_2PdCl_4 was added to cell cultures to achieve a final Pd Concentration of 50 mg/L. Methyl orange (MO) and diatrizoate were chosen as the contaminants in water. In the cultures with, and without, Pd, MO (100 mg/L) was degraded within 30 min and in over 6 h, respectively. Diatrizoate (20 mg/L) was degraded within 10 min in cultures with Pd (Shen et al. 2015). The degradation of MO and diatrizoate were both enhanced by Pd. The removal of MO was the result of the combined action of hydrogen, hydrogenase and Pd^0 nanoparticles. Pd^0 particles also played an essential role in the removal of diatrizoate. The Pd^0 particles were well dispersed by cells of *C. saccharolyticus* and showed a better catalytic activity than chemical Pd^0 without dispersant The diameter of most Pd^0 particles formed in the presence of cells is under 100 nm. These Pd^0 particles are polyporous and homogeneous (Shen et al. 2015).

3.2.1.8 *Thermoanaerobacter* Spp.

Thermoanaerobacter BKH1

A green technique of silica nanoparticles (SiO_2-NPs) formation by using a thermophilic bacterium *Thermoanaerobacter* BKH1 as biological template is demonstrated by Show et al. (2015). SiO_2-NPs are synthesized from inorganic (magnesium tri-silicate), and organic (tetraethyl orthosilicate) precursor with the help of *Thermoanaerobacter* BKH1. TEM image of a specimen revealing the separations of SiO_2-NPs from the BKH1 template with few residual particles over the surface is demonstrated in Fig. 3.5a. Similar residual particles are seen in

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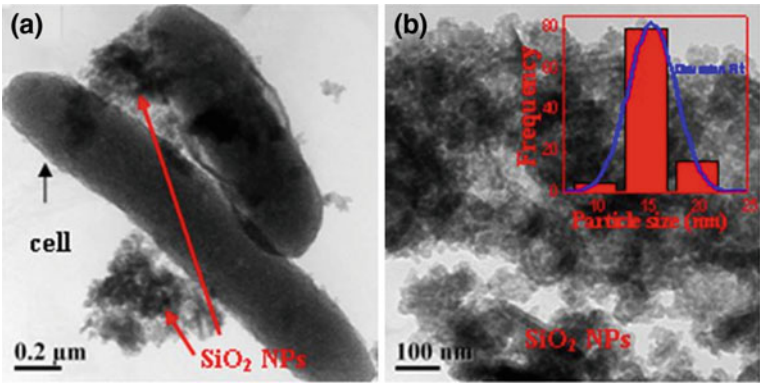

Fig. 3.5 TEM micrograph of **a** separation of the SiO_2-NPs from bacterium cell by centrifuge, **b** separated SiO_2-NPs and histogram with Gaussian curve fitting (*inset*) shows the particle size distribution where the average particles size is 15 ± 5 nm (n = 100). *Source* Show et al. (2015). Copyright © 2015, Elsevier. Reproduced with permission

Fig. 3.5b. The amorphous nature of the SiO2-NPs is also confirmed from TEM images as well. The biologically synthesized NPs are in regular spherical shapes. The histogram of the particle size distribution is shown as inset in Fig. 3.5b. The average particle size was around 15 ± 5 nm. The synthesized SiO_2-NPs can be utilized in various biomedical applications, such as, killing of unwanted bacteria with UV irradiation and subsequently use as an optical probe in medical diagnosis by using the visible emission (Show et al. 2015). The Zeta potential of the biologically derived SiO_2-NPs reveals stability of the synthesized nanoparticles in dispersed medium and impedes agglomeration. It can be logically asserted that silica leaching proteins, for instance bioremediase, may be involved in the formation of silica nanoparticles as a consequence of bacterial activity (Show et al. 2015).

Thermoanaerobacter X513

Moon et al. (2013) reported a microbially facilitated synthesis of cadmium sulfide (CdS) nanostructured particles (NP) using anaerobic, metal-reducing *Thermoanaerobacter* sp. The extracellular CdS crystallites were <10 nm in size with yields of ~3 g/L of growth medium/month with demonstrated reproducibility and scalability up to 24 L (Fig. 3.6). During synthesis, *Thermoanaerobacter* cultures reduced thiosulfate and sulfite salts to H_2S, which reacted with Cd^{2+} cations to produce thermodynamically favored NP in a single step at 65 °C with catalytic nucleation on the cell surfaces. Photoluminescence (PL) analysis of dry CdS NP revealed an exciton-dominated PL peak at 440 nm, having a narrow full width at half maximum of 10 nm. A PL spectrum of CdS NP produced by dissimilatory sulfur reducing bacteria was dominated by features associated with radiative

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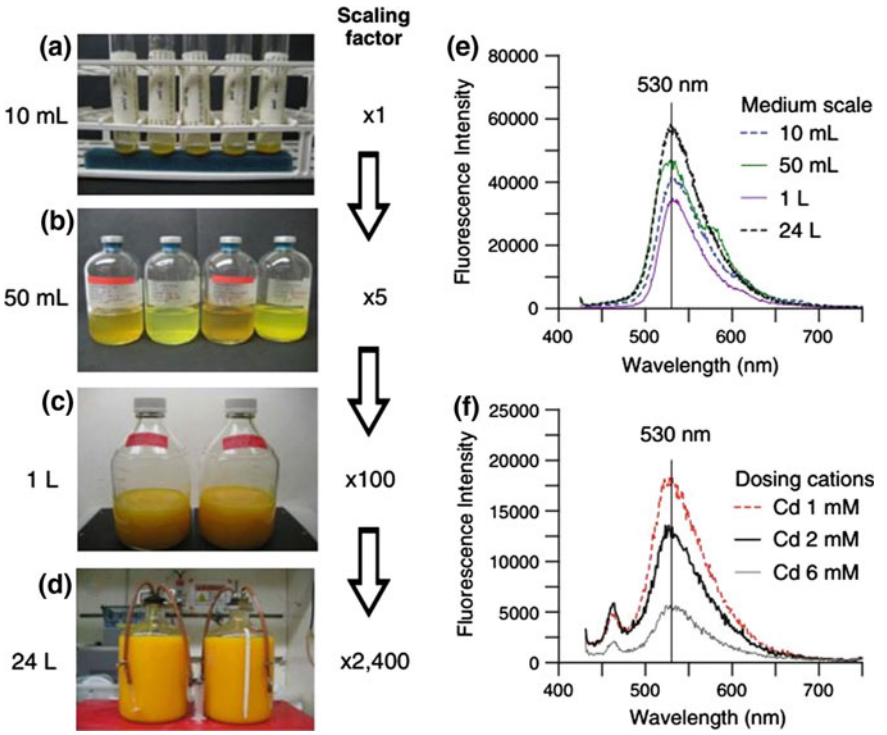

Fig. 3.6 Scale-up experiment for CdS NP from 10 mL to 24 L; **a** dosing 0.09, 0.27, 0.44, 0.62, and 0.89 mM of Cd^{2+} to 10 mL; **b** pairs of control and microbial samples of 50 mL (*left*, with cysteine S; *right*, with thiosulfate); **c** pulsed dosing of 0.4 and 0.8 mM/day for 10 days to 1 L; **d** pulsed dosing of 0.4 mM/day for 10 days to parallel reactors of 24 L. Fluorescence comparison for the scale-up experiment, **e** e dosing of Cd 0.4 mM/day into varying medium scales of 10 mL, 50 mL, 1 L, and 24 L of medium for 10 days; **f** single dosing of 1, 2, or 6 mM Cd^{2+} into the same 10 mL medium volume. *Source* Moon et al. (2013). Copyright © 2013, Elsevier. Reproduced with permission

exciton (Moon et al. 2013). CdS NPs of controlled size and crystal structure with high yield and size uniformity are produced. Controlling factors such as cell mass, dosing concentrations, type of precursors, and the basal medium composition using appropriate microbial populations as a reducing force are critical in producing copious CdS NP (Moon et al. 2013). Advantages of CdS NP synthesis using *Thermoanaerobacter* X513 include: (1) a relatively simple procedure without complicated steps (Tong and Zhu 2006), (2) low energy consumption at near room temperature compared to traditional methods requiring 250–300 °C (Yu and Peng 2002) or high mechanical energy like ball-milling up to 30 h (Patra et al. 2011), (3) easily tunable synthetic conditions for size control without implementation of additional steps like anion exchange chromatography (Kang et al. 2008),

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(4) semi-continuous production without sacrificing the culture and anaerobic system, (5) minimizing the use of organic solvents and hazardous precursors (Flenniken et al. 2004), and (6) no need for post-treatment such as a lengthy dialysis (Zhang et al. 2004a).

Thermoanaerobacter TOR-39

Synthesis of Zinc Ferrite

A biological method to produce nanometer sized Zn-ferrites from *Thermoanaerobacter* TOR-39 was described by Yeary et al. (2011). Variability of particle size within sample is obvious in the TEM (Fig. 3.7). Figure 3.7a indicated large crystalline entities of 500 nm in size while Fig. 3.7b revealed well defined particles closer to 10 nm. Figure 3.7c shows a TEM image from different sample set showing uniformed well developed crystalline particles.

Highest amounts of zinc substitution, is shown in Fig. 3.7d. The bacteria produced consistent particles of 30–50 nm in size at an incubation temperature of 65 °C (Yeary et al. 2011). Microbially-mediated synthesis of nm-sized Zn-ferrites resulted in enhanced magnetic properties relative to chemically produced nanoparticles. The increased magnetic properties may have been related to lower fabrication temperatures of the bacterial process. Furthermore, properties of

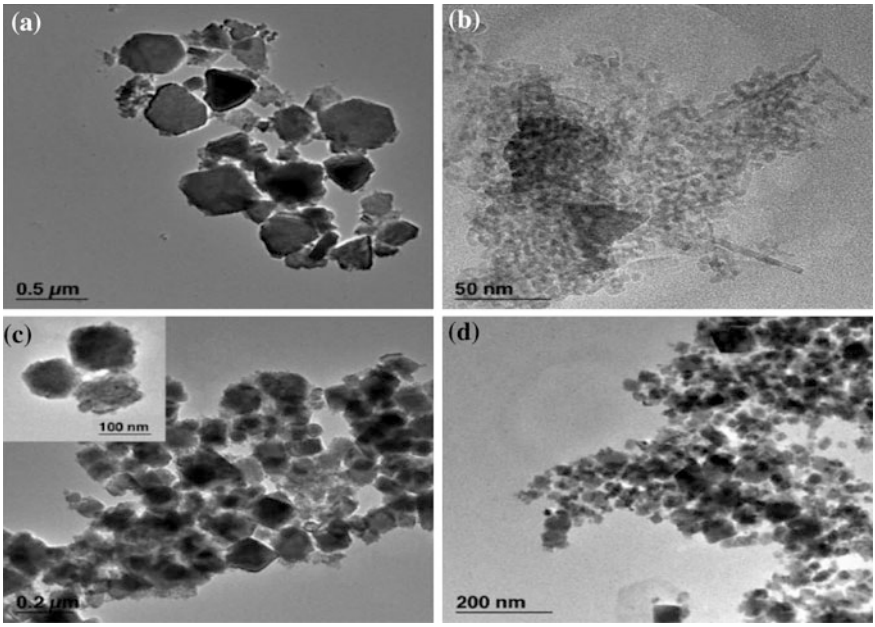

Fig. 3.7 TEM micrographs of representative Zn-ferrites. *Source* Yeary et al. (2011). Copyright © 2011, Elsevier. Reproduced with permission

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microbially synthesized zinc ferrites vary systematically with Zn content (Yeary et al. 2011). Cation site location is often influenced by synthesis temperature, therefore, the biosynthesis of Zn-ferrite occurring at a much lower temperature compared to other methods may lead to different phenomena. Formation of Zn-ferrite with the incubation at ambient or slightly above room temperature may encourage a different distribution of zinc ions between octahedral and tetrahedral sites relative to what is observed in processes that take place at elevated temperatures. It is therefore possible that low temperature incubation may enhance the magnetic properties of bacterially synthesized Zn-ferrite (Yeary et al. 2011).

Synthesis of Lanthanide-Substituted Magnetite Nanoparticles

Moon et al. (2007) proposed a new mixed-lanthanide precursor method as compared to the traditional direct addition technique. Lanthanide (Nd, Gd, Tb, Ho and Er)-substituted magnetites, $LyFe_{3-y}O_4$ were microbially produced using lanthanide-mixed precursors, $L_xFe_{1-x}OOH$, where x = 0.01–0.2. By combining lanthanides into the akaganeite precursor phase, some of the toxicity is mitigated, enabling the microbial formation of L-substituted magnetites using a metal reducing bacterium, *Thermoanaerobacter* sp. TOR-39 (Moon et al. 2007). The employment of L-mixed precursors enabled the microbial formation of L-substituted magnetite, nominal composition up to $L_{0.06}Fe_{2.94}O_4$, with at least tenfold higher L-concentration than could be obtained when the lanthanides were added as soluble salts. This mixed-precursor method can be used to extend the application of microbially produced L-substituted magnetite, while also mitigating their toxicity (Moon et al. 2007).

Thermoanaerobacter ethanolicus

Thermoanaerobacter ethanolicus is a species of thermophilic, anaerobic, non-spore-forming bacteria. This bacterium were first isolated from hot springs in Yellowstone National Park. The bacteria ferment sugars into ethanol and carbon dioxide more than other anaerobes, hence the species name ethanolicus. The growth range of *T. ethanolicus* is 37–77 °C and pH 4.4–9.9, with the optimum growth temperature at around 70 °C (Wiegel and Ljungdahl 1981).

The extracellular synthesis of magnetite nanoparticles based on the reduction of iron oxyhydroxides to magnetite nanocrystals by *Thermoanaerobacter ethanolicus* TOR 39 was elucidated by Roh et al. (2006). Magnetite yield of up to 20 g/L per day was observed in 20-L vessels. *T. ethanolicus* TOR 39 reduced iron oxyhydroxides in the presence of metals (i.e. Co, Cr, Mn, Ni) and produced metal-substituted magnetite nanoparticles. The particles for sharp well-formed octahedral crystals. These crystals are in the single-domain size range of 30–100 nm. The magnetites formed using akaganeite with Cr, Mn and Ni showed well crystalline magnetite crystals (Roh et al. 2006). The biologically facilitated formation of metal-substituted magnetite does not require the reduction agents and the addition of exogenous electron

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carrier substances such as humic acids. These biologically mediated reactions for mineral synthesis represent a novel way to make a number of inorganic materials and are potentially useful for the synthesis of nm-sized ferromagnetic materials (Roh et al. 2006). Magnetic nanoparticles synthesized by *T. ethanolicus* TOR-39 may be useful for improved magnetorheological (MR) fluids and ferrofluids for applications in active damping and for advanced power transmission devices such as fluid clutches (Roh et al. 2006).

Roh et al. (2007) examined the influence of Mn ion on the microbial synthesis of magnetite nanoparticles by *Thermoanaerobacter ethanolicus* TOR 39. The reductive biotransformation of an akaganeite (β-FeOOH) and Mn-substituted (2–20 mol%) akaganeite ($Fe_{1-x}Mn_xOOH$) by *T. ethanolicus* TOR-39 was investigated under anaerobic conditions at pH 7–8. *T. ethanolicus* TOR-39 reduced akaganeite and formed nm-sized magnetite using lactate or glucose as an electron donor. The Mn-mixed akaganeite as a magnetite precursor enabled production of microbially-synthesized Mn-substituted magnetite nanoparticles by *T. ethanolicus* TOR-39 at 60 °C rather than synthesizing a mixture of Mn-carbonate (Roh et al. 2007). Microbial formation of metal-substituted Fe(II)-minerals such as magnetite, siderite, and green rust is influenced by foreign ions (e.g. Mn), microorganisms, and incubation temperature. This method provide a means to mitigate the metal toxicity problem by incorporating or pre-alloying the substituting species into the colloidal metal oxyhydroxide phase. Besides reducing the potential toxicity, this method has further benefit of enhancing the uniform, intimate mixing of the different metal species in the final product by providing an intimately mixed starting material. This method may also allow the incorporation of metal species whose low aqueous solubility would limit the amount of the species that could be carried as soluble ions in the culture medium. Moreover, if several of the metal species are each reducible by the bacteria, the invention may help to produce a single-phase product that suppress the formation of two separate product phases. This mixed precursor method can therefore be used to extend the application for bacterial synthesis fields where there is a need for economically low-energy consumable microbial production of nm-sized materials that should involve toxic or inhibitory element to bacterial growth or by product formation in addition to magnetite synthesis (Roh et al. 2007).

Thermoanaerobacter sp. X513

Elemental copper nanoparticles with a bimodal distribution of 3 and 70 nm diameters were synthesized from inexpensive oxidized copper salts by an extracellular metal-reduction process using anaerobic *Thermoanaerobacte*r sp. X513 bacteria in aqueous solution (Jang et al. 2015). FTIR spectra indicated that chelating and capping agents coated the NP surfaces despite heterogeneous bacterial organic matter produced during the microbial activity (Jang et al. 2015). These coatings protected the surface from air oxidation under aqueous and dry lm conditions. The chelating agent, NTA, effectively facilitated the growth of particles and limited the

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size to 70 nm. Fatty acids and amines capped Cu NPs without causing an increase in size, stabilized them against oxidation and agglomeration in aqueous solution and enabled the formation of the most stable elemental Cu NP film. Compared to previously reported Cu NP syntheses, this biological process substantially reduced the requirement for hazardous organic solvents and chemical reducing agents, while reducing the levels of Cu oxide impurities in the product. This process was highly reproducible and scalable from 0.01 to 1 L batches (Jang et al. 2015).

3.2.2 Thermophilic archaea

3.2.2.1 *Sulfolobus islandicus*

The hyperthermophiles from the order *Sulfolobales-Sulfolobus* spp., grow optimally at temperatures above 80 °C and pH values below 3 (O'Connor and Shand 2002). They are metabolically dependent on sulfur oxidation when sulfur acts as the final electron acceptor. Some strains of *Sulfolobus* have technical potential for metal extraction from ore (Huber and Prangishvili 2006). The potential of the thermoacidophilic archaeon *Sulfolobus islandicus* cells to serve as macromolecular template for the formation of silver nanoparticles (AgNPs) was demonstrated by Kalabegishvili et al. (2015). At the reaction in silver nitrate solution with *S. islandicus* suspension, the aggregation of the AgNPs from Ag(I) to Ag(0) by biomolecules, proteins and enzymes of *S. islandicus.* Sonication increases nanoparticle formation as it increases total surface of the biomass by its fragmentation. The *S. islandicus* suspension grown for 4 days was sonicated at 35 kHz for 10 min, then the aqueous solution of $AgNO_3$ with a concentration of 10^{-3} M was added to this suspension. The AgNPs synthesis was carried out at 75 °C. The stable formation of small sized AgNPs in the biomass of *S. islandicus* takes place in a few hours. In the first 20 h of reaction of the *S. islandicus* biomass with silver nitrate aqueous solution, the formation of AgNPs in the range of 10–50 nm takes place. The sizes increase further with time, reaching 10-50 nm (25 nm on average). The number of AgNPs also increases with time (Kalabegishvili et al. 2015).

3.2.3 Thermophilic Fungi

3.2.3.1 *Humicola* sp

Silver Nanoparticle Synthesis

Syed et al. (2013) elucidated the biosynthesis of silver nanoparticles by the thermophilic fungus *Humicola* sp. The fungus when reacted with Ag^+ ions reduces the

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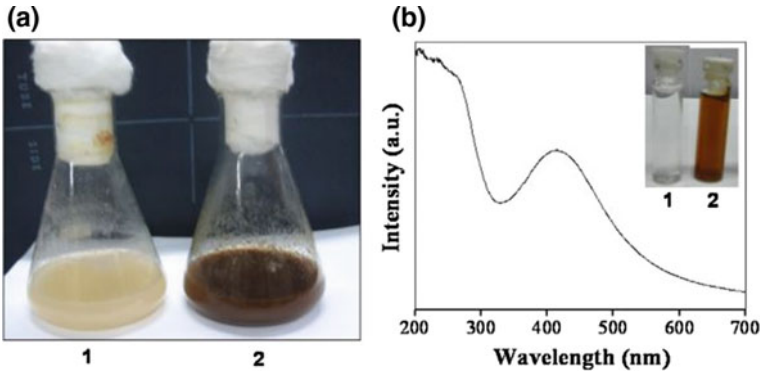

Fig. 3.8 **a** Conical flasks with fungus (Humicola sp.) before exposure (*1*) and after (*2*) exposure to $AgNO_3$ ions. **b** UV–Vis spectrum for the silver nanoparticles. Inset shows filtrate of the control flask (*1*) and filtrate of treated flask (*2*). *Source* Syed et al. (2013). Copyright © 2013, Elsevier. Reproduced with permission

precursor solution and leads to the formation of extracellular nanoparticles. The uniqueness of this study is that the investigators achieved superior control over the size of these nanoparticles, focusing upon them to be in the size range of 5–25 nm, so that these AgNPs when employed in biomedical applications will not block the glomerulus of the kidneys and will easily pass through urine within a short period of time. Figure 3.8a shows two conical flasks with the fungal biomass before (1) and after (2) exposure to 1 mM $AgNO_3$ solution at temperature 50 °C and pH 9 for 96 h under shaking condition. Formation of silver nanoparticles is clearly demonstrated by the change in color from yellow to brown. After filtration, it was observed that the aqueous solution contained the silver nanoparticles, characterized by an intense brown color (Fig. 3.8b). The AgNPs synthesised are non-toxic to cancer and normal cells up to concentrations of 50 µg/ml and thus will find various applications in drug and targeted drug delivery systems (Syed et al. 2013).

Cerium Oxide Nanoparticle Synthesis

Khan and Ahmad (2013) reported for the first time, the bio-inspired synthesis of biomedically important cerium oxide (CeO_2) nanoparticles using the thermophilic fungus *Humicola* sp. The fungus Humicola sp. when exposed to aqueous solutions of oxide precursor cerium (III) nitrate hexahydrate ($CeN_3O_{96}H_2O$) results in the extracellular formation of CeO_2 nanoparticles containing Ce(III) and Ce(IV) mixed oxidation states, confirmed by X-ray Photoemission Spectroscopy (XPS). The formed nanoparticles are naturally capped by proteins secreted by the fungus and thus do not agglomerate, are highly stable, water dispersible and are highly

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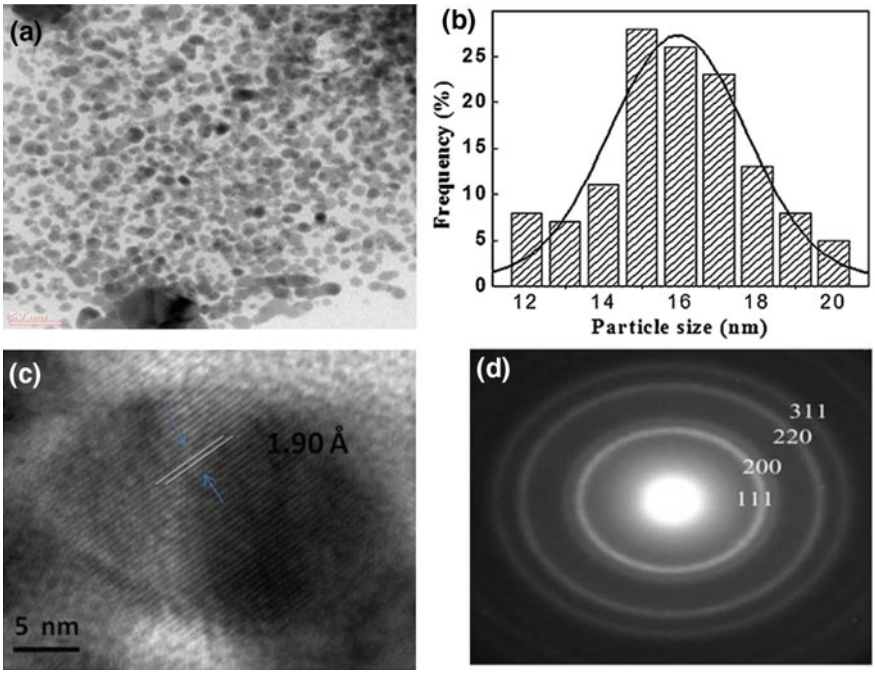

Fig. 3.9 **a** TEM micrograph recorded from drop-cast films of CeO_2 nanoparticle solution formed by the reaction of cerium nitrate with the fungal biomass of *Humicola* sp. for 96 h. **b** Particle size distribution determined from TEM microgaph. **c** HR-TEM image of CeO_2 nanoparticles showing inter planar distance. **d** Selected Area Electron Diffraction (SAED) pattern recorded from the CeO_2 nanoparticles. *Source* Khan and Ahmad (2014). Copyright © 2014, Elsevier. Reproduced with permission

fluorescent as well. The CeO_2 nanoparticles are polydispersed and spherical in shape (Fig. 3.9a). Particle size distribution analysis in Fig. 3.9b reveals that the particles are in the range of 12–20 nm with 16 nm as an average diameter. Figure 3.9c represents the HR-TEM image of one of the CeO_2 nanoparticles showing inter planar distance or 'd' value which was estimated to be 1.90 Å and corresponds to the {2 2 0} plane of CeO_2 nanoparticles. Selected Area Electron Diffraction pattern in Fig. 3.9d indicates that the CeO_2 nanoparticles are polycrystalline in nature (Khan and Ahmad 2013). Khan and Ahmad (2013) have shown a very simple fungus based method for the synthesis of biomedically important cerium oxide (CeO_2) nanoparticles. Capping protein which is involved in the capping of nanoparticles makes them water dispersible and can prove very important for their clinical applications such as treatment of diseases which involve ROS production.

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Gadolinium Oxide Nanoparticle Synthesis

Gadolinium oxide nanoparticles are very important as nuclear, electronic, laser, optical, catalyst and phosphor materials (Hussein 1994; Bhattacharyya and Agrawal 1995; Chen 1996; Gündüz and Uslu 1996). Many organic compounds use Gd_2O_3 for their dimerization (Gündüz and Uslu 1996). It is also used in imaging plate neutron detectors, as neutron reactors (Gündüz and Uslu 1996), and as an additive in ZnO_2 to enhance its toughness (Bhattacharyya and Agrawal 1995; Chen 1996). Gd_2O_3 has several potential applications in biomedicine, too. For example, it is used in magnetic resonance imaging, since it exhibits superparamagnetism and involves T1 relaxation, and can be useful as a multimodal contrast agent for in vivo imaging (Bridot et al. 2007). It can also be easily doped with other lanthanides and exploited as a fluorescent tag, thus replacing other fluorescent organic molecules. Chemical and physical protocols for the of Gd_2O_3 nanoparticles are limited. The most common methods are the thermal decomposition of precursor salts, mechanochemical processing, milling and calcinations (Matijević and Hsu 1987; Mazdiyasni and Brown 1971; Rowley and Parkin 1993). Unfortunately, these methods give agglomerated particles, occur at high temperatures, and employ harsh environments, thus rendering it difficult to find any usage of Gd_2O_3 nanoparticles in biomedical applications.

Khan et al. (2014) showed that the thermophilic fungus *Humicola* sp. can be used for the synthesis of Gd_2O_3 nanoparticles at 50 °C. As $GdCl_3$ is dissolved in water along with fungal biomass, $GdCl_3$ ionizes to Gd^{3+} and $3Cl^{-}$. The Gd^{3+} ions are then attracted toward anionic proteins, which are secreted by *Humicola* sp in solution. Reductase enzymes present in the anionic protein fraction act on Gd^{3+} and convert it to Gd^{2+}. Oxidase enzymes, which are also secreted by the fungus in the solution mixture, act on these Gd^{2+} ions resulting in the formation of Gd_2O_3 nanoparticles. The $GdCl_3$ NPs articles are irregular in shape, presenting an overall quasi-spherical morphology. Particle size distribution analysis of Gd_2O_3 nanoparticles confirmed that the nanoparticles are in the range of 3–8 nm with an average size of 6 nm. Since Gd_2O_3 nanoparticles have proved their value in site specific drug delivery systems for cancer therapy, Khan et al. (2014) extended the work of biosynthesis of Gd_2O_3 nanoparticles to bioconjugation with taxol. Bioconjugation of taxol with gold and iron oxide nanoparticles has also been reported (Gibson et al. 2007; Hwu et al. 2009). Taxol is one of the most important anticancer drugs used for breast, ovarian and lung cancers. The potent anticancer effect of taxol is mainly attributed to its mechanism of action. It stabilizes microtubules by preventing their depolymerization Khan et al. (2014). However, taxol is a hydrophobic drug and less specific to certain tumors due to its low solubility in water. To counter these problems, we carried out the bioconjugation of chemically modified taxol with biocompatible Gd_2O_3 nanoparticles, which may result in an enhancement of the hydrophilicity of taxol and may render it more potent in killing tumor/cancer cells Khan et al. (2014).

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3.3 Thermophilic Enzymes in Nanotechnology

3.3.1 Immobilization of Thermozymes with Magnetic Nanoparticles

Thermophilic microorganisms represent a novel source of highly active enzymes with attractive features for industrial bioprocesses due to their adaptability and stability under extreme conditions (Demirjiana et al. 2011). Thermoenzymes from these microorganisms allow to perform reactions at high temperatures, which result in lower medium viscosity, increased substrate diffusion coefficients and fewer microbial contamination risks (Zhou et al. 2013). Thermostable enzymes from extreme thermophiles have led to a special focus due to their intrinsic thermostability and resistance to denaturing physical and chemical factors (Li et al. 2010a). However, to carry out these bioprocesses under preparative conditions, immobilization procedures are required to enable biocatalyst recovery and reusability. Immobilized enzymes are drawing significant attention for potential commercial applications as biocatalysts by reducing operational expenses and by increasing process utilization of the enzymes. Immobilized enzymes can be recycled by utilizing the physical or chemical properties of the supporting material. Several nanoparticles have been employed to improve traditional enzyme immobilization methods in order to enhance loading, activity and stability of enzymes and to reduce the biocatalyst costs in industrial biotechnology (Abad et al. 2005; Yiu and Keane 2012). In particular, MNP-based immobilization of enzymes presents several advantages, including (i) high surface-to-volume ratio offered by nanosize magnetic beads, (ii) good dispersibility, (iii) easy separation of enzymes from the reaction mixture, and (iv) reuse by applying an external magnetic field (Johnson et al. 2011). One of the crucial points in protein immobilization on nanoscale solid surfaces is that structural modifications may occur, which could affect protein activity and stability to different extents depending on the protein and the conjugation strategy (Occhipinti et al. 2011). For this reason, there has been an increasing interest in developing new reliable approaches for the immobilization of enzymes on magnetic nanoparticles (Johnson et al. 2008; Li et al. 2010b; Yu et al. 2012). However, although great efforts have been made for this purpose, the actual effect of immobilization on enzyme functionality is still poorly understood. Furthermore, not many studies involving enzymes from extremophile microorganisms have been carried out so far.

3.3.1.1 Immobilization of α-Amylase

Magnetic nanoparticles has been used for efficient immobilization of lysine-tagged α-amylase (BACΔNC-*Lys*7) from thermophilic *Bacillus* sp. strain TS-23 (Chen et al. 2012). The carboxylated magnetic nanoparticles were prepared by the simple co-precipitation of Fe^{3+}/Fe^{2+} in aqueous medium and then subsequently modified

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with adipic acid. Transmission electron microscopy micrographs showed that the carboxylated magnetic nanoparticles remained discrete and had no significant change in size after the binding of BACΔNC-*Lys*7. Free enzyme was active in the temperature range of 45–70 °C and had an optimum of 60 °C, whereas the thermal stability of BACΔNC-*Lys*7 was improved as a result of immobilization. The immobilized BACΔNC-*Lys*7 could be recycled 20 times without a significant loss of the amylase activity and had a better stability during storage with respect to free enzyme. Taken together, the magnetic nanoparticles coated with this functional moiety can be a versatile platform for the effective manipulation of various kinds of engineered proteins (Chen et al. 2012). Surface immobilization of the enzyme on the magnetic nanoparticles increase the thermal stability, probably by increasing its molecular rigidity, thus preventing any undesirable change in the tertiary molecular structure due to heating (Chen et al. 2012).

3.3.1.2 Immobilization of Superoxide Dismutase

Superoxide dismutase (SOD) is one of the most important metalloenzymes for aerobic and anaerobic organisms in the first line of defense against oxidative stress, catalyzes the disproportionation of superoxide anion (O_2^-) to O_2 and H_2O_2 (Miller and Sorkin 1997). Due to its enzymatic activity, SOD has been widely applied in the medical treatment, cosmetic, food, agriculture, and chemical industries (Pugliese and Pugliese 2002; Zhang et al. 2004b; Kumar et al. 2006). Thermostable Fe/Mn-SODs have been identified in thermophiles including, *Aquifex pyrophilus* (Lim et al. 1997), *Chloroflexus aurantiacus* (Lancaster et al. 2004), Sulfolobus solfataricus (Yamano and Maruyama 1999), *Aeropyrum pernix* (Yamano et al. 1999), *Pyrobaculum aerophilum* (Whittaker and Whittaker 2000), *Thermus aquaticus* (Sato and Harris 1977), and *Thermus thermophilus* (Li et al. 2010a; Liu et al. 2011). These thermostable SODs have the potentials to be widely used in industry. Superparamagnetic nanoparticles, due to the remarkable properties such as superparamagnetism, high field irreversibility, and high saturation field, have shown great potential applications in various fields (Andrade et al. 2011), especially in biomedicine and bioengineering such as magnetic separation (Gupta and Gupta 2005; Lucena et al. 2011), tumor hyperthermia (Ito et al. 2005), magnetic resonance imaging (De et al. 2008), magnetically assisted site-specific drug delivery (Neuberger et al. 2005; McCarthy et al. 2007), and biomolecule immobilization (Saiyed et al. 2007).

Song et al. (2012) immobilized the thermostable Mn-SOD from *Thermus thermophilus* on supermagnetic nanoparticles. The thermostable Mn-SOD from *T. thermophilus* was covalently bound to the supermagnetic 3-APTES-modified $Fe_3O_4@SiO_2$ nanoparticles using glutaraldehyde method to prepare the Mn-SOD bound magnetic nanoparticles. The immobilization proof of Mn-SOD on the 3-APTES-modified $Fe_3O_4@SiO_2$ nanoparticles was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), X-ray diffraction (XRD), transmission electron microscopy (TEM), and vibrating sample magnetometer (VSM) observation (Song et al. 2012). By comparison with the free Mn-SOD, it

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was found that the immobilized Mn-SOD on nanoparticles exhibited better resistance to temperature, pH, metal ions, enzyme inhibitors, and detergents. The results showed that the immobilized Mn-SOD on nanoparticles could be reused ten times without significant decrease of enzymatic activity (Song et al. 2012).

3.3.1.3 Immobilization of Trelahose Synthase

Trehalose synthase (TreS) catalyzes the reversible interconversion of trehalose (glucosyl-alpha,alpha-1,1-glucose) and maltose (glucosyl-alpha1-4-glucose) (Woo et al. 2010; Schiraldi et al. 2002; Zdziebło and Synowiecki 2006). Trehalose can find applications in medicine, pharmacy, cosmetics and food chemistry as sweeter, stabilizer, antifreeze agent and humectants (Roser 1991; Richards et al. 2002). This non-reducing disaccharide protects cell proteins and biological membranes against physical and chemical stresses such as freezing, high temperature, dehydration and high osmotic pressure (Crove and Crove 2000; Benaroudi et al. 2001; Higashiyama 2002; Elbein et al. 2003). Furthermore, the mild sweetness of trehalose, its low cariogenicity, good solubility in water, high water retention capabilities, stability, reduction of water activity, depression of freezing point and protein protection properties make it a valuable food ingredient (Richards et al. 2002). Trehalose is resistant to hydrolysis at low pH values and even at elevated temperatures. Moreover, this disaccharide does not caramelize and undergo Maillard's reaction.

The immobilization on magnetic nanoparticles of the recombinant trehalose synthase from thermophilic bacterium *Deinococcus geothermalis* was demonstrated by Panek et al. (2013). In their study the gene encoding trehalose synthase from Recombinant trehalose synthase from D. *geothermalis* was covalently immobilized on magnetic support obtained by co-precipitation of Fe^{3+} and Fe^{2+} salts. The mean diameter of the prepared nanoparticles determined by SEM analysis was 30 nm. However, the size distribution of the particles seems to be wide. Before the immobilization magnetic support was silanized using 3-aminopropyltriethoxysilane which allows coupling the enzyme via glutaraldehyde. The catalytic activity of enzyme immobilized using glutaraldehyde is dependent on the amount of crosslinking reagent applied during enzyme fixation as well as other factors, such as immobilization time and enzyme concentration in the media. The most active preparations (0.134 U/g of support) were obtained at glutaraldehyde concentration of 10 mM. Increase of glutaraldehyde concentrations from 15 mM to 30 mM has no influence on the amount of bound protein. At higher glutaraldehyde concentration of 30 mM the enzyme activity decreased considerably to 63.4 % of the maximal value and was 0.084 U/g of support. The reaction of enzyme coupling is fast and preparations of the immobilized trehalose synthase achieved maximal activity after 0.5 h of the process. Further increase of immobilization time did not influence on enzyme activity. The obtained immobilized preparation has specific activity of 0.134 U/g support when measured at 40 °C using maltose as substrate (Panek et al. 2013). Immobilization process was almost fully completed after 30 min of the reaction at 30 °C. The highest immobilization yield of the enzyme

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was achieved at glutaraldehyde concentration of 10 mM. No significant differences in optimal pH and temperature were observed upon immobilization. The immobilized trehalose synthase exhibited mass transfer limitation, which is reflected by higher KM and activation energy values. In addition, immobilized trehalose synthase was easily separated from the reaction medium by an external magnetic field and retained 82 % of its initial activity after successive twelve repeated batches reaction. Stability during the operation of immobilized trehalose synthase was investigated by determination of residual activity of the enzyme after repeated batch reactions carried out for 1 h each. After each run the immobilized enzyme was recovered from the reaction mixture by magnetic separation and washed. This experiment shows the high stability of the immobilized trehalose synthase. No remarkable changes were observed on the adsorption capacity and activity recovery of the trehalose synthase during twelve reaction cycles which retained about 80 % of initial activity (Panek et al. 2013). This is the first report on such immobilization of trehalose synthase from *D. geothermalis*.

3.3.1.4 Immobilization of Carboxypeptidase

Carboxypeptidases are enzymes that remove amino acids one residue at a time from the C-termini of polypeptide chains. Three types of enzyme have been characterized which differ in the rate at which they release particular amino acids. Carboxypeptidases A (CPA) (from pancreas) release most rapidly amino acids with an aromatic or large aliphatic side chain, while carboxypeptidases B (CPB) (also from pancreas) release the basic amino acids lysine and arginine very much faster than any of the other common protein amino acids. Plants contain carboxypeptidase C, which will liberate the amino acid proline as well as being able to release many of the other protein amino acids (Ambler 1967).

Sommaruga et al. (2014) developed a nanobioconjugate of carboxypeptidase from the hyperthermophilic archaeon *Sulfolobus solfataricus* CP*Sso* immobilized on silica-coated magnetic nanoparticles, which exhibited enhanced stability in aqueous media at room temperature as well as in different organic solvents (Fig. 3.10). CP*Sso* is a heat- and pressure-resistant zinc-metalloprotease consisting of four identical 43 kDa subunits (Colombo et al. 1995; Bec et al. 1996; Tortora and Vanoni 2004; Occhipinti et al. 2006). The catalytic and kinetic mechanisms of CP*Sso* have been well established and were confirmed by a 3D model that was developed and validated in the past years (Occhipinti et al. 2003; Sommaruga et al. 2008). CP*Sso* exhibits nonconventional catalytic properties that are useful in several synthetic processes. First, it removes any amino acid from the C-terminus of short peptides, with the sole exception of proline, and also hydrolyzes N-blocked amino acids, thus acting as an aminoacylase (Sommaruga et al. 2008). Second, despite its remarkable thermophilicity, it maintains a significant fraction of its maximal activity even at room temperature. Finally, CP*Sso* maintains a significant activity in solvent mixtures even at high content of organic fraction (Colombo et al. 1992). These peculiar properties highlight the biotechnological potential of this enzyme, in

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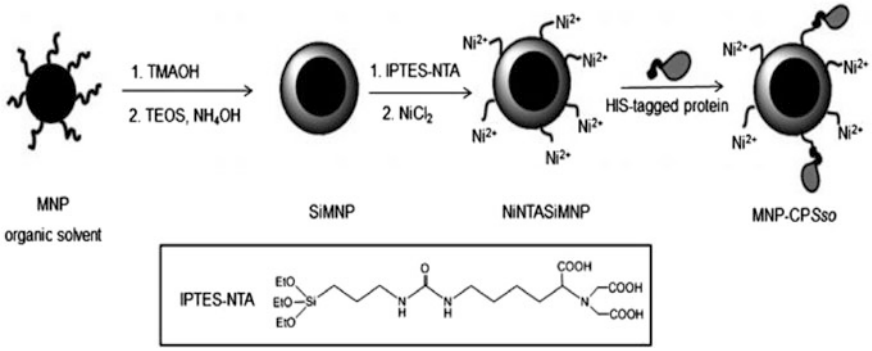

Fig. 3.10 Procedure of CP*Sso* immobilization on NiNTASiMNP. *Source* Sommaruga et al. (2014). Copyright © 2014, BioMed Central. Reproduced with permission

particular to achieve the synthesis of N-blocked amino acid in organic media. The nanobioconjugate was obtained by affinity-oriented immobilization of His-tagged CPSso on silica-coated magnetic nanoparticles functionalized with Ni-NTA groups (NiNTASiMNP). The procedure for the synthesis of NiNTASiMNP is illustrated in Fig. 3.10 and the resulting functionalized Fe_3O_4@silica core-shell nanoparticles gave a stable dispersion in aqueous environment, as determined by dynamic light scattering analysis. Following the immobilization, CP*Sso* acquired distinctly higher long-term stability at room temperature compared to the free native enzyme, which, in contrast, underwent extensive inactivation after 72 h incubation, thus suggesting a potential utilization of this enzyme under low energy consumption. CP*Sso* conjugation also resulted in a significantly higher stability in organic solvents at 40 °C, which made it possible to synthesize N-blocked amino acids in remarkably higher yields compared to those of free enzyme. Improved stability of MNP-CP*Sso* in organic solvent is relevant to possible industrial applications of the enzyme as a biocatalyst in synthetic reactions carried out in organic environment. CP*Sso* could be an ideal candidate as a biocatalyst for the synthesis of N-blocked amino acids in water-cosolvent mixtures following the thermodynamic method (Sommaruga et al. 2014).

3.4 Synthesis of Nanoparticles by Psychrophiles

3.4.1 Synthesis of Nanoparticles by Psychrophilic Bacteria

3.4.1.1 *Morganella Psychrotolerans*

Ramanathan et al. (2011) showed for the first time that by controlling the growth kinetics of *Morganella psychrotolerans*, a silver-resistant psychrophilic bacterium,

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Table 3.2 Psychrophiles in biosynthesis of nanoparticles

Psychrophile	NP	References
Bacteria		
Morganella psychrotolerans	Ag	Ramanathan et al. (2011)
Phaeocystis antarctica	Ag	Shivaji et al. (2011)
Pseudomonas proteolytica	Ag	Shivaji et al. (2011)
Pseudomonas meridiana	Ag	Shivaji et al. (2011)
Arthrobacter kerguelensis	Ag	Shivaji et al. (2011)
Arthrobacter gangotriensis	Ag	Shivaji et al. (2011)

the shape anisotropy of silver nanoparticles can be achieved (Table 3.2). This is particularly important considering that there has been no report that demonstrates a control over shape of Ag nanoparticles by controlling the growth kinetics of bacteria during biological synthesis. This is most possibly because most of the previous studies in this field had only reported the outcomes of exposure of Ag^+ ions to bacteria, without making any deliberate efforts to control the bacterial growth kinetics to achieve shape control. To this end, recent study by Fayaz et al. (2009) in which the effect of temperature on the size of Ag nanoparticles formed during a fungus-mediated biosynthesis process was investigated is particularly notable. It is also notable that controlling reaction kinetics and altering reaction parameters via photochemical, microwave, and ultrasound-assisted techniques are known to achieve anisotropic growth in conventional chemical synthesis.

Ramanathan et al. (2011) utilized *Morganella psychrotolerans* as a model organism, which is a close relative of silver resistant bacteria *Morganella morganii*. *M. morganii* RP42 strain was recently reported for its specificity toward sustaining high concentrations of Ag^+ ions via extracellular synthesis of spherical Ag nanoparticles (Parikh et al. 2008). Ramanathan et al. (2011) chose *M. psychrotolerans* as a model organism for controlling shape anisotropy of Ag nanoparticles due to its tolerance for lower temperature (psychros: cold) and its capability to grow at a wider temperature range of typically 0–30 °C with 20 °C as the optimum temperature (Emborg et al. 2006). At the optimum growth temperature of 20 °C, predominantly spherical Ag nanoparticles of ca. 2–5 nm diameter along with relatively few nanoplates of 100–150 nm edge length were observed during TEM imaging (Fig. 3.11b) (Ramanathan et al. 2011). However, in contrast to previous biosynthesis studies, when *M. psychrotolerans* bacteria was used in this study for biosynthesis of Ag nanoparticles at temperatures different from its optimum growth temperature, formation of Ag nanoplates was observed (Fig. 3.11a, c and d). For instance, at 25 °C, which is 5 °C higher than the optimum growth temperature of bacteria, a mixture of triangular and hexagonal nanoplates along with spherical nanoparticles was obtained (Fig. 3.11a). Similarly, at 15 °C, which is 5 °C lower than the optimum growth temperature, again a mixture of nanoplates and spherical particles was obtained (Fig. 3.11c). Further reduction in bacterial physiological

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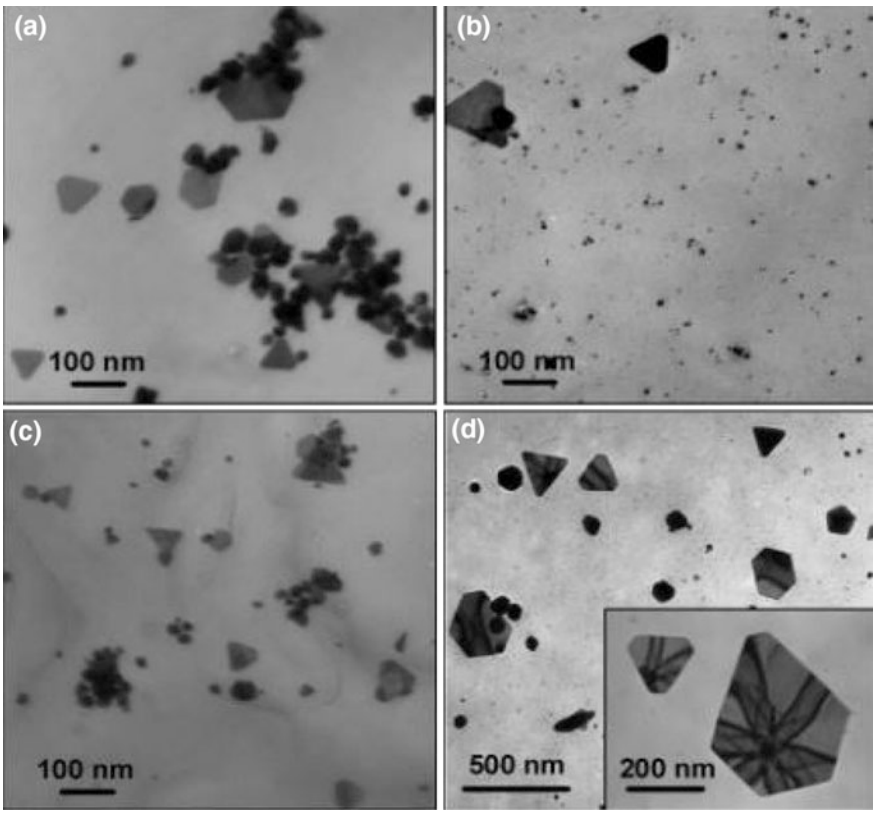

Fig. 3.11 TEM images of biogenic Ag nanoparticles biosynthesized by *M. psychrotolerans* at **a** 25, **b** 20, **c** 15, and **d** 4 °C after 20 h, 24 h, 5 days, and 15 days of reaction, respectively. *Source* Ramanathan et al. (2011). Copyright © 2011, American Chemical Society. Reproduced with permission

activity and growth by reducing its growth temperature to 4 °C results in a significant increase in the number of nanoplates, whereas only a relatively smaller proportion of spherical nanoparticles were formed (Fig. 3.11d) (Ramanathan et al. 2011). It is however notable that although the proportion of spherical Ag particles formed at 4 °C is lower than that observed at other temperatures; the spherical particles formed at 4 °C are larger in size (ca. 70-100 nm). Typically, *M. psychrotolerans* was found to synthesize Ag nanoplates with 50-150 nm edge length at 25 and 15 °C; however, biosynthesis at 4 °C resulted in larger nanoplates with 150-450 nm edge length (Ramanathan et al. 2011). The possibility to achieve nanoparticle shape control by using a green biosynthesis approach is expected to open up new exciting avenues for eco-friendly, large-scale, and economically viable shape-controlled synthesis of nanomaterials (Ramanathan et al. 2011).

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3.4.1.2 *Phaeocystis antarctica*, *Pseudomonas proteolytica*, *Pseudomonas meridiana, Arthrobacter kerguelensis*, and *Arthrobacter gangotriensis*

Cell-free culture supernatants of five psychrophilic bacteria *Phaeocystis antarctica*, *Pseudomonas proteolytica, Pseudomonas meridiana, Arthrobacter kerguelensis,* and *Arthrobacter gangotriensis* were used to synthesize AgNPs (Shivaji et al. 2011). AgNPs synthesized are spherical in shape and the average size of the AgNPs varied from minimum of 6.2 ± 2.4 nm to a maximum of 12.2 ± 5.7 by TEM. The cell-free culture supernatants formed AgNPs only if the reaction was incubated in the light. These results are similar to the earlier observations using culture supernatants of *B. subtilis* (PTCC 1023), *L. acidophilus* (PTCC 1608), *K. pneumoniae* (PTCC 1053), *E. coli* (PTCC 1399), *E. cloacae* (PTCC 1238) Staphylococcus aureus (PTCC 1112) and *Candida albicans* (PTCC 5011) (Minaeian et al. 2008; Natarajan et al. 2010), but differ from that of Saifuddin et al. (2009) who demonstrated that the culture supernatant of *B. subtilis* produces AgNPs in the dark. Two other studies dealing with extracellular biosynthesis of AgNPs using culture supernatants of bacteria did not mention whether the synthesis was achieved in the light or dark (Shahverdi et al. 2007; Kalimuthu et al. 2008). It is still not known as to how light or darkness influences the formation of AgNPs. Shivaji et al. (2011) observed that AgNPs formed using cell-free supernatants of *A. kerguelensis* and *P. antarctica* were stable up to 8 months if stored in dark but not in the light. The cell free culture supernatants of *A. kerguelensis* and *P. antarctica* started synthesis of AgNPs after 2 h of incubation. This rate was faster than that reported in most of the earlier studies, where synthesis was slow as in *B. licheniformis* (Kalimuthu et al. 2008), *B. subtilis* (Saifuddin et al. 2009) and *F. oxysporum* (Durán et al. 2005) but was comparable to the synthesis in *Morganella* sp. (Parikh et al. 2008) where it took place in 1 h. Further, AgNPs from *A. kerguelensis* were more stable (no change up to 24 h) compared to the AgNPs from *P. antarctica*, as judged by the decrease in the characteristic peak at 410 nm and formation of a new peak at 524 nm by 6 h, probably due to the aggregation of AgNPs. The ability of the cell-free culture supernatant of *A. kerguelensis* to synthesize AgNPs did not depend on the temperature (8, 22 and 30 °C), pH (pH 5, 7 and 10) or the phase of growth (early log, mid log and late log phase of growth) of the culture or on the temperature at which the AgNPs were synthesized. In contrast, when the cell-free culture supernatant of *P. antarctica* was used, the stability of the AgNPs was compromised irrespective of the growth temperature of the bacterium (8, 22 and 30 °C), when the culture was grown at pH 7 and the AgNPs were synthesized either at 25 or 37 °C. These study indicates that the factors in the cell-free culture supernatants that facilitate AgNPs synthesis and stability vary from bacterial species to species (Shivaji et al. 2011). The AgNPs exhibited antibacterial activity and this confirms earlier studies on the bactericidal activity of silver nanoparticles (Sondi and Salopek-Sondi 2004; Li et al. 2005; Panacek et al. 2006).

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3.5 Psychrophilic Enzymes in Nanotechnology

Pioneering studies of psychrophiles at the molecular level were mainly focused on cold-active enzymes because this aspect was regarded as a prerequisite to the environmental adaptation (Feller 2003, 2008; Feller and Gerday 2003). It has been shown that the high level of specific activity at low temperature of cold-adapted enzymes is a key adaptation to compensate for the exponential decrease in chemical reaction rates as the temperature is reduced. Such high biocatalytic activity arises from the disappearance of various noncovalent stabilizing interactions, resulting in an improved flexibility of the enzyme conformation. It should be noted that this adaptive feature is genetically encoded within the protein sequence and results from a long-term selection. As a general picture, psychrophilic enzymes are all faced to a main constraint, to be active at low temperatures, but the ways to reach this goal are quite diverse. The main functional and structural adaptive properties of cold-active enzymes as well as the recent advances related to their synthesis, folding and biotechnological applications are presented by Feller (2013), and Margesin and Feller (2010).

3.5.1 Pectate Lyase

Pectate lyase is one of the enzymes of the pectinase group of enzymes. The enzyme exhibits a β-elimination mechanism in the cleavage of α-1,4-glycosidic bonds of polygalacturonic acid producing unsaturated Δ 4,5 bond at the non reducing end of the polysaccharide and generates 4,5-unsaturated oligogalacturonates, which results in the formation of a double bond between C4 and C5 at the non-reducing end via E2 elimination mechanism and an elimination of CO_2 (Mukhopadhyay et al. 2012). The pectate lyase enzymes have enormous applications in the textile, food and beverages and paper industries, in wastewater treatment and bioremediation. These enzymes have the capacity to degrade pectin, which is a component of plant biomass, which pectin is often the raw material for industrial usage. Pectate lyase also has the ability to degrade or the waste material obtained after utilization of plant biomass. The basic principle of solid state functionalization at nanoscale had first been explored by Bhattacharyya et al. (2014). The theoretical insight of lipid functionalized SWNT self-assembly (nanorope design) which serve as a molecular machine has been reported previously by Bhattacharyya et al. (2013) for developing a reusable glucose sensor.

Mukhopadhyay et al. (2015a) investigated the use of nanoparticles to stabilize a psychrophilic pectate lyase (optimally active at 10 °C) at a temperature as low as 4 °C. Pectate lyase from a pyscrophilic bacterium was supplemented with calcium hydroxyapatite nanoparticles (NP-PL) as a substitute for Ca, (the cationic activator of this enzyme) and entrapped in single walled nanotube (SWNT) based molecular self-assembly. The retention of enzymatic activity (more than 70 %) after repeated freezing and thawing at 25 °C in presence of the specific nanoparticle offers a novel

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use of such nano-enzyme systems. The activity and stability of PL was enhanced both at temperatures as low as 4 °C and as high as 80 °C in presence of NP and SWNT. The enzyme could be repeatedly released and re-trapped (in SWNT based molecular self-assembly) while retaining significant activity (Mukhopadhyay et al. 2015a). The immobilized PL (in SWNT based molecular self-assembly), retained its activity after repeated freezing and thawing. This unique capability of SWNT to activate and stabilize a cold active enzyme at temperatures much lower or higher than its optimal range may be utilized for processes that require bio-conversion at low temperatures while simultaneously allowing for shifts to higher temperature (Mukhopadhyay et al. 2015a).

3.5.2 *Laccase*

Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of ortho and para-substituted monophenolic and polyphenolic substrates and from aromatic amines by one-electron abstraction, to form free radicals, capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation (Gianfreda et al. 1999). Laccase enzymes have enormous applications in the textile, dye and paper industries, in wastewater treatment and bioremediation. These enzymes have the capacity to degrade lignin (in lignocellulose) thereby enhancing its potential as raw material for industrial usage. There has been a limited study of psychrophilic pectinolytic, cellulolytic and xylanase secreting bacteria. In fact, reports on psychrophilic laccase secreting bacteria in literature are lacking. A marine Antarctic, psychrotrophic bacterium (*Pseudoalteromonas haloplanktis*, strain ANT/505), isolated from sea ice covered surface water from the Southern Ocean, showed pectinolytic activity on citrus pectin agar. Isolated enzymes (pelA and pelB) from this strain represented the first pectate lyase isolated and characterized from a cold-adapted marine bacterium (Truong et al. 2001). Reports on pectinases from cold-adapted microorganisms have been restricted to psychrotrophic spoilage bacteria such as different strains of *Pseudomonas fluorescens* (Schlemmer and Ware 1987).

Mukhopadhyay et al. (2015b) demonstrated a simple nanotechnology based immobilization technique for imparting psychrostability and enhanced activity to a laccase from a psychrophilic bacterium obtained from Himalayan (Pindari glacier) soil. Laccase from the psychrophilic bacterium was supplemented with copper oxide nanoparticles (NP) corresponding to copper (NP-laccase), the cationic activator of this enzyme and entrapped in single walled nanotube (SWNT). The activity and stability of laccase was enhanced both at temperatures as low as 4 °C and as high as 80 °C in presence of NP and SWNT. The enzyme could be released and re-trapped (in SWNT) multiple times while retaining significant activity. Laccase, immobilized in SWNT, retained its activity after repeated freezing and thawing. This unique capability of SWNT to activate and stabilize cold active enzymes at temperatures much lower or higher than their optimal range may be utilized for

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processes that require bio-conversion at low temperatures while allowing for shifts to higher temperature if so required (Mukhopadhyay et al. 2015b).

3.6 Future Perspectives

Major drawbacks associated with the biosynthesis of nanoparticles using thermophiles and psychrophiles include tedious purification steps and poor understanding of the mechanisms. For instance, the extraction and purification of the produced metal nanoparticles from the extremophilic microbes (intracellular or extracellular synthesis) for further applications are not well investigated. Many bacterial species have been reported to synthesize nanoparticles very quickly and efficiently but intracellularly. However, because of the current unavailability of an efficient method for intracellular NP recovery and associated agglomeration issues (Shakibaie et al. 2010), researchers cannot utilize the potential of these highly efficient microorganisms and are more focused on extracellular NP-synthesizing microorganisms. In order to release the intracellularly produced NPs, additional processing steps such as ultrasound treatment or reaction with suitable detergents are required (Sonkusre et al. 2014). This can be exploited in the recovery of precious metals from mine wastes and metal leachates (Iravani 2014). Biomatrixed metal nanoparticles could also be used as catalysts in various chemical reactions (Castro et al. 2014). This will help to retain the nanoparticles for continuous usage in bioreactors. Physicochemical methods including freeze-thawing, heating processes, and osmotic shock can be used to extract the produced NPs from the cells. But, it seems that these methods may interfere with the structure of NPs, and aggregation, precipitation, and sedimentation could happen. These may change the shape and size of NPs and interfere with the suitable properties of them. Moreover, enzymatic lysis of the microbial cells containing intracellular NPs can be used, but this method is expensive and it cannot be used in up-scalable and industrial production of NPs. It seems that surfactants and organic solvents can be used for both extraction and stabilization of NPs, but these chemical materials are toxic, expensive, and hazardous. It should be noted that in case of extracellular production of nanoparticle, centrifuge could be used for extraction and purification of NPs, but aggregation might happen (Iravani 2014; Sonkusre et al. 2014).

The elucidation of the biochemical pathways is necessary to develop a rational approach to nanoparticles biosynthesis. A number of issues need to be addressed from the nanotechnology and microbiological points of view before such biosynthetic procedures can compete with the conventional protocols. Frequently encountered in the biosynthesis of nanoparticles are the challenges to control the shape and size of the particles; to achieve the monodispersity in solution phase, and to scale up for production level processing. Furthermore, little is known about the mechanistic aspects, and information in this regard is necessary for economic and rational development of nanoparticle biosynthesis. These important technical challenges must be overcome before this bio-based method with extremophiles will be a successful and competitive alternative for industrial synthesis of nanoparticles.

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