Chapter-6

Production of

*Lactobacillus rhamnosus* Biomass Utilizing Whey

as Medium

6.1 Prologue

The previous chapter summarized the role of cell wall components (Biomass) in AgNP’s synthesis. Biosorption and bioreduction of silver metal
ions by cell wall (particularly EPS) of the biomass is the proposed mechanism involved in the AgNP’s synthesis. The present chapter refers to the upgradation of the factors that influence the cell-wall to increase its capacity to reduce and adsorb silver ions, finally, producing AgNP’s of desired shape and size. The process development is the key step in bioreduction of silver using biomass of *L. rhamnosus*. It involves optimizing the production parameters such as (i) initial silver ion concentration, (ii) temperature and (iii) pH. All these parametric conditions need to be in place, in its effective form for an organism’s physiology to efficiently interact or react with its production environment and comes out with the desired nanoparticle being produced at its best quality and quantity. A variety of relatively simple physico-chemical processes should and do effect the observed behavior of nanoparticle production.

The biosorption process involves a solid phase (sorbent or biosorbents; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, metal ions). Due to higher affinity of the sorbent for the sorbate species, the later is attracted and removed by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phase (Das *et al.*, 2008).

The mechanism of metal biosorption is complicated process. The status of biomass (living or non-living), types of biomaterials, properties of metal solution chemistry, ambient/environmental conditions such as pH, influence the mechanism of metal biosorption (Das *et al.*, 2008). The metal ions are absorbed to the surface of cells by interactions between metals and functional groups displayed on the surface of cells (Lin *et al.*, 2005; Liesje Sintubin *et al.*, 2009).

All metal ions before gaining access to cell membrane and cell cytoplasm come across the cell wall. The cell wall consists of variety of polysaccharides
and proteins and hence offers a number of active sites capable of binding metal ions. Difference in the cell wall composition among the different groups of microorganisms, viz., algae bacteria cyanobacteria and fungi, cause significant differences in the type and amount of metal ion binding to them. The potential metal binding groups in microbes are carboxylates, amines, imidazoles, phosphates, sulfhydryls, sulfates and hydroxyls. These positively charged protonated binding groups may build negatively charged metal complexes (Crist et al., 1981).

Cell walls of bacteria are principally composed of peptidoglycans, which consists of linear chains of the disaccharide N-acetylglucosamine-β 1, 4-N-acetylmuramic acid with peptide chains. In E. coli K12, peptidoglycan was found to be a potent binder of the metals tested and carboxylate groups were the principal components involved in metal binding (Hoyle and Beveridge, 1983).

The major advantages of biosorption include low cost, high efficiency, minimization of chemical and, regeneration of biosorbents and possibility of metal recovery (Kratchovil and Volesky 1998). Research over the past decade has provided a better understanding of metal biosorption by certain potential biosorbents Numerous studies have identified a number of potent microbial species capable of accumulating metals from aqueous environment. Gram positive bacteria have been identified for having a high potential of metal sequestering ability, which makes them competent enough to include them in commercial biosorbents based formulations (Brierley et al., 1986).

This chapter describes the optimization and development of a method to produce AgNP’s using the biomatrix/biomass of L. rhamnosus as a reducing and capping agent since the cell wall controls the growth of the nanoparticles and prevents their agglomeration. The main objectives of using optimizing parameters are to produce a small/desired sized AgNP’s in greater amounts at lower costs and to do so at faster rates than enzymatic reactions can.
6.2 Materials and Methods

Studies were conducted to know the effects of pH, temperature and concentration of AgNO₃ on biosynthesis of AgNP’s by *L. rhamnosus*.

6.2.1 Influence of pH on Silver Reduction

Biomass was cultured, harvested (5000 rpm for 10 min), after incubating in MRS broth for 48 hrs at 37°C. The cell pellets were washed three times by deionized water to eliminate the possible biosorption by the medium. As ionic silver potentially forms oxide and precipitates at high pH, thus silver was added as a diamine complex for future experiments. Diamine silver complex ([Ag(NH₃)₂]⁺) solution was prepared by adding ammonia solution (NH₃.H₂O, 25% w/w) into aqueous solution of AgNO₃ until the precipitate of AgOH was transformed to soluble diamine silver complex. Nitric acid (65%) and ammonia solution were used to adjust the pH values of AgNO₃ and diamine silver complex solutions, respectively (Zhang *et al*., 2005). The Ag concentration in diamine silver complex was adjusted by controlling the initial additions of silver nitrate (AgNO₃) into deionized water for following optimization studies.

Briefly, 200 ml of 10 mM concentration of ionic silver was added to 1g of biomass. The reduction of ionic silver was performed at varying pH’s (2.0, 4.0, 6.0, 8.0, and 10.0), by preparing diamine complex separately for each pH.

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\text{(AgNO}_3 + 3 \text{NH}_3 + \text{H}_2\text{O} \rightarrow [\text{Ag(NH}_3)_2\text{OH} + \text{NH}_4\text{NO}_3)}
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Harvested biomass of *L. rhamnosus* was re-suspended in aqueous solutions of diamine silver complex at different pH values to obtain a concentration of 1g of dried biomass and incubated in dark at 35°C. After biosorption reached equilibrium, the bacterial cells were separated from suspension by centrifugation (5000 rpm, 10 mins at room temperature) and then assayed using Uv-Vis spectroscopy. The concentration of Ag in the sample
and size of AgNP’s were measured by Atomic Emission Spectrophotometer (AES) and Transmission Electron Microscopy (TEM) respectively.

6.2.2 Influence of Temperature on Silver reduction

The effect of varying temperatures on AgNP’s production by L. rhamnosus was evaluated. Different temperatures ranging from 25°C to 45°C (with a difference of 5°C) were checked for ionic silver reduction. After 12 hrs of incubation the biocomposite i.e. biomass and reduced silver was harvested by centrifugation. Obtained biocomposite was subjected to AES and TEM analysis.

6.2.3 Influence of Concentration of AgNO₃ on Silver Reduction

Optimized conditions of pH and temperature were employed for studying the effect of varying concentration of AgNO₃ ranging from 7 to 11 mM. All other conditions remained constant as described previously. Individual samples were harvested by centrifugation and employed for AES and TEM analysis.

6.3 Results and Discussions

6.3.1 Influence of pH on Silver Reduction

The influence of pH on silver reduction was performed with L. rhamnosus. There was a clear influence of the pH on the amount of silver associated with the biomass. When the pH increased, more silver was recovered. The highest recovery of silver was obtained at an alkaline pH 10.0. Additionally, pH influenced the rate of the reduction reaction. The suspension turned brown when silver was reduced, and the suspension coloring accelerated when increasing pH. At pH 10.0, the suspension turned dark brown within an hour. These observations were supported by Uv–Vis analysis (Fig. 6.3a). This plasmon resonance band was centered at around 425 nm, and the intensity increased with time which indicated the rapid formation of AgNP’s. No such plasmon resonance bands were observed at pH 2.0. At pH 4.0,
broadening of absorbance indicate the aggregation of particles. The study demonstrated that the recovery of silver and the reduction rate were pH dependent.

AES analysis revealed 136 mg/g of adsorption of silver on the biomass of L. rhamnosus at pH 10.0, and the least adsorption of 17 mg/g was observed at pH 2.0. While the adsorption of silver for pH 4.0 (73.6 mg/g) and 6.0 (94 mg/g) were found intermediate. However there was not much noticeable difference in AgNP’s recovery between pH 8.0 (135mg/g) and 10.0 (Fig-6.3b), but at pH 10.0 silver reduced and precipitated within 5 hr, where as pH 8.0 precipitated Ag after 24 hr. When pH increased, both recovery and the reduction rate increased (Fig-5.3a & b).

TEM demonstrated that the localization of AgNP’s on the cell wall and average particle size distribution were pH dependent. TEM analysis at varying pH’s 2.0, 4.0 and 10.0 with biomass harvested after the reduction and biosorption of Ag were well in agreement with the Uv-Vis spectral interpretations. Figure 6.3c (pH 2.0) shows no detectable nano particle biosorbed over the cell wall and figure-6.3d (pH 4.0) revealed AgNP’s ranging from 35 to 89 nm with average size of 56 nm. Biosorption conducted at pH 10.0 produced smallest AgNP’s size ranging from 5-16 nm having average particle size of 11 nm entrapped over the bacterium clearly revealed in figure-6.3e. Thus pH was found critical in silver ion reduction. pH seems to be the most important parameter in the biosorption processes. It affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of the metallic ions (Aksu et al., 1991).

Several Gram-positive and Gram-negative bacteria were tested to reduce silver in a non-enzymatic way after application of a high Ag+ concentration in alkaline conditions. Liesje Sintubin et al., (2009) suggested a reduction mechanism (Fig-6.3f (a)) which is quite agreeable for the present production method of AgNP’s. Whenever pH increases, more competition occurs between
protons and metal ions for negatively charged binding sites. Lin et al., (2005) discovered a slight drop in pH after biosorption of Ag+ on the biomass of *Lactobacillus A09*, which indicated the competition between protons and metal ions. High pH catalyzes the ring opening of monosaccharides such as glucose to their open chain aldehyde (Fig-6.3f (b)). It is the aldehyde which delivers the reducing power. And when metal ions are present, the aldehyde will be oxidized to the corresponding carboxylic acid and at the same time the metal ions will be reduced (Fig-6.3f (c)).

In the present study silver reduction was rapid when compared to other biological precipitation processes. For example, *Enterobacteria* could synthesize AgNP’s rapidly: *K. pneumoniae* reduced silver in less than 5 min (Shahverdi et al., 2007). *Bacillus licheniformis* required 6 hrs before silver was reduced (Kalimuthu et al., 2008), whereas 12 and 72 hrs, were required before silver reduction by *Aeromonas* spp. SH10 (Nair and Pradeep 2002) and *Aspergillus flavus* respectively, (Vigneshwaran et al., 2007) was observed. Our results with *L. rhamnosus* biomass which is generally regarded as safe microorganism produced AgNP's within 5 hrs.

### 6.3.2 Influence of Temperature on Reduction of Silver

The effect of varying temperatures on AgNP’s production by *L. rhamnosus* is having a greater impact. The size and rate silver reduction varied with different temperatures. Maximum biosorption of Ag was obtained at 35°C (137
Fig-6.3a Influence of pH on silver reduction based on time duration

Fig-6.3b Influence of pH on reduction of silver ions based on AES analysis
Fig-6.3c No detectable AgNP’s over the biomass at pH 2.0

Fig-6.3d Varying size of silver crystal formed on cell wall at pH 4.0
(a) Bacterial cell with reducing sugars such as glucose and protonated anionic functional groups (–RH). (b) When pH increases, protons dissociate and create negatively charged adsorption sites for Ag+. The reducing sugars turn into their open-ring structure and are now able to reduce Ag+. (c) The aldehyde function of the reducing sugar is oxidized to its carboxylic acid, while Ag⁺ is reduced to Ag⁰.

**Fig-6.3f** Suggested bacterial reduction of Ag⁺ to Ag⁰ at the cell surface
mg/g), followed by 30°C (136.4 mg/g) (Fig-6.3 g). As temperature increased the size of AgNP’s also increased (Fig-6.3 h & i). At 35°C the average size of AgNP’s was recorded as 11 nm (ranging from 5 to 16 nm) where as at 45°C the size drastically increased to 44 nm (ranging from 11 to 55 nm). Moreover increase in temperature was inversely proportional to the absorption of silver on biomass. Thus temperature plays a curial role in AgNP’s synthesis.

Numerous investigations have been carried out to better understand and define conditions, which would lead to higher nanoparticle production efficiency. Gil-Jae Lee et al., (2004) demonstrated the effect of temperature and pH over the preparation of silver nanorods. However control over temperature, for the synthesis of AgNP’s using microorganism has been rarely reported. Aksu et al., (1991) reported that temperature does not influence the biosorption processes in the range of 20°C-35°C, which is similar to our finding, thus showing 35°C to be optimum.

6.3.3 Influence of AgNO₃ concentration

Highest recovery of AgNP’s (136.9 mg/g biomass) was obtained from 11 mM of initial concentration of AgNO₃, followed by 10 mM and 9 mM with 136.5 mg/g and 136 mg/g biomass respectively. While it was 115 mg and 102 mg recovery of silver per gram biomass at 8 mM and 7 mM respectively (Fig-6.3j). However the process was found economical at 9mM initial concentration of AgNO₃. Moreover the broadening of Uv-Vis absorbance peak for added 11 mM AgNO₃ concentration to the biomass indicate the aggregation/ Polydispersion of AgNP’s (Fig-6.3k). The non-enzymatic production of AgNP’s by Lactobacillus spp. allowed the application of high concentrations silver solution i.e. upto 1 g/L for 5 g/L of biomass, which is much higher applied concentration of silver when compared to enzymatic processes. Whereas in enzyme catalyzed silver reduction it is restricted to lower silver concentrations ranging between 10 and
Fig-6.3g Influence of the temperature on the silver reduction based on AES

Fig-6.3h Influence of temperature at 35°C on the size and distribution of AgNP’s
Fig-6.3i Influence of temperature at 45°C on the size and distribution of AgNP’s
Fig-6.3j Influence of the varying conc. of AgNO$_3$ on the silver reduction based on AES

Fig-6.3k Sharp peak of AgNP's at 9 mM concentration of AgNO$_3$
100 mg/L Ag⁺ (Kalimuthu et al., 2008; Kowshik et al., 2003; Shahverdi et al., 2007; Vigneshwaran et al., 2007).

Parametric optimization studies revealed that temperature of 35°C, pH 10.0 and 9mM concentration of AgNO₃ was favorable for the production of AgNP's using biomass of *L. rhamnosus*. Since such rapid silver reduction rates are typically achievable only while using chemical processes, our method combines the eco-friendliness of biological AgNP's production with the speed of chemical processes. Moreover, the chemically synthesized nanoparticles require another step for the prevention of aggregation of the particles (Venkataraman et al., 2011). But, biological synthesis of AgNP's uses harmless, eco-friendly reducing agents and the nanoparticle structure is stabilized by the biomolecules present in the environment eliminating the extra step in preventing the aggregation of chemically synthesized nanoparticles by stabilizers. Since the biological molecules are eco-friendly, the pollution due to chemicals and byproducts can be prevented from reaching the environment (Venkataraman et al., 2011). The size and shape of the particle can also be completely regulated by controlling the environment where the nanocrystal growth occurs (pH and temperature). Furthermore, biological methods have the greater advantage of easy bulk synthesis which can be exploited for industrial scale production too.

Increasing pH enhanced the silver recovery and the reduction rate, creating a process which is fast compared to other biological production methods as reported by Nair and Pradeep (2002), Kalimuthu et al., (2008) and Vigneshwaran et al., (2007). Moreover, the association of the nanoparticles with the bacterial biomass facilitates the concentration of the nanoparticles by centrifugation and further these biocomposite can be used in various applications.