EFFICACY OF SILVER NANOPARTICLES AS DELIVERY SYSTEM IN GENETIC TRANSFORMATION

by

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THESIS

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2018

DECLARATION

I, hereby declare that this thesis entitled **"Efficacy of silver nanoparticles as delivery system in genetic transformation"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other university or society.

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CERTIFICATE

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LIST OF ABBREVIATIONS

E.coli	*Escherichia coli*
EC_{50}	Effective Concentration 50
LB medium	Luria Bertani medium
AgNPs	Silver nanoparticles
h	Hour
min	Minutes
cfu	Colony Forming Units
ng	Nanogram
OD	Optical Density
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
IPTG	Isopropyl β-D-1-thiogalactopyranoside

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Introduction

1. INTRODUCTION

Genetic engineering is a powerful technique for the transfer and introgression of the gene of interest into the host genome. Bacterial transformation is an important is an important step in the cloning process (Kotnik *et al.*, 2015). *Escherichia coli*, the universal host for cloning is not assumed to be naturally transformable; it develops high genetic competence only under artificial conditions. The most widely used methods for bacterial artificial transformation are electroporation and chemical transformation.

Electroporation create holes in the bacterial cell membrane by application of external electric pulse for the uptake of extracellular DNA with high efficiency. However, its application is limited by cost of the equipment and species dependent optimization of parameters. Chemical transformation is marked by two phases, the first phase involving the uptake of the DNA across the cellular envelope and the second phase involving the setting up of the DNA in the cell as a stable genetic material (Hanahan, 1983). The cell membrane is manipulated with calcium cations and temperature imbalances to render them competent to uptake foreign DNA. The drawback of chemical transformation is the low transformation efficiency. So, efficient gene delivery systems possessing high transformation efficiency and low cytotoxicity is a major challenge in different gene transfer experiments (Dizaj *et al.*, 2014).

Nanobiotechnology is an emerging area of intense scientific research due to its wide potential applications in biological fields. Nanoparticles are reported to improve the bacterial transformation efficiency due to its ability to overcome physiological barriers (Dizaj*et al.*, 2014). Reducing the particle size from micro to nano scale aid in overcoming the hindrance of physiological barriers such as the cell wall. Iron oxide nanoparticles, gold nanoparticles, chitosan-DNA nanoparticles, graphene oxide

nanoparticles etc. are being used for transformation (Berry *et al.,* 2003; Varela *et al.*, 2014).

Silver has been used as an antimicrobial agent since ancient civilizations (Alexander *et al.*, 2009). Silver nanoparticles are also reported to affect the integrity of biological membranes, causing toxicity to the cells. Size of the nanoparticles used as well as its concentration is found to be critical in determining its toxicity (Kim *et al.*, 2012; Ivask*et al.*, 2014). Silver ions released by the nanoparticle are crucial in causing the cytoxicity (Reidy*et al.*, 2013). Silver nanoparticles are reported to bind to both double stranded and single stranded DNA (An and Jin, 2012).

There are no reports on the use of silver nanoparticles for gene transfer in microbes. As silver nanoparticles affect the membrane integrity of microbes they have the potential to be used in gene delivery system, with increased transformation efficiency.

Hence, the present study was undertaken with an objective to:

Evaluate the efficiency of silver nanoparticles for gene delivery in microbes.

Review of literature

2. REVIEW OF LITERATURE

Genetic engineering or recombinant DNA technology holds great potential for addressing various challenges faced by mankind such as meeting the food or nutritional demands of increasing population. The process of transformation holds key step in genetic engineering. Effective bacterial transformation is necessary for recombinant DNA research, gene cloning and complementation, quantification of gene expression and tagging with reporter proteins. Translocation of foreign genetic material across bio membranes plays important role in recombinant DNA technology since natural genetic transformation is not present in majority of bacteria (Kotnik *et al.*, 2015).

2.1 TRANSFORMATION

The idea of transformation originated in 1928, when microbiologist Fred Griffith was conducting experiments on the infection of mice with *Streptococcus pneumoniae* or *Pneumococcus*. Bacteria like *Pneumococcus, Micrococcus, Haemophilus, Bacillus* etc., have natural ability to take up extracellular DNA due to presence of proteins on their exterior surface that binds to DNA and transport it inside the cell. Even after several years of Avery's identification of DNA as the transforming principle, all efforts failed to transform *Escherichia coli* DH5α, a largely used cloning host and many other bacteria due to the absence of transfer proteins in them. Hence, they are being transformed artificially either by manipulating physically or chemically (Michod *et al.*, 1988).

2.1.1 *Escherichia coli* DH5α – Universal Host for Cloning

Escherichia coli DH5α, a universal host for molecular cloning is an engineered *E.coli* strain to enhance the transformation efficiency and is named after Douglas Hanahan an American biologist. The cells are defined by three mutations *recA1* and *endA1* which help plasmid insertion and lacZM15 which

enable blue white screening (Hanahan *et al.*, 1983). It has a three-walled membrane *i.e.* outer membrane, inner membrane and in between them the periplasmic space containing peptidoglycan layer (Tu *et al.*, 2005).

Raghav *et al.* (2011) opined that porins, the pore forming proteins present in high copy number (approximately 105 porin molecules per cell) in the outer membrane, behaves as a molecular sieve for the passive transport of hydrophilic molecules of maximum molecular weight up to 5,000 Da. Therefore, macromolecules like DNA and proteins are not likely to pass through *E. coli* cell wall. So there is need for artificial transformation in cloning with vectors to achieve high transformation efficiency.

2.1.2 Vectors for Transformation

Many vectors (plasmids, phages, cosmids etc.) are used for cloning. Among these vectors, pBR322 and pUC18 are the commonly used vectors for cloning in *E.coli* DH5α. pCAMBIA is used for transformation in both *E.coli* DH5 alpha and *Agrobacterium* strains.

2.1.2.1 pBR322

It is one of the widely used cloning vectors (4361bp)in *E.coli* DH5α strain. It has ampicilin resistance gene and tetracycline resistance gene as the selectable markers and pMB1 origin of replication (Brown *et al.*, 2010) (Appendix IV).

2.1.2.2 pUC18

It is a small sized (2686 bp) and high copy number cloning vector for replication. It has ampicilin resistance gene as selectable marker and LacZ alpha as scorable marker and pMB1 origin of replication that lack *rop* genes (Brown *et al.*, 2010). Multiple cloning site (MCS) is placed within the scorable marker (Appendix IV)

2.1.2.3 pCAMBIA 1305.2

It is of 11,921 bp length of having high copy number for cloning in *E.coli* and pVS1 replicon for high stability in *Agrobacterium* strains. It is having kanamycin resistant gene as selectable marker for bacterial selection, Hygromycin B resistant gene (hptII) as plant selectable markers and gus A as reporter gene (Brown *et al.*, 2010)

2.1.3 Artificial Transformation

Artificial transformation includes various approaches such as electrical and chemical processes to attain ability to take up extracellular DNA (Ren *et al.*, 2015). The two main methods for the induction of competency in bacterial cells for the uptake of foreign genetic material are electroporation and calcium chloride method.

2.1.3.1 Electroporation

In electroporation, cells are permeabilized by the application of very short or high-voltage electric pulses to create holes in the cell membrane. It is used to transform novel strains of microbes previously recalcitrant and resistant to transformation (Rhee *et al.*, 2007). Molecules ranging in size from small organic metabolites and reporter dyes to large macromolecules including antibodies and plasmids can be introduced into cells. Electroporation generally shows high transformation efficiency.

Okamoto *et al.* (1997) reported high efficiency transformation of *Bacillus brevis* by electroporation. Gonzales *et al.* (2013) reported that transformation efficiency of $3x10^7$ transformants (cfu) per µg DNA was obtained when *E.coli* DH5α cells were subjected to electroporation.

Jimenez- Antano *et al.* (2018) published a simple method for the production of highly competent cells of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* for transformation by electroporation.

Although electroporation seems to be a simple and effective method, its application is limited to only a few species. Electric pulses may also damage the

gene, leading to altered codons and wrong translational end product. Several parameters including impedance and capacitor discharge must be optimized to achieve high efficiency. It requires very high cell density and is also expensive (Rebersek *et al.*, 2011). So there is a need for simple and cost effective technique with simple apparatus.

2.1.3.2 Chemical Method

Induction of competency using chemicals that weaken the cell membrane is a major technique for bacterial transformation. Divalent ions (Ba^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Sr^{2+}) are more efficient in the induction of competency compared to monovalent ions and trivalent ions. Among the different divalent ions Ca^{2+} is reported to be very efficient in competency induction (Panja *et al.*, 2008).

Panja *et al.* (2006) reported that the standard method of transformation of *E. coli* involves two steps, suspending the cells in ice-cold $CaCl_2$ (100 mM), to make the cells competent for DNA uptake and subjecting the DNA bound competent cells to a heat shock from 0 to 42 ℃ for 90 seconds to facilitate the entry of adsorbed DNA into the cell cytoplasm.

2.1.3.3 Mode of action of calcium chloride

Weston *et al.* (1981) has reported that negatively charged exogenous foreign DNA was electrostatically repelled by the negatively charged bacterial membrane and dissolved calcium ions in the calcium chloride heat-shock method will neutralize the repulsive negative charges in order to allow the plasmid to interact with the cell membrane.

Asif *et al.* (2017) reported that calcium cations form strong covalent bonds with the phosphate groups in the double stranded DNA. So, once neutralized, the DNA is no longer repelled from the membrane, and then during the heat-shock step at

42 ° C, the fluidity of the membrane is altered which may release membrane lipids resulting in generation of membrane pores through which the plasmid enters.

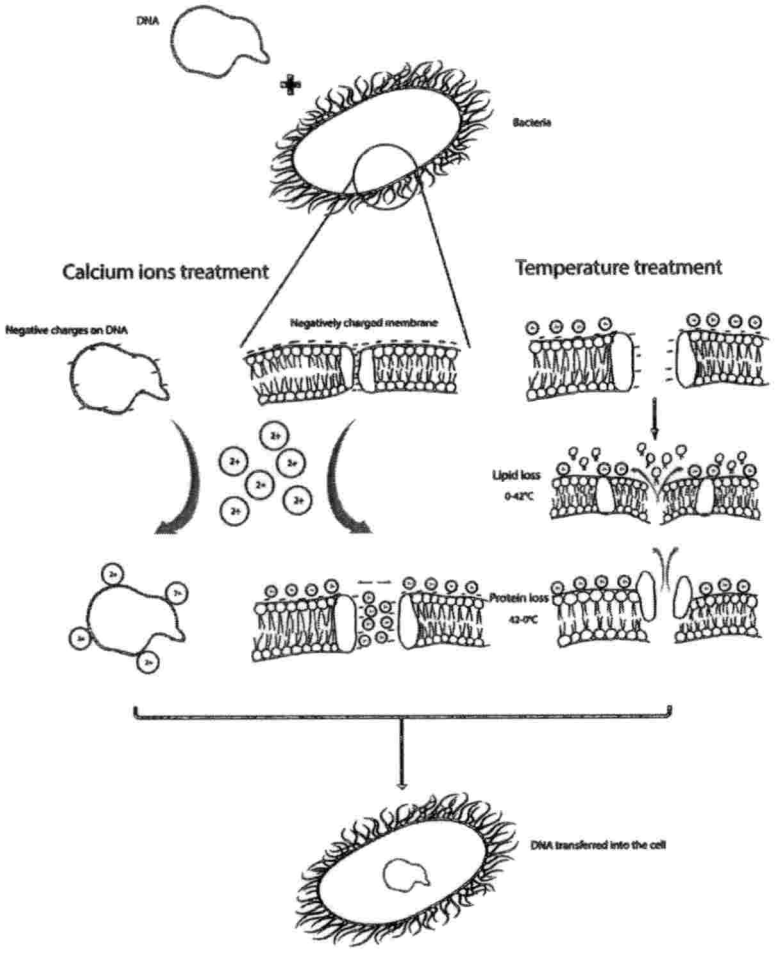

Fig.1 Manipulation using chemical and heat treatment of the bacterial membrane for increased porosity and DNA uptake (Asif *et al* 2017).

Li *et al.* (2010) have reported that factors such as growth rate can affect the calcium chloride mediated transformation efficiency. Growing cells in nutrient limitations of carbon, nitrogen and phosphorous followed by nutrient rich medium increases the growth rate and transformation efficiency. However, this increase in transformation efficiency is due to more transformable cells in the medium, than due to more DNA uptake.

Chemical transformation of competent cells by $CaCl_2$ solution is a simple and cost-effective choice that does not require any specialized equipment. However, competent cells formed by these transformation methods have transformation efficiencies of about 10^6-10^7cfu/ug plasmid DNA which is highly inefficient. The majority of DNA molecules added, does not enter any cell and majority of bacterial cells, receive no DNA (Nishimura *et al.*, 1990; Chan *et al.*, 2013).

Transformation methods developed to date are optimized to specific bacterial species for high efficiency. Given the aforementioned limitations of conventional genetic transformation in microbes, recombinant DNA technology can benefit from gene transfer technology that is inexpensive, robust and facilitate transfer of genes with high efficiency.

Nanotechnology is predicted to be one of the key technologies of the 21st century having applications in diverse branches of science and technology including biotechnology.

2.2 NANOTECHNOLOGY

Nanotechnology is a novel, innovative, interdisciplinary scientific approach that involves designing, development and application of materials and devices at molecular level in nanometer scale (Ali *et al.*, 2014). Physicist, Nobel Laureate and father of nanotechnology, Richard Feynman defined nanotechnology as

"A biological system can be exceedingly small. Many of the cells are very tiny, but they are very active; they manufacture various substances; they walk around; they wiggle; and they do all kinds of marvelous things- all on a very small scale. Also they store information. Consider the possibility that we too can make a thing very small which does what we want – that we can manufacture an object that maneuvers at that level."

Richard Feyman, 1959.

2.2.1 Properties and Applications of Nanoparticles

Particles/globules having the size range of 1-100 nm are considered as nanoparticles. Compared to their bulk counterparts, nanoparticles exhibit different physical and chemical properties. Physical characteristics such as surface area, magnetic, mechanical, optical as well as chemical properties such as reactivity, thermal properties, and so forth many properties are found to change at the nanoscale (Pandiarajan *et al.*, 2017).

New properties of many of these newly developed nanomaterials are already used in different industries (Sambale *et al.* 2015). Nowack and Bucheli (2007) have highlighted the role of modified nanomaterials for improving many sectors of the economy, such as consumer products, pharmaceutical materials, cosmetic products, transportation, energy and agriculture.

Given the inherent nanoscale functions for the biological components of living cells, it was inevitable that nanotechnology would be applied to the life science and gave rise to the term "Nanobiotechnology".

2.2.2 Nanobiotechnology

Nanobiotechnology is the combination of engineering and molecular biology that is leading to a new class of multifunctional devices and systems for

biological and chemical analysis with better sensitivity, specificity and a higher rate of recognition (deMorais *et al.*, 2014).

Nanobiotechnology has widened its application in fields like pharmacy, medicine for detection of pathogens, antimicrobial agents, drug delivery, gene therapy, tissue engineering and genetic engineering for gene delivery (Singh *et al.*, 2015)

A new era of novel nanobiotechnology was successfully adopted as main or adjuvant technologies in genetic transformation and antimicrobial technology. Numerous recent reports are indicative of significant contributions of this novel technology in genetic transformation and antimicrobial activity (Nagamune *et al.*, 2017).

2.2.3 Gene Transformation Studies Using Nanoparticles

Nanoparticles-mediated gene transfer methods are new and have the potential to directly transfer DNA into the cells, achieving stable integration and rapid expression of the transgene. Nanoparticles like gold, mesoporous silver, starch and carbon nanotubes are being used as vehicles for gene delivery in gene therapy, animals and plant genetic engineering and to little extent in microbes (Cunnigham *et al* ., 2015).

Bozkir *et al.* (2004) reported that the efficiency of chitosan nanoparticle mediated transformation in *Escherichia coli* cells was significantly higher than naked DNA or poly-L-lysine (PLL)–DNA polycation complexes and a threefold increase in gene expression was produced by nanoparticles as compared to the same amount of naked plasmid DNA (pDNA) in transfection studies using COS-7 cells.

Karekar *et al.* (2008) reported that induction of competency in cells by gold nanotriangles and transformation using plasmid showed higher transformation efficiency as compared to that with competent cells transformed using only plasmid by approximately 15 - 17 folds.

Al-Jailawi *et al.* (2014) reported that transformation efficiency was increased 1.3 and 2.1 folds with TiO_2 (0.2 mg /ml) and ZnO (0.4 mg /ml) nanoparticles respectively and concluded that use of ZnO and TiO_2 nanoparticle solutions could be one of the factors that help to promote the competent cells by increasing their permeability through cell membrane. Chatterje and Sarkar (2014) synthesized glutathione-surface-functionalized gold nanoparticles, characterized them and applied them for the transfer of pUC 19 gene into non-competent *E. coli* DH5α and successful transformation was indicated by the growth of ampicilin resistant colonies in medium containing the antibiotic. The transformation efficiency of the method was evaluated and compared with a conventional method by checking biocompatibility of the synthesized nanoparticle on bacteria.

Kumari *et al.* (2017) reported that gold nanoparticles can be effectively used as gene delivery vehicles. Bacterial cells when treated with gold nanoparticles showed an increase in transformation efficiency and even gram positive bacteria were able to take up DNA due to ability of gold nanoparticles to readily cross the cell membrane barrier and pull the conjugated DNA also inside the cell without the need of heat shock.

Abyadeh *et al.* (2017) evaluated the transformation efficiency of chitosan nanoparticles by electrospray method and found that transformation efficiency increased with decreasing molecular weight, amount of nanoparticle/plasmid (N/P ratio) and nanoparticle size. Transformation efficiency of 1.7×10^8 CFU/μg plasmid was obtained with chitosan molecular weight (30kDa), N/P ratio (1) and nanoparticles size (125nm).

Ding *et al.* (2017) reported that nano Al_2O_3 could significantly promote plasmid-mediated antibiotic resistant gene transformation in Gram-negative *Escherichia coli*

strains and in Gram-positive *Staphylococcus aureus*. According to them under suitable conditions, 7.4×10^6 transformants of *E. coli* and 2.9×10^5 transformants of *S. aureus* were obtained from 100 ng of a PBR322 plasmid.

Wang *et al.* (2018) reported that bacterial exposure to ZnO nanoparticles (nZnO) at sublethal concentrations (1 to 10 mg l^{-1}) for 24 h significantly increased the conjugative frequency of antibiotic resistance plasmid RP4 by 24.3-fold in *E. coli* pure cultures, and an 8.3 fold increase occurred in a mixed culture of indigenous aquatic microbiota and in addition, nZnO increased by three fold the transformation efficiency of *E. coli* via the uptake of naked plasmid pGEX4T-1.

2.2.4 Antimicrobial Activity of Nanoparticles

Nanoparticles (NPs) are increasingly used as an alternative antibiotics, particularly advantageous in treating bacterial infections. Nanoparticles are used in antibacterial coatings for implantable devices and medicinal materials to prevent infection and promote wound healing, in antibiotic delivery systems to treat disease, in bacterial detection systems to generate microbial diagnostics, and in antibacterial vaccines to control bacterial infections (Wang *et al.*, 2017).

Different types of nanoparticles interact differently with bacterial cells, thereby influencing their growth, viability and physiology. It has been reported that biophysical interactions that occur between nanoparticles and bacteria include biosorption, nanoparticle breakdown or aggregation, and cellular uptake, with effects including membrane damage and toxicity (Brayner *et al.*, 2006).

Nanoparticles of made of calcium oxide, magnesium oxide, iron oxide, zinc oxide, titanium dioxide, gold and silver are used as antimicrobial agents. Currently, silver nanoparticles (AgNPs) are the most widely commercialized NPs that are used in various applications such as catalyst, jewellery, dentistry, photography, medicine, cosmetics, textile, and food package (Buzea *et al.*, 2007). Worldwide production of

AgNPs is over 400 tonnes per year and 30% of which are used in medical applications (Pourzahedi and Eckelman, 2014) due to their antimicrobial activities towards a broad spectrum of microorganisms (Li *et al.*, 2013).

AgNPs have been incorporated into numerous food products and medical applications (Kim and Ryu, 2013) where they have been used for preventing fungal growth in fruits, surface coatings for various textiles and medical implants, treatment of wounds and burns, application in water disinfectants and food containers (Chen and Schluesener, 2008).

2.2.4.1 Toxicity of Silver Nanoparticles

Belluco *et al.* (2016) reported that the amount of silver ions released increased over time and they found that both ionic and nanoform of silver are having antibacterial activity against *Listeria monocytogenes*, a serious food borne pathogen.

Buszewski *et al.* (2018) reported that nanoparticles produced by a *Streptacidiphilus durhamensis* strain ranged in size from 8 nm to 48 nm and showed highest antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus mirabilis*, followed by *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus subtilis*.

Dash *et al.* (2012) reported that exposure of increasing concentrations of silver nanoparticles to algal thalli resulted in progressive depletion in algal chlorophyll content, chromosome instability and mitotic disturbance, associated with morphological malformations in algal filaments. SEM micrographs revealed dramatic alterations in cell wall in nanoparticle-treated algae, characterized with cell wall rupture and degradation in *Pithophora*.

Navarro *et al.* (2008) reported that increasing concentrations of AgNPs reduced the algal (*Chlamydomonas reinhardtii*) photosynthetic yield and the toxicity of AgNPs was found time-dependent.

Li *et al.* (2013) reported that the Gram-positive bacteria *S. aureus* and *B. subtilis* were more sensitive to AgNPs than Gram negative bacterium *E. coli* and they found that 5nm size of silver nanoparticles had more antibacterial activity due to small size and large surface area and hence enhanced the membrane leakage. AgNPs attach to the cell membrane and penetrate inside the bacteria and destroys the structure and permeability bacterial membranes.

Nalwade and Jadhav (2013) reported that biosynthesized silver nanoparticles from the leaves of *Datura albanees* have antibacterial activity against *Chlostridium diptheriae* and they found that silver nanoparticles release ions into the nutrient media and they attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death.

Elechiguerra *et al.* (2005) investigated the interaction between Ag-NPs and HIV-1and reported that Ag-NPs undergo a size-dependent interaction, with NPs in the range of 1–10 nm attached to the virus. It was also suggested that Ag-NPs interact with the HIV-1 virus via preferential binding to the exposed sulfur-bearing residues of the gp120 glycoprotein knobs, resulting in the inhibition of the virus from binding to host cells.

Ivask *et al.* (2014) reported the toxicity of silver nanoparticles increased with decreasing particle size of 20–80 nm due to release of Ag ions and biological effects of silver NPs indicated the crustacean are the most sensitive organism to silver NPs, followed by algae, *E. coli* and *Pseudomonas fluorescens*, yeast *Saccharomyces cerevisiae*, and finally mammalian fibroblasts *in vitro*.

Lu *et al.* (2013) studied the antibacterial activity of Ag Nps with different sizes (5, 15 and 55 nm) against five anaerobic oral pathogenic bacteria such as *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis*, *Actinomycete mcomitans*, *Fusobacterium. nuceatum* and aerobic bacteria *Escherichia coli* and found that the antibacterial effect was less in anaerobic bacteria than that of aerobic bacteria and different sizes of Ag NPs exhibited different antibacterial activity against bacteria i.e. 5nm Ag NPs presented the best antibacterial activity by completely inhibiting the growth at 25 µg/mL.

According to Kim *et al.* (2011) the cytotoxic effect of silver nanoparticles on MC3T3-E1 and PC12 cell lines was size and dose dependent. AgNPs of 10nm was more toxic compared to 50nm and 100nm.

Gliga *et al.* (2014) reported that among the different sized citrate coated AgNPs (10, 40 and 75 nm) studied, particles of 10 nm size were more cytotoxic for human lung cells. The toxicity was associated with the rate of intracellular Ag release and all AgNPs tested caused an increase in the overall DNA damage after 24 h.

Mazumder *et al.* (2011) reported that the accumulation of nanoparticles and their toxic effects on rice plant depends upon the concentration and exposure time. Nanoparticles are reported to penetrate inside root cells by damaging cell wall and causing adverse effect on external and internal portions of cell.

Haase *et al.* (2010) reported that even low dose of silver nanoparticles exerted adverse toxicity against human macrophages isolated from human monocytic leukemia cell line THP-1 when exposed for 24h to 48 h with increasing concentrations of silver nanoparticles of size 20 and 40 nm. Both types of AgNPs displayed strong cytotoxicity, depending on dose and time of treatment. The AgNPs of 20nm were more toxic compared to the AgNPs of 40nm. The inhibitory concentration 50 (IC50) values after 24 h of exposure was 110µg/ml and 140 µg/ml

for 20nm and 40nm respectively. After 48 h of exposure the IC (50) values were 18µg/ml for 20 nm and 30µg/ml for 40nm.

Krishanan *et al.* (2015) reported that when bacterial growth at different concentrations of AgNPs was assessed after 24 hours, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *E. faecalis* were observed at concentration of 5 mg/L, and 10 mg/L respectively, indicating that they have both bacteriostatic and bactericidal activity.

Park *et al.* (2011) reported the induced effect of different sizes (20, 80, 113 nm) of silver nanoparticles on cellular metabolic activity and membrane damage using *in vitro* assay. They found that silver nanoparticles of 20 nm were more toxic than the larger nanoparticles (80 and 113nm). Agnihotri *et al.* (2014) reported that TEM analysis of AgNPs treated bacterial cells showed the presence of AgNPs on the cell membrane and internalized within the cells. Cvjetko *et al.* (2017) reported that the toxicity of AgNPs is directly correlated with their size, overall surface charge and surface coating.

2. 2. 4.2 Mode of Action of Silver Nanoparticles on Microbes

Silver nanoparticles are being used as antimicrobial agents. The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known and is a debated topic. There are however various theories on the action of silver nanoparticles on microbes to cause the microbicidal effect.

The most critical physical and chemical parameters that affect the antimicrobial potential of AgNPs include size, shape, surface charge, concentration and colloidal state. AgNPs exhibits their antimicrobial potential through multifaceted mechanisms

such as adhesion and penetration to microbial cells, ROS and free radical generation and modulation of microbial signal transduction pathways (Dakal *et* al., 2016).

Morones *et al.* (2005) reported that the pronounced antibacterial activity of AgNPs is due to their large surface area to volume ratio providing better contact with the microorganisms and to their interaction with the functional groups on the microbial cell surface.

Danilczuk *et al.* (2006) reported the formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death.

Feng *et al.* (2000) proposed the release of silver ions by the nanoparticles. These ions can interact with the thiol groups of many vital enzymes and make them inactive. The bacterial cells in contact with silver, take in silver ions which inhibit several functions in the cell and damage the cells. Then, there is the generation of reactive oxygen species, which are produced possibly through the inhibition of a respiratory enzyme by silver ions that attack the cell.

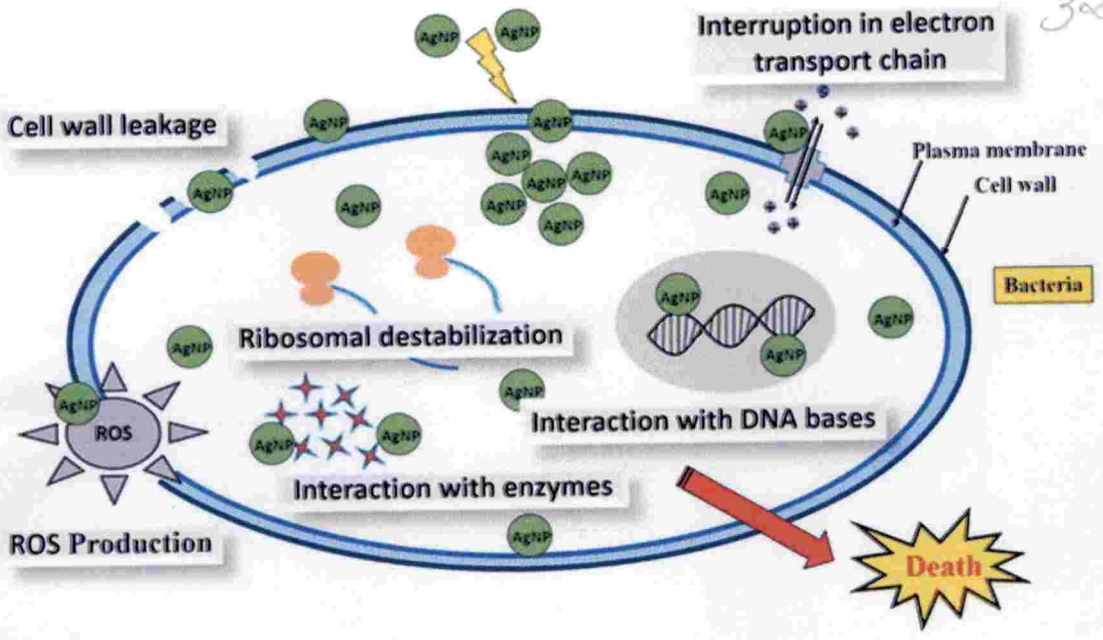

Fig.2 Mode of action of silver nanoparticles on microbes (Dhakal *et al.*, 2016)

Prabhu and Poulose (2012) reported the interaction of the silver nanoparticles with phosphorus in the DNA lead to problems in the DNA replication of the bacteria and thus terminated the microbes.

Studies of Hwang *et al.* (2008) using high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) described the morphological, structural, and physiological changes that occur when silver nanoparticles are attached to the surface of the cell membranes of bacteria. The nanoparticles penetrated inside the cell and generated Ag^{+} ions that are partially responsible for the biocidal effects.

Raffi *et al.* (2008) reported that opposite charges of bacteria and nanoparticles are attributed to their adhesion and electrostatic forces play a role in the adhesion of silver particle to the bacterial cell wall surface.

Yan *et al.* (2018) reported that the silver nanoparticles interacts with cell membrane, regulates protein expression (27 up-regulated and 32 down-regulated proteins) and generates intracellular reactive oxygen species (ROS) by releasing silver ions.

Although there are reports on the use of gold, iron and silica nanoparticles in bacterial transformation, there are no reports on the use of silver nanoparticles in bacterial transformation. So, the present study was undertaken to study the efficiency of silver nanoparticles in bacterial transformation.

Material and methods

3. MATERIALS AND METHODS

The study entitled "Efficacy of silver nanoparticles as delivery system in genetic transformation" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016 to 2018. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 TOXICITY ASSAY OF SILVER NANOPARTICLES ON *E.COLI* CELLS.

Escherichia coli (*E.coli*) strain DH5α in the exponential phase of its growth was exposed to different concentrations of silver nanoparticles of 100 nm particle size and the colony forming ability of the treated cells was checked using the protocol of Ivask *et al.* (2014). The half maximal effective concentration (EC_{50}) value was estimated by using untreated bacterial cells as treatment control.

3.1.1 Culture Medium

3.1.1.1 Chemicals

All the chemicals used for the preparation of the culture medium were of analytical grade and procured from Sisco Research laboratories (SRL), India. The antibiotics were purchased from Himedia Laboratories, India. Silver nanoparticles of 100 nm particles size and citrate stabilized of stock concentration 20 mg L^{-1} were bought from Sigma Aldrich chemicals. They were diluted into different concentrations by using sterile autoclaved water for further studies.

3.1.1.2. Composition and preparation of medium

Luria Bertani (LB) broth and LB agar were used in microbial experiment (Appendix I). Both LB broth and LB agar were prepared as per the manufactures instructions. Media were sterilized by autoclaved at a pressure of 1.06 Kg cm $^{-2}$ and temperature of 121°C for 20 min. The medium was then stored at 25±2°C.

3.1.3 Growth Curve Analysis of *E.coli* DH5α

Single colony of the *E. coli* was inoculated into 10 ml LB broth and kept at 37 °C in rotary shaker of 120 rpm and incubated overnight. Next day optical density (OD) at 600 nm of the culture was measured using spectrophotometer and value was adjusted to 0.05 by adding sterile LB broth and was dispensed into test tubes. These diluted cultures were used for estimating growth kinetics by measuring OD_{600} value at every 30 minutes for 13h. OD value was used to plot the growth curve of the organism. (Absorbance verses Time).

3.1.4 Correlation between Optical density and Bacterial Population

An overnight culture of *E.coli* DH5α was serially diluted using sterile fresh LB broth to 10^{-8}. Optical density of different dilutions was measured by spectrophotometer at 600nm. Serially diluted bacterial cultures were plated on plain LB agar plated and incubated at 37°C for overnight. The colony forming units (population) were counted by counting number of colonies on plate per mL and regression analysis was carried to establish relationship between population and optical density.

3.1.5 EC_{50} value for Silver Nanoparticles on *E coli DH5α*

E coli strain DH5α in the exponential phase of their growth was exposed to different concentrations of silver nanoparticles (Table 1) and the colony forming ability of the treated cells was checked to find EC_{50}. Overnight culture of *E. coli* was added to fresh LB broth in 1:50 ratio and cultured till exponential phase and population of bacteria was adjusted to 3×10^7 cfu/mL. Bacterial cells were pelleted out

at 5000 rpm for 5 minutes and 200 µl of sterile autoclaved water was added to cells. Bacterial suspension (200µl) was mixed with 200 µl each of different concentrations of silver nanoparticles in microcentrifuge tubes and incubated for 4h at 37°C. After incubation serial dilutions of the cultures were made and 20µl was used to spread on LB agar plates. Colony forming units were counted from different treatments. EC 50 value was estimated by using the statistical package SPSS.

Table1. Concentrations of silver nanoparticles used for toxicity study

Treatment	Silver nanoparticles (mg L^{-1})
T1	Control
T2	0.01
T3	0.1
T4	1
T5	5
T6	10
T7	15
T8	20

3.2 INDUCTION OF COMPETENCY IN BACTERIAL CELLS

E. coli (strain DH5α) cells in the exponential phase were treated with different concentrations of silver nanoparticles alone or in combination of both silver nanoparticles and calcium chloride and calcium chloride alone (Table 2). The range of concentrations of silver nanoparticles which caused minimal toxicity were used for treating bacterial cells. Competent *E. coli* cells prepared using $CaCl_2$ following the standard protocol (Sambrook and Russell, 2001) was used for ascertaining treatment efficacy. Bacterial cells without any treatment with either calcium chloride or silver nanoparticles were used as negative control.

3.2.1 Induction of Competency using Calcium Chloride

Competent cells of *E.coli* DH5α were prepared by calcium chloride ($CaCl_2$) method (Maniatis *et al.* 1989). The *E. coli* cells were grown in 10 ml of the Luria Bertani (LB) broth (Maniatis *et al.*, 1982) (Appendix) overnight at 37°C on rotary incubator at 120 rpm. Overnight grown culture (500 µL) was transferred to 50 ml of fresh LB broth and incubated at 37°C until the OD value reached 0.2-0.3 at 600 nm. Then the culture was transferred to fresh 2ml tubes and kept on ice. The cells were pelleted at 5000g for 5 minutes at 5-10°C. The supernatant was decanted off. The pellet was resuspended in 1mL of 0.1M $CaCl_2$ solution and pelleted at 5000g for 5minutes. Further 1mL of 0.1M $CaCl_2$ solution was added and incubated on ice for 30 minutes. The cells were pelleted at 5000g for 10 minutes at 5-10°C. The supernatant was discarded. The pellet was resuspended in 200 µl of 0.1 M $CaCl_2$ and stored in ice prior to use.

3.2.2 Induction of Competency using Silver Nanoparticles

E.coli DH5α was grown in10 mL LB broth (Maniatis *et al.*, 1982) (Appendix I) overnight at 37 °C on rotary incubator at 120 rpm. Overnight grown culture (500 µL) was transferred to 50 ml of fresh LB broth and incubated at 37 °C until the OD value reached 0.2- 0.3 at 600 nm. Then the culture was transferred to fresh 2ml tubes. The cells were pelleted at 5000 g for 5 min at 5-10°C. The supernatant was decanted off and bacterial cells were resuspended in 200µL of sterile autoclaved water. Bacterial cells were mixed with 10µL each of different concentrations of silver nanoparticles in the range of concentrations which caused minimal toxicity and incubated for 30 min, 60 min and 120 min in a rotary shaker at 37 °C and 180 rpm.

Table: 2 Different treatments for competency induction in *E.coli* DH5α cell

Sl. No.	Treatment
1	0.01 mg L^{-1} silver nanoparticles (30min)
2	0.01 mg L^{-1} silver nanoparticles (60min)
3	0.01 mg L^{-1} silver nanoparticles (120 min)
4	0.1 mg L^{-1} silver nanoparticles (30 min)
5	0.1 mg L^{-1} silver nanoparticles (60 min)
6	0.1 mg L^{-1} silver nanoparticles (120 min)
7	1 mg L^{-1} silver nanoparticles (30min)
8	1 mg L^{-1} silver nanoparticles (60min)
9	1mg L^{-1} silver nanoparticles (120min)
10	2 mg L^{-1} silver nanoparticles (30min)
11	2 mg L^{-1} silver nanoparticles (60min)
12	2 mg L^{-1} silver nanoparticles (120min)
13	4 mg L^{-1} silver nanoparticles (30min)
14	4 mg L^{-1} silver nanoparticles (60min)
15	4 mg L^{-1} silver nanoparticles (120min)
16	0.01 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$
17	0.1 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$
18	1mg L^{-1} silver nanoparticles+ 0.1 M $CaCl_2$
19	2 mg L^{-1} silver nanoparticle+0.1 M $CaCl_2$
20	4 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$
21	0.1 M $CaCl_2$
22	Control

3.2.3 Induction of Competency using Combination of Calcium Chloride and Silver Nanoparticles

E.coli DH5α were grown in10 mL LB broth (Maniatis *et al.*, 1982) (Appendix I) overnight at 37°C on rotary incubator at 120 rpm. Overnight grown culture (500 µL) was transferred to 50 mL of fresh LB broth and incubated at 37°C until the O.D. value reached 0.2- 0.3 at 600 nm. Then the culture was transferred to fresh 2ml tubes and stored in ice. The cells were pelleted at 5000 g for 5 minutes at 5-10°C. The supernatant was decanted off. The pellet was resuspended in 1mL of 0.1M $CaCl_2$ solution and again pelleted at 5000g for 5minutes. Further 1mL of 0.1M $CaCl_2$ solution was added and incubated on ice for 30 minutes. The cells were pelleted at 5000g for 10 minutes at 5-10 °C. The supernatant was discarded. The pellet was resuspended in 200 µl of 0.1 M $CaCl_2$ and stored in ice prior to use. Silver nanoparticles (10 µl) in the range of concentrations which caused minimal toxicity were added to the competent cells prepared by the above method prior to transformation.

3.3. ISOLATION OF PLASMID DNA FROM HOST

E.coli DH5α cells harbouring vectors pUC18 and pBR322 and *Agrobacterium* cells harbouring pCAMBIA were inoculated in LB media supplemented with appropriate antibiotics. Antibiotics used were ampicilin (60mg/L) for pUC18, ampicilin (60mg/L) and tetracycline (20mg/L) for pBR322 and kanamycin (50mg/L) for pCAMBIA. Cultures were incubated overnight at 37°C with shaking. *Agrobacterium* transformed with pCAMBIA were incubated at 28°C. Plasmid was isolated by following the alkaline lysis method (Birnboim *et al.*, 1979). Bacterial culture (2ml) was taken in microcentrifuge tubes and the cells were pelleted at 5000g for 5 minutes at 4°C. The cells were pelleted and supernatant was discarded. The bacterial pellet was resuspended completely in 200µl of GET buffer (Appendix) by vortexing and incubated in ice bath for 5 minutes. 300µl of lysis buffer (Appendix) was added,

mixed gently by inverting and kept on ice for 2-3 minutes. To this 300µl of neutralizing solution was added and mixed by inverting and kept in ice for 5 minutes. The white precipitate formed was centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and 200µL of chloroform was added and mixed by inverting. The mixture was centrifuged at 12,000g for 5 minutes at 4°C. This step was repeated once again. The supernatant was transferred to a fresh tube and an equal volume of chilled isopropanol was added, mixed by inverting and incubated at -20°C for 20 minutes. Pellet was obtained by centrifuging at 10,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed in 500µL of 70 per cent ethanol. After centrifugation at 8000g for 5 minutes at room temperature, the supernatant was discarded and the pellet was thoroughly dried and dissolved in 50µl Tris EDTA (TE) buffer (pH 8.0) or sterile water (Appendix IV). The plasmid was quantified and stored at -20°C for further usage.

3.3.1 Quantification of Plasmid DNA

Quantification of DNA was carried out using UV-visible spectrophotometer (Spectronic Genesys 5). The optical density of the DNA samples was recorded at both 260 and 280 nm. The concentration of DNA was calculated using the following formula:

Amount of DNA (µg/mL) = A_{260}x 50 x dilution factor

Where A_{260}= absorbance at 260 nm

The quality of the DNA was judged from A_{260}/A_{280} value. A_{260}/A_{280} values between 1.8 and 2 indicate high quality of DNA.

3.4 TRANSFORMATION OF COMPETENT CELL

3.4.1 Transformation of Competent Cells Prepared by using Calcium Chloride

Competent cells prepared using calcium chloride were transformed using vectors pUC18, pBR322 and pCAMBIA. To 100 µl of the competent cells, 1 µl (1µg) of the plasmid DNA was added and mixed gently. The tubes were kept on ice for 30 minutes and then transferred to a water bath and incubated at 42°C for 2 minutes. After incubation the tubes were quickly plunged in ice bath and chilled for 5 minutes. LB broth (1 mL) was added to the tube and incubated at 37°C for 1 hr with gentle shaking. The cells were plated on LB agar with ampicilin 60 mg L^{-1}, X-GAL (30 mg L^{-1}) and IPTG (24 mg L^{-1}) when pUC 18 vector was used. Ampicilin (60 mg L^{-1}) and tetracycline (20 mg L^{-1}) were used for pBR322 vector and for kanamycin (50 mg L^{-1}) when pCAMBIA vector was used.

3.4.2Transformation of Competent Cells Prepared by using Silver Nanoparticles

To100µL of competent cells prepared by silver nanoparticles, 1µL (1µg) plasmid DNA of pUC18 vector was added and shaken gently. To the cells 1mLof LB broth was added and incubated at 37°C for 1hour with gentle shaking. The cells were plated on LB agar with ampicillin (60 mg L^{-1}), X-GAL (30 mg L^{-1}) and IPTG (24 mg L^{-1}). The best treatment from the competent which gave highest transformation efficiency was transformed with pBR322 and pCAMBIA vectors. The transformed colonies were selected on LB agar media containing ampicillin (60 mg L^{-1}) and tetracycline for pBR322 (20 mg L^{-1}) and kanamycin (50 mg L^{-1}) for pCAMBIA.

3.4.3 Transformation of Competent Cells Prepared by using Silver Nanoparticles and Calcium Chloride

Competent cells prepared using combination of silver nanoparticles and calcium chloride were transformed using vector pUC18. To 100 µl of the competent cells, 1 µl (1µg) of the plasmid DNA was added and mixed gently. The tubes were kept on ice

for 30 minutes and then transferred to a water bath and incubated at 42°C for 2 minutes. After incubation the tubes were quickly plunged in ice bath and chilled for 5 minutes. LB broth (1 mL) was added to the tube and incubated at 37°C for 1 hr with gentle shaking. The cells were plated on LB agar with ampicilin 60 mg L^{-1}, X-GAL (30 mg L^{-1}) and IPTG (24 mg L^{-1}).

3.5 CHECKING FOR TRANSFORMATION EFFICIENCY

Efficiency of transformation with pUC 18 vector was checked by culturing the transformed cells on selective medium and by blue white screening following the standard protocol (Sambrook and Russell, 2001). For confirmation of transformation, plasmid was isolated from the colonies grown in the selection medium, by alkaline lysis method (Birnboim *et al.*, 1979) and subjected to electrophoresis in 1% agarose gel. Transformation efficiency with pBR322 and pCAMBIA was estimated by counting the number of transformed colonies in appropriate selective antibiotic media.

3.6 COMPARISON OF TRANSFORMATION EFFICIENCY

Transformation efficiency of different treatments was compared by estimating the number of transformed cells per unit weight of the vector used. Transformation efficiency is defined as the number of transformant colony forming units (cfu) obtained per µg of plasmid DNA and is calculated as follows.

$$\text{Transformant cfu} = \frac{[\text{Number of bacterial colonies} \times \text{dilution ratio} \times \text{Original transformation volume}]}{\text{Plated volume}}$$

Transformation efficiency = Transformant cfu / Plasmid DNA (µg)

Results

4. RESULTS

The results of the present study on "Efficacy of silver nanoparticles as delivery system in genetic transformation'' was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016-2018 are presented below.

4.1 TOXICITY ASSAY OF SILVER NANOPARTICLES ON *E.COLI* CELLS

4.1.1 Growth Kinetics of *E.coli* strain DH5α

E.coli DH5α growth curve analysis identified all the phases of bacterial growth *viz.* lag phase, log phase, stationery phase and death phase. Lag phase was observed for the initial 2h followed by log phase for nearly 6 h and then stationary phase for 4h and finally a decline in the bacterial population was noted.

4.1.2 Correlation between Bacterial Population and Optical Density

The absorbance was measured at 600 nm for different dilutions of *E.coli* DH5α bacterial cultures. At 10^{-1}, 10^{-2} and 10^{-3} dilutions only smear of bacterial colonies were observed. From 10^{-4} dilutions to till 10^{-8} dilutions countable colonies were observed. Regression analysis was performed for number of colonies as X-axis and optical density (OD600) on Y-axis (Table 4). From regression analysis r^2 was observed as 0.85.

4.1.3 EC_{50} value of silver nanoparticles for *E coli* DH5α Cells

There was a considerable variation in the response of *E.coli* cells to silver nanoparticles in the different treatments tried (Table1). Among the treatments, the highest inhibition of growth (100% death of bacterial cells) was obtained in treatments T_6, T_7, and T8 where *E.coli* cells were treated with 10 mg L^{-1}, 15 mg L^{-1} and 20 mg L^{-1} of silver nanoparticles respectively. Inhibition of growth was minimum in treatment T_2 where *E.coli* cells were treated with 0.01mg L^{-1} of silver

nanoparticles. In treatment T_5 with 5mg L^{-1} of silver nanoparticles, approximately 50% of the population of *E. coli* cells was inhibited (Table 5).

4. 2 Induction of competency in bacterial cells

Competency of cells prepared by different treatments (Table1) was assessed by checking transformation efficiency (Table 7).

4.3. Isolation of plasmid DNA

Plasmid isolated from *E.coli* cells having pUC18 and pBR322 and from *Agrobacterium* cells having pCAMBIA were used for transformation. The quantity and quality of plasmid was checked by measuring absorbance at 260nm and 280nm (Table 4).

Table: 6 Quantity of isolated plasmid

Sl.no	Vector	A_{260}	A_{280}	A_{260}/A_{280}	Quantity (µg/mL)
1	pUC18	0.1485	0.1226	1.2112	4462.42
2.	pBR322	0.0934	0.0890	1.049	2802.00
3.	pCAMBIA	0.0628	0.0523	1.2007	1887.13

Plasmids isolated from cells were subjected to agarose gel electrophoresis. The gel analysis showed three bands in each of the plasmids isolated (Plate 2).

Table: 3 Optical density at 600 nm of *E. coli* DH5α at different time periods

Time period (minutes)	OD at 600 nm
0	0.05
30	0.11
60	0.23
90	0.36
120	0.55
150	0.80
180	1.04
210	1.16
240	1.37
270	1.48
300	1.56
330	1.65
400	1.69
430	1.69
470	1.71
500	1.72
530	1.73
600	1.73
630	1.7
700	1.65
730	1.60
800	1.50
830	1.45

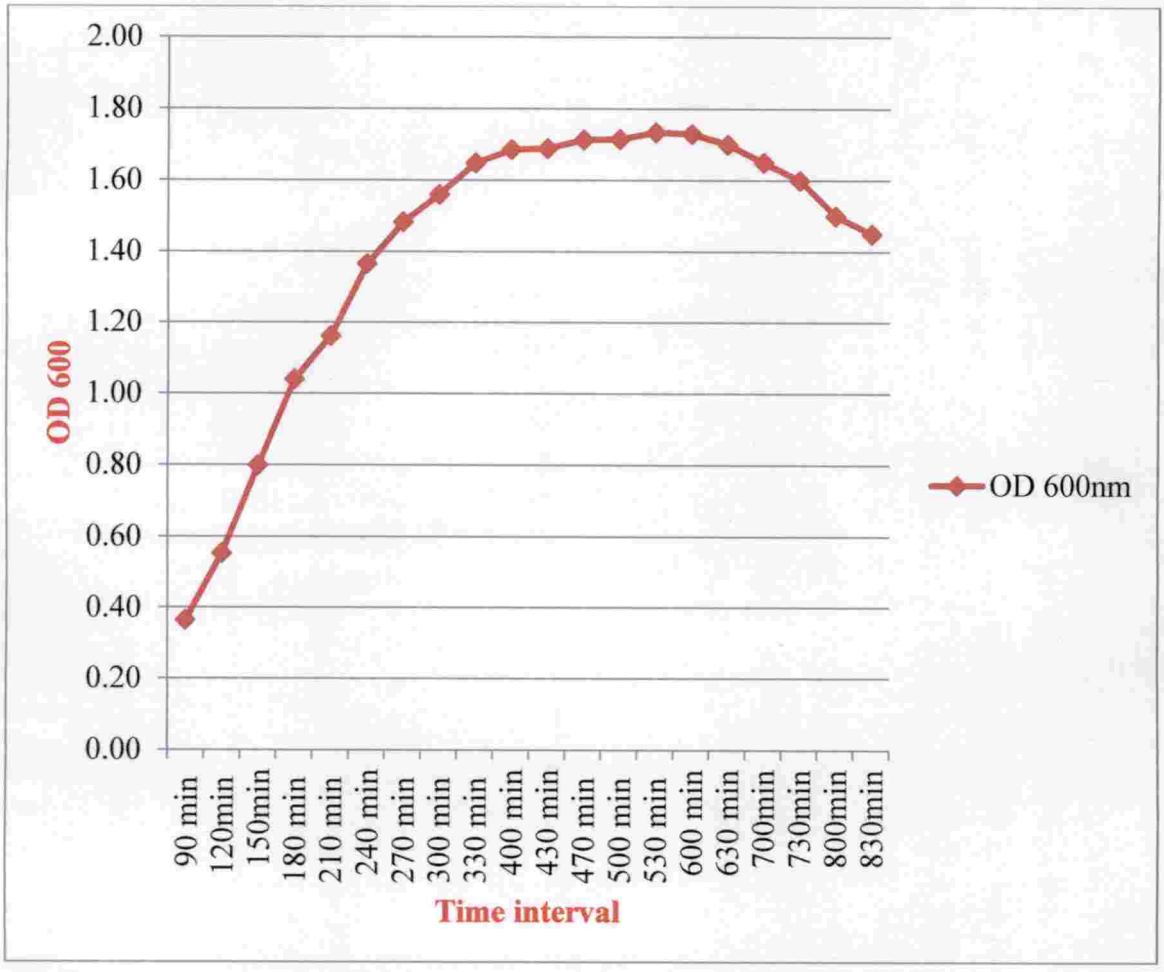

Fig3. Growth Kinetics of *E.coli* DH5α

50

Table 4 Correlation between bacterial population and optical density

Sl.No	Dilution	Absorbance (OD_{600})	Population (cfu/mL)
1	10^{0}	2.118	Smear
2	10^{-1}	0.527	Smear
3	10^{-2}	0.115	Smear
4	10^{-3}	0.063	Smear
5	10^{-4}	0.065	$3.5x\ 10^{7}$
6	10^{-5}	0.063	$3x10^{7}$
7	10^{-6}	0.061	$2x10^{7}$
8	10^{-7}	0.061	$1x10^{7}$
9	10^{-8}	0.056	$0.3x10^{7}$

The regression analysis showed r^2 value as 0.85. There was 85% correlation between absorbance and optical density. The above data was used for adjusting the population density in toxicity studies

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Table: 5 Dose response of *E.coli* DH5α against silver nanoparticles

Concentration of AgNPs(mg L^{-1})	Initial population (cfu/mL)	Final population (cfu/mL)	Inhibition (%)
0	$3x10^7$	$3x10^7$	0
0.01	$3x10^7$	$3x10^7$	0
0.1	$3x10^7$	$2.6x10^7$	13.4
1	$3x10^7$	$2.5x10^7$	16.6
5	$3x10^7$	$1.5x10^7$	50
10	$3x10^7$	0	100
15	$3x10^7$	0	100
20	$3x10^7$	0	100

On plotting the graph of inhibition *vs.* concentration of silver nanoparticles and carrying out the probability analysis using the statistical package SPSS the EC 50 was estimated at 4.49 mg/L.

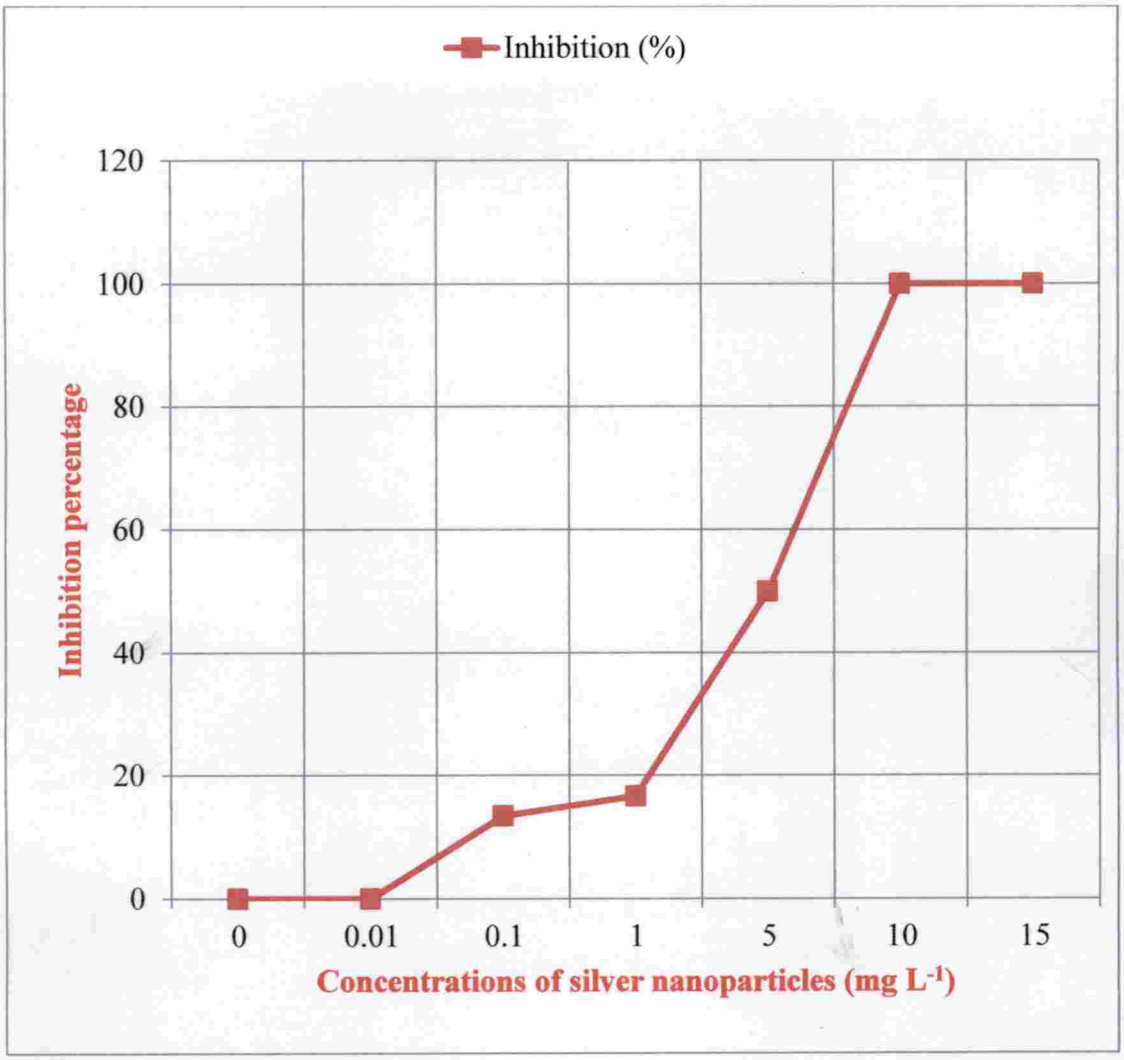
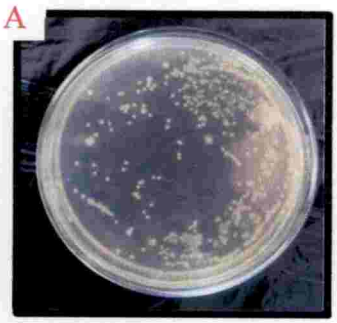
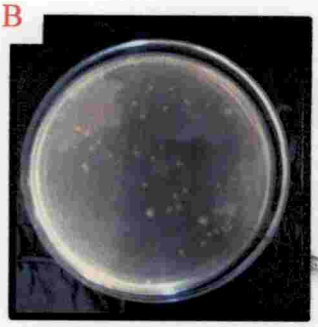


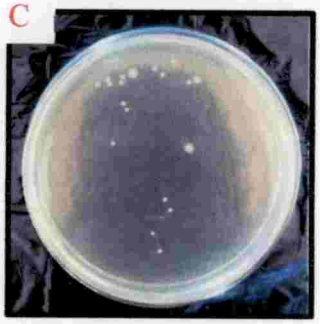
Fig.4 Dose response curve of *E.coli* DH5α against silvernanoparticles



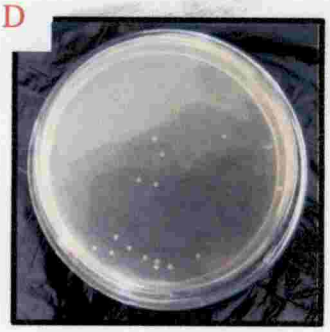
0.01mg L^{-1} silvernanoparticles



0.1mg L^{-1} silver nanoparticles

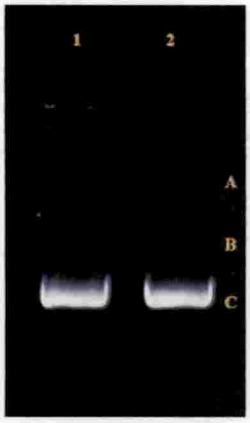


1mg L^{-1} silvernanoparticles



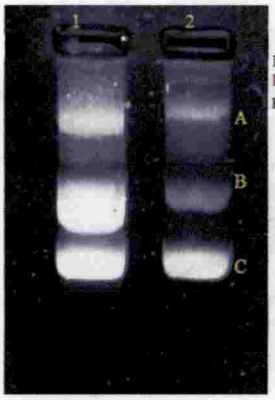
5 mg L^{-1} silver nanoparticles

Plate 1 Dose response of *E.coli* DH5α against different concentrations of silver nanoparticles



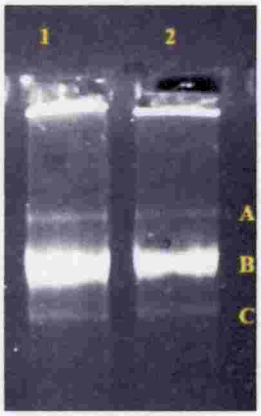
Lane 1and 2
Replications of
pUC18

pUC18



Lane 1 and 2
Replications of
pBR322

pBR 322



Lane 1 and 2

Replications of
pCAMBIA

pCAMBIA

A. Linear

B. Super coiled

C. Circular, Single stranded

Plate 2 Agarose gel profile of plasmid vectors isolated for transformation

4.4 TRANSFORMATION OF COMPETENT CELL

4. 4.1 Transformation Efficiency of Competent Cells Transformed using pUC18

Transformation efficiency was checked by counting the number of transformed colonies grown per weight of vector DNA used. Transformation efficiency estimated showed wide variation in the different treatments. Transformation efficiency was higher in all the treatments with silver nanoparticles compared to calcium chloride alone. Maximum transformation efficiency was observed when cells were treated with 1mg L^{-1} of silver nanoparticles. Average efficiency values observed for different treatment time period of 30, 60 and 120 min using 1mg L^{-1} silver nanoparticles were $6.03x10^4$, $8.3x10^4$, and $4.5\ x10^4$ cfu/ng of vector DNA respectively. Silver nanoparticles at a concentration of 0.01 mg L^{-1} for 30 min, 60 min, and 120 min yielded a transformation efficiency of $4x10^3$, $4.5x10^3$, $3x10^3$cfu/ng of DNA respectively. Silver nanoparticles at a concentration of 4 mg L^{-1} at different time periods of 30min 60min and 120min showed a transformation efficiency of $1x10^4$, $1.5x10^4$, $1x10^4$ cfu/ng of DNA respectively. Silver nanoparticles in combination with 0.1M calcium chloride increased the transformation efficiency compared to 0.1 M calcium chloride alone. However, it was lesser compared to treatment with silver nanoparticles alone. Transformation efficiency of $2.6x10^3$, $1.65x10^4$, $2.55x10^4$, $8x10^3$ and $7.01x10^3$ cfu/ng of vector DNA were obtained with respect to different concentration of silvernanoparticles *viz*., 0.01, 0.1, 1, 2 and 4 mg L^{-1} when used in combination with 0.1M calcium chloride.

The minimum transformation efficiency ($2.3x10^3$ cfu/ng of DNA) was observed when 0.1M calcium chloride alone was used for competency induction. No colonies were observed in the control. ANOVA single factor analysis of the data also showed significant difference between all the different treatments studied. Treatment with 1mg L^{-1} silver nanoparticles showed significantly higher transformation efficiency compared to all other treatments. Economic analysis also showed

feasibility of use of silver nanoparticles in genetic transformation. Silver nanoparticle treated *E. coli* cells on transformation with pUC18 did not yield blue colonies on LB plates supplemented with ampicilin, X-Gal and IPTG as expected. *E. coli* cells treated with both silver nano and calcium chloride produced a mixture of both blue and white colonies whereas competent cells prepared by treating with 0.1 M calcium chloride yielded blue colonies (Plate 3). For confirmation of transformation plasmid were isolated from the white colonies grown in the selection medium, by alkaline lysis method (Birnboim *et al.*, 1979) and subjected to electrophoresis in 1% agarose gel (Plate 4).

4.4.2 Transformation of Competent Cells using pBR322 vector

The best treatment (1mg L^{-1} silver nanoparticles for 60 min) which yielded maximum transformation efficiency was employed for transforming *E. coli* with two other vectors viz., pBR322 and pCAMBIA. *E. coli* cells treated with silver nanoparticles (1mg L^{-1} silver nanoparticles for 60 min) and transformed using pBR322 did not form colonies on LB agar plates supplemented with ampicillin (60 mg L^{-1}) and tetracycline (20 mg L^{-1}) (Plate 5) and tetracycline alone (Plate 6) but colonies were formed when ampicillin (60 mg L^{-1}) alone was used (Plate 7). Efficiency of transformation on ampicillin supplemented plates was $8x10^4$ cfu/ng of vector DNA. Control cells treated with 0.1M calcium chloride yielded a transformation efficiency of $2.3x10^3$cfu/ng of DNA.

4.4.3 Transformation of Competent Cells using pCAMBIA vector

Silver nanoparticle treated (1mg L^{-1} for 60 min) *E. coli* cells when transformed with pCAMBIA vector showed a higher transformation efficiency of 7.9 $x10^4$ cfu/ng of DNA compared to the traditional method (0.1 M calcium chloride and heat shock) which yielded an efficiency of $2.3x10^3$ cfu/ng of DNA when plated on kanamycin supplemented LB agar plates (Plate 8).

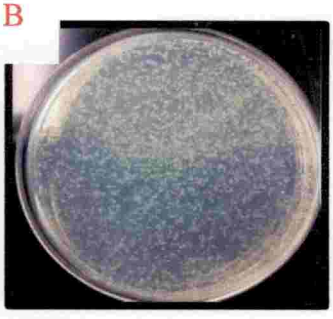
Table: 6 Transformation efficiency of competent cells induced by different treatments

Treatment	Transformation efficiency (cfu/ ng of DNA)
0.01 mg L^{-1} silver nanoparticles (30min)	$1.4x10^3$
0.01 mg L^{-1} silver nanoparticles (60min)	$4.5 x10^3$
0.01 mg L^{-1} silver nanoparticles (120 min)	$3.0x10^3$
0.1 mg L^{-1} silver nanoparticles (30 min)	$1.5x10^4$
0.1 mg L^{-1} silver nanoparticles (60 min)	$1.7x10^4$
0.1 mg L^{-1} silver nanoparticles (120 min)	$1.3x10^4$
1 mg L^{-1} silver nanoparticles (30min)	$6.03x10^4$
1 mg L^{-1} silver nanoparticles (60min)	$8.3x10^4$
1mg L^{-1} silver nanoparticles (120min)	$4.5 x10^4$
2 mg L^{-1} silver nanoparticles (30min)	$2x10^4$
2 mg L^{-1} silver nanoparticles (60min)	$2.5x10^4$
2 mg L^{-1} silver nanoparticles (120min)	$2x10^4$
4 mg L^{-1} silver nanoparticles (30min)	$1x10^4$
4 mg L^{-1} silver nanoparticles (60min)	$1.5x10^4$
4 mg L^{-1} silver nanoparticles (120min)	$1x10^4$
0.01 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$	$2.6x10^3$
0.1 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$	$1.65x10^4$
1mg L^{-1} silver nanoparticles+ 0.1 M $CaCl_2$	$2.55x10^4$
2 mg L^{-1} silver nanoparticle+0.1 M $CaCl_2$	$8x10^3$
4 mg L^{-1} silver nanoparticles+0.1 M $CaCl_2$	$7.01x10^3$
0.1 M $CaCl_2$	$2.3 x10^3$
Control	0

A

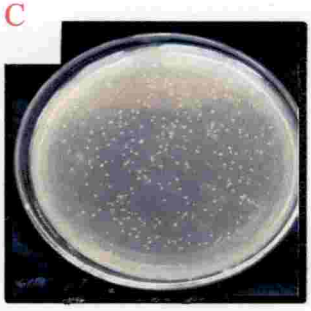
0.1M Calcium chloride

B



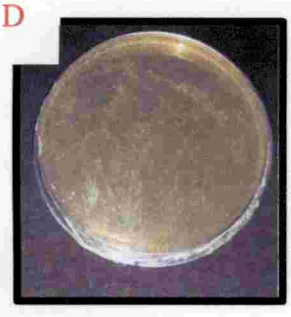
1mg L^{-1} silver nanoparticles for 60min

C



0.1mg L^{-1} silver nanoparticles for 60min

D

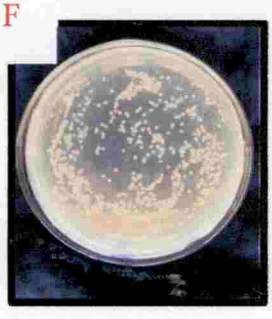


2 mg L^{-1} silver nanoparticles for 60min

E

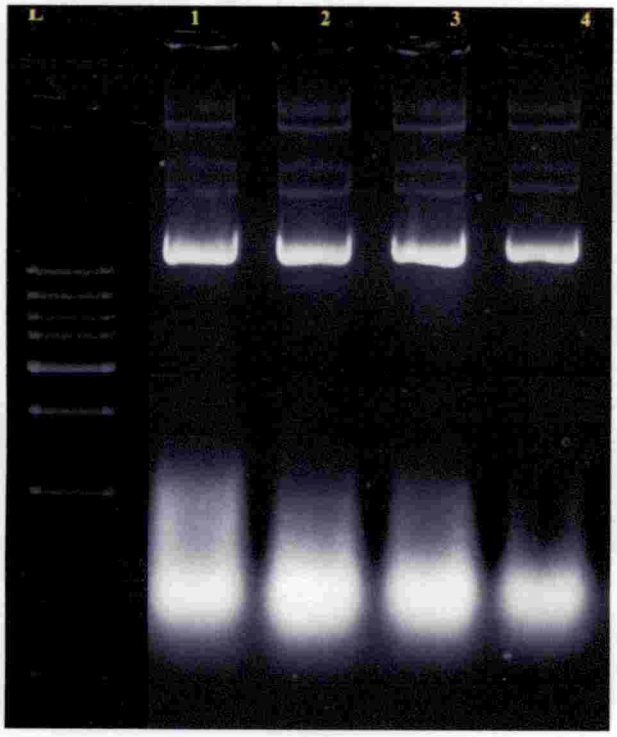
0.01mg L^{-1} silver nanoparticles + 0.1M calcium chloride

F



0.1mg L^{-1} silver nanoparticles 0.1M calcium chloride

Plate 3 *E.coli* DH5α cells subjected to different treatments for competency induction and transformed using pUC18 vector grown in selection media



L: 1kb DNA ladder

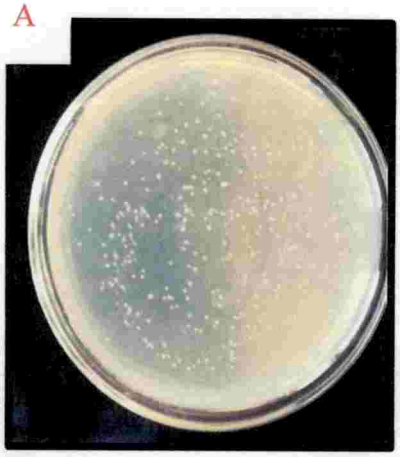
Lane 1: Commercial pUC18 Plasmid

Lane 2: Cells treated with O.1M calcium chloride

Lane 3: Cells treated with Silver nanoparticles

Plate 4 Agarose gel profile of plasmid isolated from the colonies grown in selection medium

A



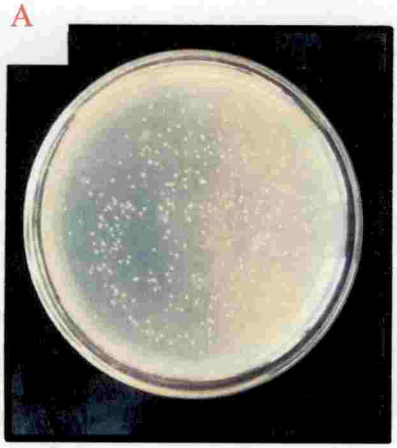
0.1M calcium chloride

B

1mg L^{-1} silver nanoparticles for 60min

Plate 5 Competent *E.coli* DH5α cells transformed using pBR322 vector and grown on LB agar plates containing ampicillin and tetracycline

A

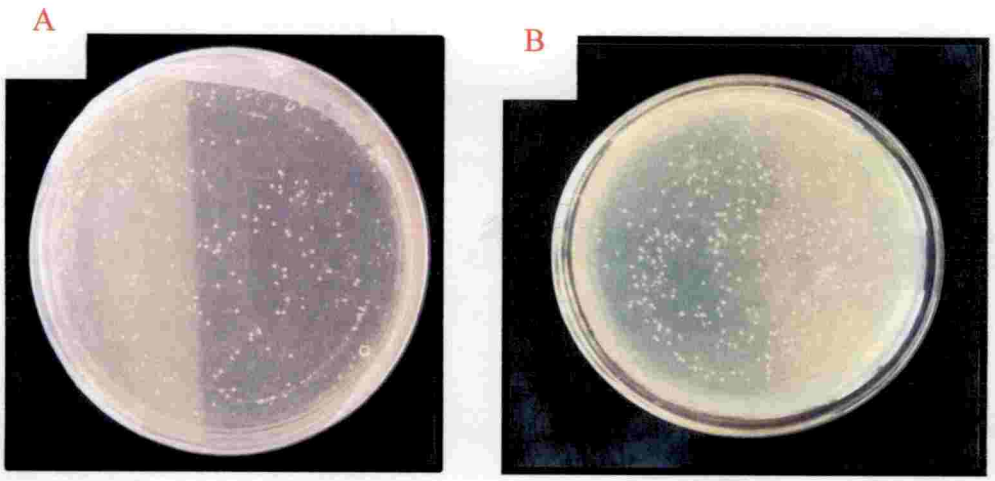


0.1M calcium chloride

B

1mg L^{-1} silver nanoparticles for 60min

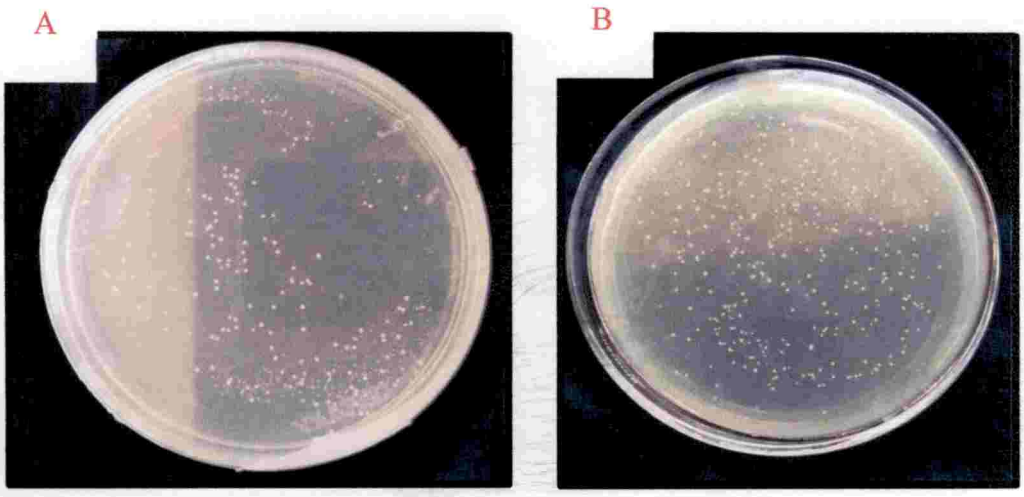
Plate 6 Competent *E.coli* DH5α cells transformed using pBR322 vector and grown on LB agar plates containing tetracycline



0.1M calcium chloride

1mg L^{-1} silver nanoparticles for 60min

Plate.7 Competent *E. coli* DH5α cells transformed using pBR322 and selected on LB agar media containing ampicillin



0.1M calcium chloride

1mg L^{-1} silver nanoparticles for 60min

Plate.8 Competent *E.coli* DH5α cells transformed using pCAMBIA vector and grown on LB agar media containing kanamycin

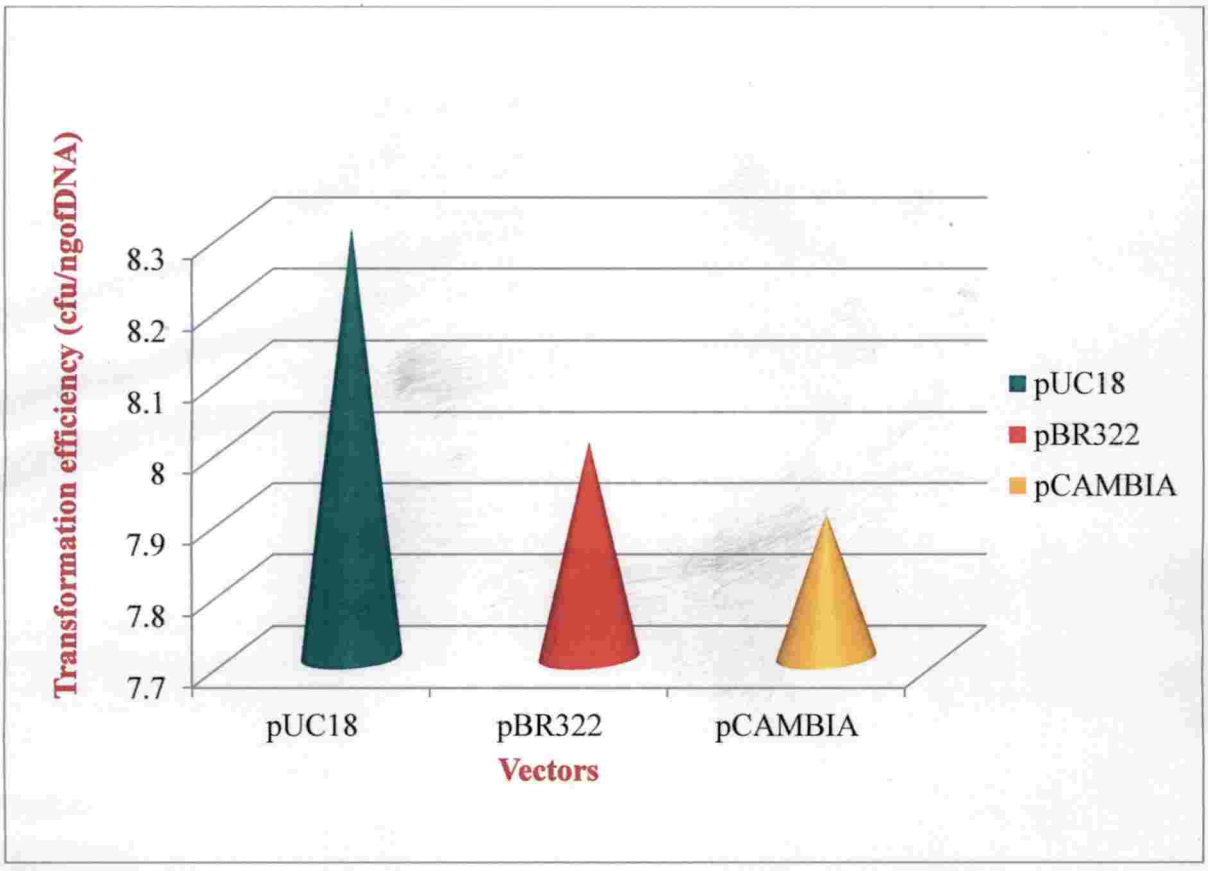


Fig.5 Transformation efficiency of competent cells induced by silver nanoparticles and transformed with different vectors

Discussion

5. DISCUSSION

Efficient introduction of plasmid DNA into cells by transformation plays a significant role in DNA cloning and molecular genetic manipulation. Natural genetic transformation is absent in many bacteria mainly due to repulsions caused by the negative charges of both DNA and bacterial membrane and less porosity of membrane (Asif *et al.*, 2017). *Escherichia coli*, a common host for cloning has three-walled membrane. Pore-forming proteins (porins), which are present in high copy number within the outer membrane, behave as a molecular sieve for the passive transport of hydrophilic and high molecular weight molecules like DNA and proteins (Raghav *et al.*, 2011). So, there is ample motivation for controlled artificial transformation and many approaches have been attempted.

Several techniques such as electroporation, chemical transformation, electron spray and sonoporation have been widely used for transformation studies (Okubo *et al.* 2008; Yoshida and Sato, 2009). Cost of the equipment and requirement of very high cell density are the main limitations of electroporation. Chemical transformation methods are simple and cost effective (Rebersek *et al.*, 2011). Competent cell preparation using calcium chloride is the critical step in transformation and has great impact on transformation efficiency. The major limitation in the technique of chemical transformation is the low transformation efficiency (Liu *et al.*, 2017). Therefore, current emphasis is on devising more efficient methods for the introduction of DNA into host.

Nanotechnology has evolved as a major interdisciplinary science and is being explored for various biological processes (Pinto-Alphandary *et al.* 2000). Nanoparticles are typically smaller (nanosacle10^{-9} m) compared to large biological molecules, such as enzymes, receptors and antibodies and thus offer unprecedented interactions with biomolecules both on the surface and inside the cell (Cai *et al.*, 2008).

Numerous recent reports are indicative of significant contributions of nanotechnology in genetic transformation and antimicrobial action (Nagamune *et al.*, 2017). Nanoparticle mediated gene delivery is widely used in the medical field for gene therapy, but its application in genetic transformation is less exploited. Gold, iron oxide, zinc oxide and silica nanoparticles are the most widely used nanoparticles in molecular biology. Silver nanoparticles are widely used in medical, pharmacy, food and cosmetic industries due to its antimicrobial property (Iravani *et al.*, 2014). However, its application in molecular cloning is not yet reported.

Silver nanoparticles interact with microbial membrane, penetrate inside the cell and increase the porosity of the cell membrane (Dakal *et al.*, 2016). Hence, it can also facilitate the uptake of extracellular DNA similar to the process of bacterial artificial transformation induced by electroporation, calcium chloride and heat shock method. So, in the present study an attempt was made to evaluate the efficiency of silver nanoparticles as delivery system for microbial genetic transformation.

Toxicity of silver nanoparticles is dependent on the size of the nanoparticles. Ivask *et al.* (2014) reported that toxicity of silver nanoparticles decreased with increasing particle size and differently sized particle exhibited different levels of toxicity. According to them, 10 nm particles exhibited maximum inhibition at 0.5mg L^{-1} and 80 nm particles exhibited maximum inhibition at 10mg L^{-1}. Cho *et al.* (2018) reported that silver nanoparticles of 100 nm particle size was less toxic compared to particles of size 10 nm and 60 nm and hence silver nanoparticles of 100 nm size commercially purchased and was used for further studies.

Transformation studies were carried out using *E.coli* DH5α strain as host with vectors *viz.*, pUC18, pBR322 and pCAMBIA. Growth curve of *E.coli* DH5α was analysed by growing the bacteria and measuring absorbance at 600nm (OD_{600}) at every 30min. The growth curve exhibited the different growth phases *viz.*, lag phase, log phase, stationary phase and death phase. The cells remained in the lag phase for 2 h wherein it was preparing to grow and hence no growth in cell volume and number

was noticed. Further, an increase in cell volume and cell density was noticed for 6 h indicating the log phase. From log phase it entered to stationary phase wherein there was no increase in cell density and subsequently it entered into the death phase. Similar pattern in growth curve was reported by Chotinantaku *et al.* (2014).

Significant correlation was observed between the population of bacterial cells and optical density in the present study. In regression analysis, r^2 value was 0.85 indicating 85% correlation between population and optical density. Hence, the population of bacterial cells used for the toxicity assay of silver nanoparticles was determined based on OD values. Similar method was followed by Dorobantu *et al.* (2015) for adjusting bacterial population in toxicity assay of silver nanoparticles against bacteria.

Silver nanoparticles of different concentrations (Table1) were used for testing their toxicity on *E.coli* DH5α cells in exponential phase. Among them, 0.01mg L^{-1} of silver nanoparticles exhibited no toxicity on bacterial cells and concentration of 10mg L^{-1} and above showed100% inhibition of growth with respect to initial bacterial concentration (10^7cfu/mL). The half maximum effective concentration (EC50) was calculated as 4.49 mg L^{-1}. Increase in the concentration of silver nanoparticles exhibited increased toxicity. Navarro *et al.* (2008) have also reported that an increase in the concentration of silver nanoparticles resulted in decrease in the photosynthetic yield and increased toxicity in algae. Kim *et al.* (2011) reported that in *Staphylococcus aureus* and *Escherichia coli*, toxicity of silver nanoparticles increased with increase in incubation time and concentration of silver nanoparticles. According to Acharya *et al.* (2018) the minimum inhibitory concentration of silver nanoparticles (10 nm size) was 188ug/mL in *Pseudomonas aeruginosa* at an initial bacterial concentration of 10^5cfu/mL.

Based on the results of the toxicity assay in the present study, silver nanoparticles of concentrations less than 4.5mg L^{-1} were used for further studies on induction of competency and assessment of transformation efficiency using vectors pUC18, pBR322 and pCAMBIA.

Competent cells produced by different treatments (Table 2) were transformed with vectors pUC18 to assess the transformation efficiency. Silver nanoparticles at a concentration of 1 mg L^{-1} exhibited maximum transformation efficiency compared to lower (0.01mg L^{-1} and 0.1mg L^{-1}) and higher concentrations (2mg L^{-1} and 4mg L^{-1}) of silver nanoparticles. The small size of the silver nanoparticles might have facilitated them to readily cross the cell membrane barrier, pulling the DNA also inside the cell (Gunashekaran *et al.*, 2012). In the present study heat shock treatment could be eliminated. Disruption of the cell membrane would have increased their porosity, thereby facilitating increased uptake of extracellular DNA resulting in increased transformation efficiency. At low concentrations of silver nanoparticles, the interaction between the silver nanoparticles and the cell membrane may be comparatively less and hence the induction of porosity might also be lesser. This leads to comparatively lower transformation efficiency. Low transformation efficiency while using higher concentrations of silver nanoparticles may be due to increased interaction between the silver nanoparticles with cell membrane thereby causing increased disruption of the cell (Wang *et al*, .2015).

Combination of calcium chloride and silvernanoparticles for competency induction and transformation with vector pUC18 resulted in lower transformation efficiency ($2.6x10^3$- $2.5x10^4$) compared to the transformation efficiency exhibited when silver nanoparticles alone ($8.3x10^4$) were used. This may be due to more damage caused in the cell membrane by the interaction of both silver nanoparticles and calcium chloride. Transformation using competent cells prepared by 0.1M

calcium chloride and heat shock method of transformation resulted in less transformation efficiency (Das *et al.*, 2015).

Chatterje and Sarkar (2014) have also reported higher transformation efficiency while using glutathione-surface-functionalized gold nanoparticles for transformation of non-competent *E. coli* DH5α cells with pUC 19, compared to transformation using 0.1M calcium chloride and non competent cells.

In the present study, time of exposure of cells to silver nanoparticles was found to have an effect on transformation efficiency. Among the treatments tried for competency induction, 1mg L^{-1} silver nanoparticles for a period of 1hr exhibited maximum transformation efficiency ($8.3x10^4$cfu/ng of DNA). This may be the optimum period for the interaction of silver nanoparticles with cell membrane and the induction of porosity without much cell disruption. Fehaid *et al.* (2016) reported that the toxicity of silver nanoparticles was dose and time dependent. Toxicity increased with increase in time and dose. Lesser transformation efficiency noticed in the present study on increased period of exposure to silver nanoparticles may be due to the release of silver ions and thereby cell damage as reported by Juan *et al.* (2017) in *Nitrosomans europaea*.

Kittler *et al.* (2010) reported that the dissolution of citrate-stabilized and poly vinyl pyrrolidone stabilized silver nanoparticles in water into ions increased with storage. Release of silver ions led to a considerably increased toxicity of silver nanoparticles towards human mesenchymal stem cells due to the increased concentration of silver ions.

Barkhodari *et al.* (2014) reported the variation in toxicity of silver nanoparticles against the suspensions of blood mononuclear cells on exposure periods of 6 and 24 hrs. According to them, on exposure for 6 hrs, 10.9% to 48.4% of the

cells died and after 24 hours of exposure, cell death increased significantly (56.8% to 86.3%).

The competent cells transformed with pUC18 are usually selected by blue white scrrening due to alpha complementation. A difference in the colour of transformed colonies grown in the selection media (ampicillin+ X-Gal + IPTG) was noticed in the cells subjected to different treatments for competency induction and transformed using pUC18 vector. The cells transformed by the conventional method using calcium chloride and heat shock formed blue colonies. When silver nanoparticles alone were used for competency induction and transformed using pUC18, only white colonies were formed in the selection media indicating no β galactosidase activity. When both silver nanoparticles and calcium chloride were used for competency induction there was formation of both blue and white colonies. The absence of blue colonies indicates an inhibition of β galactosidase enzyme activity by silver nanoparticles. The plasmid isolation from the transformed colonies showed the presence transformation.

Enzyme inhibition by metal ions and nanoparticles are reported. Chudasama *et al* (2010) reported that heavy metals are toxic, bind to protein molecules and strongly interacts with thiol groups of vital enzymes and inactivates them. Greulich *et al.* (2012) reported that silver nanoparticles releases silver ions and they not only interact with a variety of biomolecules within a cell such as nucleic acids and cell wall components but also with the sulfhydryl groups of metabolic enzymes and sulfur-containing cell components like glutathione. Ahmed *et al.* (2016) have also reported the inhibition of enzyme activity of β-galactosidase by zinc oxide nanoparticles in methicillin resistant *Staphylococcus aureus*. Khan *et al.* (2017) reported the inhibition of DNA polymerase by nanoparticles of silver (Ag), silicon dioxide (SiO_2), titanium dioxide (TiO_2) and zinc oxide (ZnO). According to them, silver nanoparticles

exhibited maximum inhibition of polymerase activity. This was followed by zinc oxide, silicon dioxide and titanium dioxide respectively. Raffi *et al.* (2008) reported that treatment of *E. coli* cells with silver nanoparticles affects the DNA replication by inhibiting DNA polymerase enzyme.

β-galactosidase belongs to the enzyme class hydrolases. There are reports on the inhibition of hydrolase enzyme activity by silver nanoparticles. Shin *et al.* (2012) reported the inhibition of enzyme activity of urease, acid phosphatase, arylsulfatase and β-glucosidase in the soil microbes on treatment with silver nanoparticles. According to them inhibition was more in the case of urease (hydrolases) and dehydrogenase activities. Borase *et al.* (2015) reported the inhibition of urease (hydrolases) enzyme activity by silver nanoparticles. According to Cao *et al.* (2017) silver nanoparticles are capable of inhibiting soil exoenzymes such as dehydrogenase, urease (hydolases), acid phosphatase, neutral phosphatase and alkaline phosphatase.

In the present study, on competency induction by silver nanoparticles in bacterial cells and transformation using pBR322 vector the transformed bacterial cells failed to grow in the selection medium containing both ampicillin and tetracycline. However, they could form colonies in media containing only ampicillin. This may be attributed to the inhibition of tetracycline resistance gene activity by silver nanoparticles.

The tetracycline resistance gene of pBR322 encodes a 41-kDa inner membrane protein (TetA) that acts as a tetracycline/H+ antiporter (Allard *et al.*, 1992). The TetA (C) protein, encoded by the *tetA (C)* gene of plasmid pBR322, is a member of a family of membrane-bound proteins that mediate energy-dependent efflux of tetracycline from the bacterial cell (Mc Nicholas *et al.*, 1992). Stensberg *et al.* (2011) reported that uptake of silver nanoparticles can stimulate the production of reactive oxygen species (ROS), resulting in oxidative stress and genotoxic effects.

Production of ROS results in disruption in the flux of ions and electrons across the mitochondrial membrane. In the present study, the disturbance in the bacterial cell membrane when treated with silver nanoparticles might have disrupted the efflux of ions in the transformed cells. This would have resulted in the failure of growth of the transformed cells in the selection medium containing tetracycline. Dien *et al.* (2017) have reported that silver nanoparticles are having synergistic effect in inhibition of growth of tetracycline resistant bacteria *Klebsiella*.

Mc Shan *et al.* (2015) reported that silver nanoparticles affect the growth of *Salmonella* cells in medium containing tetracycline. According to them the silver nanoparticles are capable of forming a complex with tetracycline in the medium. The tetracycline and silver nanoparticles complex interacts more strongly with the tetracycline resistant *Salmonella* cells and causes more release of silver ions, thus creating a temporal high concentration of silver ions near the bacteria cell wall. This leads to growth inhibition of the bacteria.

In the present study, although the cells treated with silver nanoparticles and transformed using pBR322 failed to grow in selection medium containing tetracycline, they could grow in the medium containing ampicillin. Ampicillin and penicillin belongs to the same group of antibiotics. Mc shan *et al.* (2015) have reported similar results in case of pencillin. According to them, Penicillin did not affect the interaction of silver nanoparticles with the bacteria, and there is no synergistic effect.

Bhaskar *et al.* (2016) reported that synergetic effect of silver nanoparticles and antibiotics was highest in case of tetracycline followed by streptomycin and vanomycin.

In the present study, treatment with 1mg L^{-1} silver nanoparticles for 60 min on bacterial cells and transformation with pCAMBIA vector also showed increased transformation efficiency($7.9x10^4$) in selection medium containing kanamycin (50 mg L^{-1}) compared to calcium chloride and heat shock method ($2.3x10^3$). Similar results are reported by Kumar *et al.* (2017) while using gold nanoparticles for competency induction and transformation by pCAMBIA 1304 into *E.coli* DH5α cells. They have reported tenfold increase in transformation efficiency while using gold nanoparticles compared to calcium chloride method.

Basu *et al.* (2018) reported that when plasmid DNA pCAMBIA1302 harboring *gfp* marker gene and coated with gold nanoparticles were used to treat Sarcoma 180 cells, the cells exhibited green fluorescence indicating the uptake of plasmid DNA/gold nanoparticle complex and subsequent expression of the gene in the cancer cells. The untreated cells treated did not show the fluorescence indicating failure of naked DNA uptake.

Results of the present study indicated that silver nanoparticles aid in bacterial transformation. Silver nanoparticles of 100 nm size at a concentration of 1mg L^{-1} showed ten fold increase in the transformation efficiency of *E. coli* DH5α cells with the vectors pUC18, pBR322 and pCAMBIA compared to the conventional method using 0.1 M calcium chloride and heat shock.

summary

SUMMARY

The study entitled "Efficacy of silver nanoparticles as delivery system in genetic transformation" was carried during 2016-2018 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to evaluate the efficiency of silver nanoparticles for gene delivery in microbes.

The study envisaged efficient gene delivery into microbes by the use of silver nanoparticles. Vectors *viz.*, pUC18, pBR322 and pCAMBIA vectors were used for transformation of the bacterium *E.coli* DH5α. Different concentrations of silver nanoparticles of 100 nm particle size were used for the study.

In the present study, an assay was conducted in the bacterial strain *Escherichia coli* DH5α to find out the toxicity of silver nanoparticles. Different concentrations of silver nanoparticles (100 nm size) *viz.*, 0.01, 0.1, 1, 5, 10, 15 and 20 mg L^{-1} were tried. Among different concentration of silver nanoparticles, maximum inhibition (100%) of growth was observed at the concentrations of 10 mg L^{-1} and above. Inhibition of growth was minimum, when *E.coli* cells were treated with 0.01mg L^{-1} of silver nanoparticles. EC_{50} of silver nanoparticles was estimated as 4.49 mg L^{-1} by plotting the graph of inhibition of bacterial cells vs. concentration of silver nanoparticles and carrying out the probability analysis using the statistical package SPSS.

Competency was induced in *E.coli* DH5α cells using different concentrations of silver nanoparticles below the EC_{50} value *viz.* 0.01, 0.1, 1, 2 and 4 mg L^{-1} at different time periods (30min, 60min and 120min), silver nanoparticles in combination with 0.1M calcium chloride and 0.1 M calcium chloride alone (conventional method). Bacterial cells without any treatment served as control. Competency induction was assessed by transforming using pUC 18 plasmid vector.

Among the different treatments tried for competency induction, Transformation efficiency was higher in all the treatments with silver nanoparticles compared to calcium chloride alone. Maximum transformation efficiency was observed when cells were treated with 1mg L^{-1} of silver nanoparticles ($8.3x10^4$ cfu/ng of DNA). Silver nanoparticles showed an increase in transformation efficiency ranging from 2.6 $x10^3$ to $8.3x10^4$ cfu/ng of vector DNA compared to the conventional method using 0.1M calcium chloride (2.3×10^3 cfu/ng of vector DNA). Combination of calcium chloride and silver nanoparticles for competency induction and transformation with pUC18 vector exhibited lesser transformation efficiency compared to transformation efficiency exhibited by silver nanoparticles alone. The present study, the optimum period for the interaction of 1mg L^{-1} of silver nanoparticles with cell membrane and the induction of porosity for facilitating extracellular DNA uptake without cell disruption was recorded as 60 min. ANOVA single factor analysis of the data also showed significant difference between all the different treatments studied.

Blue white selection of transformed cells was not possible when silver nano particles were used. Silver nanoparticle treated *E. coli* cells on transformation with pUC18 did not yield blue colonies on LB plates supplemented with ampicilin, X-Gal and IPTG as expected. *E. coli* cells treated with both silver nano and calcium chloride produced a mixture of both blue and white colonies whereas competent cells prepared by treating with 0.1 M calcium chloride yielded blue colonies. Transformation was reconfirmed by isolation of plasmid from the colonies grown in selection medium and checking on agarose gel electrophoresis. Treatment with silver nanoparticles for competency induction inhibited the lac Z gene expression of pUC 18 plasmid vector. The treatment which yielded maximum transformation efficiency (1mg L^{-1} silver nanoparticles for 60 min) was employed for transforming *E. coli* cells with two other vectors *viz*. pBR322 and pCAMBIA.

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E. coli cells treated with silver nanoparticles (1mg L^{-1} silver nanoparticles for 60 min) and transformed using pBR322 did not form any colonies on LB agar plates containing ampicillin (60 mg L^{-1}) and tetracycline (20 mg L^{-1}) and tetracycline alone. However, the cells treated with silver nanoparticles, grown on LB agar plates containing only ampicillin (60 mg L^{-1}) confirming transformation. Treatment with silver nanoparticles showed an increase in transformation efficiency ($8x10^4$ cfu/ng of vector DNA) compared to cells treated with 0.1M calcium chloride ($2.3x10^3$cfu/ng of DNA).

E. coli cells treated with silver nanoparticles (1mg L^{-1} for 60 min) and transformed with pCAMBIA vector showed a higher transformation efficiency ($7.9 x10^4$ cfu/ng of DNA) compared to calcium chloride method ($2.3x10^3$ cfu/ng of DNA) when plated on LB agar plates containing kanamycin (50mg L^{-1}) .

There is need for further evaluation of efficacy of silver nanoparticles for gene delivery in other hosts such as *Agrobacterium* for use in plant genetic transformation. Ecotoxicity studies of silver nanoparticles are to be evaluated for their large scale.

Competent cell preparation using calcium chloride is the most commonly used method for microbial genetic transformation. The main disadvantage of this method is less transformation efficiency. The results of the present study exhibited a tenfold increase in the transformation efficiency on treatment with silver nanoparticles for competency induction in *E. coli* DH5α cells compared to the calcium chloride method indicating the potential of using silver nanoparticles as a promising alternative to calcium chloride in microbial transformation.

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Appendices

APPENDIX I

Chemical composition of Luria Bertani (LB) media for the culturing of *E.coli* DH5α

Ingredients	g / L
Casein hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C)	7.5±0.2

In case of LB agar media, agar of 15g/L of agar was added and dissolved before sterilization.

APPENDIX II

Buffers for isolation of plasmid DNA from host cells

1. **Glusose EDTA Tris (GET) buffer (10mL)**

Glucose	90.1mg
EDTA (0.5M)	200µL
Tris- Cl (0.5M)	500µL

The pH was adjusted to 8.0 and volume made upto 10mL, autoclaved and stored at 4°C

2. **Lysis Buffer (10mL)**

NaOH (1N)	2mL
SDS (10%)	0.2mL

It is to be freshly made using sterile autoclaved water (Do not autoclave)

3. **Neutralizing solution (10mL)**

Potassium acetate (5M)	6mL
Glacial acetic acid	1.15mL
Water	2.85mL

Do not autoclave store at room temperature

APPENDIX III

Preparation of stock solution of chemicals

X-Gal stock solution (60mg/mL) was prepared by dissolving in N, N dimethyl formadide and it was then filtered using bacterial membrane filters (Axiva) of 25 mm diameter and 0.2 µm pore size and covered with aluminum foil and stored at 4°C. 50µL of stock solution was used in100 mL of media. IPTG stock (20mg/mL) was prepared by dissolving water and it was then filtered using bacterial membrane filters (Axiva) of 25 mm diameter and 0.2 µm pore size and stored at -20°C as 100µL aliquots.

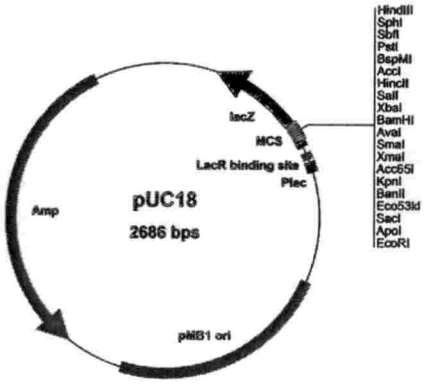
Preparation of stock solution of antibiotics

Sl.No	Antibiotic	Solvent
1	Ampicillin	water
2	Tetracycline	Water
3	Kanamycin	Water

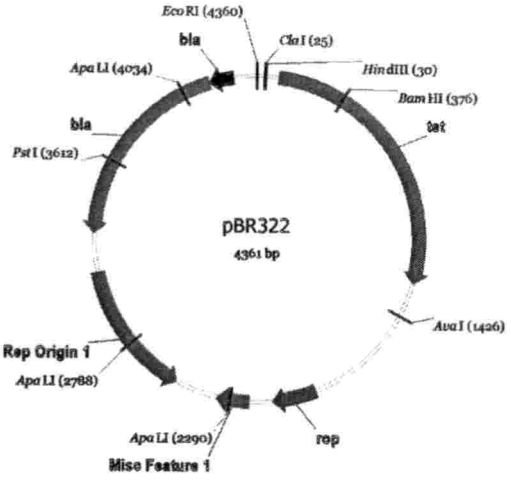
Stock solution was prepared by dissolving water and it was then filtered using bacterial membrane filters (Axiva) of 25 mm diameter and 0.2 µm pore size and stored at -20°C as aliquots.

APPENDIX IV

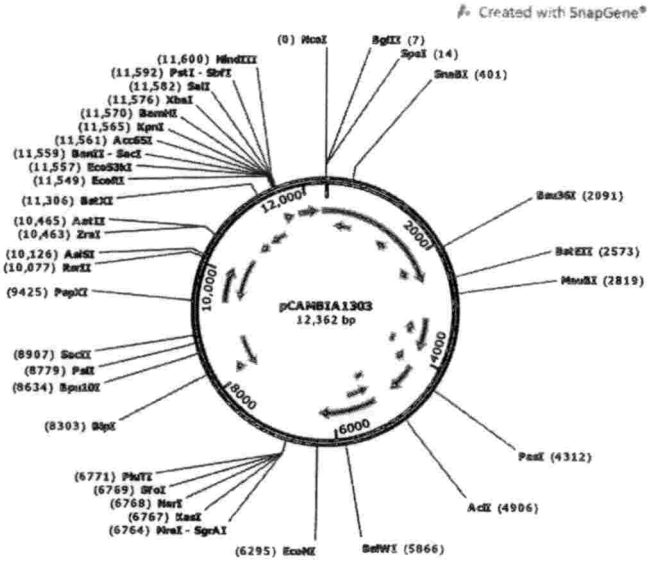
Physical map of vectors used in transformation



Physical map of vector pUC18



Physical map of vector pBR322



Physical map of vector pCAMBIA1303

EFFICACY OF SILVER NANOPARTICLES AS DELIVERY SYSTEM IN GENETIC TRANSFORMATION

100

by

GORANTLA NAGAMANI
(2016-11-096)

Abstarct of the

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COLLEGE OF AGRICULTURE

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KERALA, INDIA

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ABSTRACT

The study entitled "Efficacy of silver nanoparticles as a delivery system in genetic transformation" was carried out during 2016-2018 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to evaluate the efficiency of silver nanoparticles for gene delivery in microbes.

In the present study, an assay was conducted in the bacterial strain *Escherichia coli* DH5α to find out the toxicity of silver nanoparticles. Different concentrations of silver nanoparticles (100 nm size) *viz*., 0.01, 0.1, 1, 5, 10, 15 and 20 mg L^{-1} were tried. Maximum inhibition (100%) of growth was observed at the concentrations of 10 mg L^{-1} and above. 0.01 mg L^{-1} of silver nanoparticles showed no inhibition of bacterial growth. EC_{50} of silver nanoparticles was estimated as 4.49 mg L^{-1} by plotting the graph of inhibition of bacterial cells *vs*. concentration of silver nanoparticles and carrying out the probability analysis using the statistical package SPSS.

Competency was induced in bacterial cells using different concentrations of silver nanoparticles below the EC_{50} value *viz*. 0.01, 0.1, 1, 2 and 4 mg L^{-1} at different time periods (30min, 60min and 120min), silver nanoparticles in combination with 0.1M calcium chloride and 0.1 M calcium chloride alone (conventional method). Bacterial cells without any treatment served as control. Competency induction was assessed by transforming using pUC 18 plasmid vector.

Among the different treatments tried for competency induction, silver nanoparticles showed an increase in transformation efficiency ranging from 2.6 $x10^3$ to $8.3x10^4$ cfu/ng of vector DNA compared to the conventional method using 0.1M calcium chloride (2.3 x 10^3 cfu/ng of vector DNA). Maximum transformation efficiency ($8.3x10^4$ cfu/ng of vector DNA) was observed when cells were treated with 1mg L^{-1} of silver nanoparticles for 60 min.

Treatment with silver nanoparticles for competency induction inhibited the lac Z gene expression of pUC 18 plasmid vector. All the cells treated with silver

nanoparticles showed only white colonies on Lauria Bertani (LB) agar plates containing ampicillin (60mg L^{-1}), X-GAL (30mg L^{-1}) and IPTG (80mg L^{-1}). The colonies from cells treated with calcium chloride alone were blue in colour, whereas colonies from cells treated with silver nanoparticles and calcium chloride showed the presence of both white and blue colour. Transformation was reconfirmed by isolation of plasmid from the transformed colonies and checking on agarose gel electrophoresis.

The treatment which yielded maximum transformation efficiency (1mg L^{-1} silver nanoparticles for 60 min) was employed for transforming *E. coli* cells with two other vectors *viz*. pBR322 and pCAMBIA.

E. coli cells treated with silver nanoparticles (1mg L^{-1} silver nanoparticles for 60 min) and transformed using pBR322 did not form any colonies on LB agar plates containing ampicillin (60 mg L^{-1}) and tetracycline (20 mg L^{-1}). However, the cells treated with silver nanoparticles grew on LB agar plates containing only ampicillin (60 mg L^{-1}) confirming transformation. Treatment with silver nanoparticles showed an increase in transformation efficiency ($8x10^4$ cfu/ng of vector DNA) compared to cells treated with 0.1M calcium chloride ($2.3x10^3$cfu/ng of DNA).

E. coli cells treated with silver nanoparticles (1mg L^{-1} for 60 min) and transformed with pCAMBIA vector showed a higher transformation efficiency ($7.9 x10^4$ cfu/ng of DNA) compared to calcium chloride method ($2.3x10^3$ cfu/ng of DNA) when plated on LB agar plates containing kanamycin (50mg L^{-1}) .

Results of the present study indicated that silver nanoparticles aid in bacterial transformation. Silver nanoparticles of 100 nm size at a concentration of 1mg L^{-1} showed ten fold increase in the transformation efficiency of *E. coli* DH5α cells with the vectors pUC18, pBR322 and pCAMBIA compared to the conventional method using 0.1 M calcium chloride. Competency induction by silver nanoparticles is economically feasible.