BIOSYNTHESIS OF GOLD NANOPARTICLES BY BACTERIA FROM HYPERALKALINE SPRING AND EVALUATION OF THEIR INHIBITORY ACTIVITY AGAINST PYOCYANIN PRODUCTION

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ABSTRACT

Nanoparticles are used in wide range of applications given their unique optical, chemical and electronic properties. Microbial biosynthesis of nanoparticles has offered a milder and eco-friendly alternative to physical and chemical methods of synthesis. One potential use of nanoparticles is for the inhibition of quorum sensing-mediated processes by microorganisms during pathogenic colonization and infection. In this study, bacteria from hyperalkaline spring (pH 11) were isolated using various enrichment media. The isolates, phylogenetically related to *Lysinibacillus* sp. and *Pseudomonas stutzeri,* were investigated for their ability to biosynthesize gold nanoparticles (AuNPs) via reduction of chloroauric acid (HAuCl$_4$) at pH 9. Extracellular synthesis of gold nanoparticles was confirmed by UV-Vis absorption analysis which showed a peak at 500-600 nm wavelength range corresponding to the surface plasmon resonance of AuNPs. The AuNPs synthesized were of spherical and irregular shapes as revealed by SEM analysis. The presence of elemental gold was further confirmed by EDX analysis. FTIR results showed that various functional groups are possibly involved in the reduction of HAuCl$_4$ and stabilization of synthesized gold nanoparticles. The inhibitory effect of biosynthesized AuNPs was tested on the growth and pyocyanin production of *Pseudomonas aeruginosa* PA01 during a 72-hour incubation period. Although the biosynthesized AuNPs showed no toxicity to the test organism, an increasing inhibition level of pyocyanin production was observed with increasing volumes of nanoparticles used. The levels of pyocyanin in setups treated with biosynthesized gold nanoparticles at 10, 30 and 50 μL were significantly lower compared to the untreated setups (P ≤ 0.05). Our findings demonstrate that bacteria adapted to alkaline conditions can be used for efficient biosynthesis of AuNPs, which exhibited potential biomedical application in inhibiting pyocyanin production.

Keywords: hyperalkaline spring, chloroauric acid, gold nanoparticles, *Lysinibacillus* sp. *Psedomonas stutzeri,* pyocyanin production

INTRODUCTION

Due to their unique optical, chemical, photoelectrochemical, and electronic properties, there is a rising interest in the synthesis of nanoparticles (**Krolikowska *et al.,* 2003; Kumar *et al.,* 2003**). Specifically, gold nanoparticles are utilized in a plethora of industrial applications including optics, electronics, sensors, coatings, and medical diagnostics and treatments particularly in cancer due to their stability, oxidation resistance, and biocompatibility (**Corti and Holliday, 2004**).

Generally, the synthesis of gold nanoparticles has been carried out via chemical and physical processes to produce nanoparticles with controllable sizes. However, the synthesis of nanoparticles via these processes is difficult, expensive, and generates potential hazardous waste limiting their applications (**Dehnad *et al.,* 2015**). There are also problems in terms of the stability of the nanoparticle preparations, control of crystal growth, poor monodispersity, and aggregation of the particles in chemical and physical methods currently employed (**Gericke and Pinches, 2006a**). Hence, there is a heightened need to develop high-yield, low-cost, non-toxic and environmentally benign procedures for metallic nanoparticle synthesis (**Thakkar *et al.,* 2010**). Biosynthetic process for nanoparticles using plants and microorganisms serves as valuable alternative to physical and chemical methods due to milder process conditions and reduced use of toxic chemicals (**Li *et al.,* 2016**). Furthermore, manipulation of certain growth parameters may contribute to the development of techniques for the controlled synthesis of nanoparticles conferring unique properties useful in potentially new applications (**Shah *et al.,* 2014**).

Recently, production of gold nanoparticles by means of microbial technologies has made impact in areas such as materials science and industry. The affinity of microorganisms to metal ions, tolerance to high metal concentrations, and the ability of microbial systems to reduce and precipitate otherwise toxic inorganic ions into metal nanoparticles enable the extracellular or intracellular production of metal nanoparticles with varying morphologies (**Narayanan and Sakthivel, 2010**). A number of microorganisms including bacteria, actinomycetes, and fungi have been investigated for AuNPs synthesis. For example, the intracellular synthesis of gold nanoparticles via reduction of AuCl$_4^-$ has been reported in

Shewanella algae (**Konishi *et al.,* 2006**) and *Bacillus licheniformis* (**Kalishwaralal *et al.,* 2009**). In a study by **Nair and Pradeep (2002),** it was found out that common *Lactobacillus* strains in buttermilk were able to intracellularly produce microscopic crystals of gold, silver, and gold-silver alloy with distinct morphology without decreasing viability. In addition, production of monodispersed gold nanoparticles under alkaline condition has been documented in some alkalitolerant actinomycetes such as *Rhodococcus* sp. (**Ahmad *et al.,* 2003a**) and *Thermomonospora* sp. (**Ahmad *et al.,* 2003b**). Recently, the ability of the extreme bacterium *Deinococcus radiodurans* to reduce Au (III) in aqueous solution has also been reported (**Li *et al.,* 2016**). At present, there is an increasing interest in understanding the efficiency and reduction mechanism of direct AuNP synthesis by obligate alkaliphilic and alkalitolerant bacteria. Hence, there is a demand for the screening of extremophiles such as alkaliphiles with natural resistance to in-situ stresses and ability to produce AuNPs. The use of these unique biological systems may allow greater control in synthesis even at extreme conditions such as high pH (**Beeler and Singh, 2016**).

One potential application of biosynthesized gold nanoparticles is the inhibition of quorum sensing, a cell density-dependent phenomenon utilized by microbes to synchronize metabolic activities. Several microbial processes are controlled by quorum sensing such as horizontal gene transfer, multicellular behaviors, and microbe-microbe and host-microbe interactions. More importantly, quorum sensing is employed by bacteria during infection and in-host survival. However, quorum quenching activities of certain compounds interfere with this bacterial cell-to-cell communication attenuating the expression of several virulence factors (**Dong *et al.,* 2007**). Many organic compounds and enzymes from both prokaryotic and eukaryotic organisms have been extensively studied for potential quorum quenching activities. Conversely, the use of inorganic compounds or materials for quorum quenching is underexplored. The QS-regulated formation of biofilm by *Staphylococcus aureus* has been successfully quenched by silver nanoparticles synthesized by *Bacillus cereus* (**Chaudhari *et al.,* 2012**). Silver nanoparticles synthesized by mutant *Klebsiella pneumoniae* and *Sargassum polyphyllum* have been proven to exhibit quorum quenching activity against the production of virulence factors in *Pseudomonas aeruginosa* (**Arunkumar *et al.,* 2013**). *Pseudomonas aeruginosa* is an opportunistic pathogen with the ability to

develop resistance to antibiotics, form impenetrable biofilms, and release various virulence factors. Pyocyanin, a redox-active virulence factor, has diverse effects on cellular systems which are mostly associated with lung infections. In particular, pyocyanin can induce oxidative stress and premature senescence in mammalian cells, and can inhibit cellular respiration, ciliary functions and epidermal growth (**Lau *et al.*, 2004; Muller *et al.*, 2009**). An alternative strategy to combat the toxicity of pyocyanin is to devise strategies to inhibit its biosynthesis which is regulated by quorum sensing. **Garcia-Lara et al. (2015)** demonstrated the quorum quenching effect of ZnO nanoparticles against production of virulence factors in clinical and environmental strains of *P. aeruginosa*. In another study, treatment of mycofabricated AgNPs using metabolites of *Rhizopus arrhizus* showed inhibition of virulence factor production in the same pathogen (**Singh *et al.*, 2015**). Despite the unique properties and diverse applications of gold nanoparticles synthesized by bacteria, studies on their quorum quenching activity against pigment pyocyanin in *Pseudomonas aeruginosa* have been limited.

This study aimed to isolate and identify bacterial strains from a hyperalkaline environment, and to determine their ability for extracellular synthesis of gold nanoparticles for potential application in the inhibition of quorum sensing-mediated pyocyanin production in *P. aeruginosa* PA01.

MATERIALS AND METHODS

Isolation, screening and identification of gold nanoparticle-synthesizing bacteria

Sample collection

Water samples were collected from Poon Bato Hyperalkaline Spring in Botolan, Zambales, Philippines (15° 17' 22.5600" N, 120° 1' 28.2000" E) using sterile bottles. Temperature, pH, salinity, and dissolved oxygen were measured using water quality multisensory equipment during sample collection. Enrichment cultures were prepared by adding 10 mL water sample to 90 mL alkaline broth media adjusted to pH 11 by adding 1 M Na_2CO_3. The Na_2CO_3 was autoclaved separately from the other components of the medium and was mixed after cooling to 50°C. The various media used in enrichment and isolation were enumerated in Table 1. Enrichment solutions were put in a shaker incubator for 72 hours at 28 ± 0.2°C.

Serial dilutions of the enrichment were prepared up to a factor of 10^6 and spread plating was done by inoculating 100 μL of each sample onto respective solid media. All plates were incubated for 72 hours at 28 ± 0.2°C until growth of colonies was visible. Morphologically distinct colonies growing on the different solid media were selected and purified via successive streak plating. These isolates were examined for their cell morphology, Gram status, and were subjected to biochemical tests such as catalase and oxidase tests. Pure isolates were then transferred onto respective medium slants and were maintained for further use in glycerol stocks (15 % v/v).

Table 1. Various growth media used in enrichment and isolation of bacteria from hyperalkaline spring.

Medium no.	Medium composition (L^{-1})
1	10 g soluble starch, 5 g peptone, 5 g yeast extract, 1 g K$_2$HPO$_4$, 0.2 g MgSO$_4$· 7H$_2$O, 10 g Na$_2$CO$_3$, and 20 g agar (**Joshi *et al.*, 2008**)
2	5 g peptic digest of animal tissue, 1.5 g yeast extract, 1.5 g beef extract, 5 g NaCl, 20 g agar; pH adjusted to 10 with 1N NaOH solution (**Joshi *et al.*, 2008**)
3	6 mL glycerol, 1 g arginine, 1 g K$_2$HPO$_4$, 0.5 g MgSO$_4$·7H$_2$O, and 15 g agar (**Mwirichia *et al.*, 2010**)

Screening of isolates for AuNP biosynthesis

Synthesis of gold nanoparticles was done following the method described by **Gericke and Pinches (2006b)** with some modifications. Cultures grown for 96 hours at 28 ± 0.2°C in their respective enrichment broth media adjusted to pH 11 were used in the screening for gold accumulation and nanoparticle synthesis. After 96 hours of incubation, microbial biomass was harvested by centrifugation at 7500 rpm for 10 minutes. Cell pellets (200 mg, wet weight) obtained from the culture were washed three times with sterile distilled water to remove components of the broth medium used. The obtained biomass was resuspended in a flask containing 50 mL of aqueous $1x10^{-3}$ M HAuCl$_4$ and the was pH adjusted to pH 9 by addition of 1.0 M NaOH. The mixture was incubated with shaking for 168 hours at 28 ± 0.2°C and maintained in the dark. The formation of AuNPs in the suspension was monitored by the color change of the reaction mixture and the absorption spectrum of the sample aliquots at 500 – 600 nm was measured by Hanon i8 UV/Vis absorption spectroscopy (Jinan Hanon Instruments Co. Ltd.,

China). The solution containing only chloroauric acid served as the control and was incubated at the same conditions as the treated set-ups.

Sequencing and analysis of the 16S rRNA genes of AuNP-producing isolates

Bacterial samples were sent for amplification and sequencing of 16S rRNA genes to Macrogen Inc., Seoul, Korea. Amplification of 16S rRNA gene of the isolates was performed using the bacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGGTTACCTTGTTACGACTT) (Lane 1991). Sequencing was carried out by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). ChromasPro software (*http://www.technelysium.com.au/)* was used to manually evaluate the sequences and to remove low quality regions usually at the start and end of the fragment. DNA sequences were analyzed using the BLAST tool at the National Centre for Biotechnology Information (NCBI) server (*http://blast.ncbi.nlm.nih.gov*). The sequences were submitted for multiple alignments with reference sequences from the GenBank database using Clustal W. Phylogenetic trees were constructed with neighbor-joining algorithm of MEGA 6 software (**Tamura *et al.* 2013**) with evolutionary distances calculated according to Kimura's two-parameter correction method. The phylogenetic tree was evaluated through bootstrap analysis from 1000 bootstrap replicates. All sequences generated in this study were deposited in the GenBank database under accession numbers MG994984 -MG994985.

Recovery of extracellularly synthesized AuNPs

Reaction mixtures that indicated positive result for gold nanoparticle synthesis based on visible color change and UV-Vis analysis were centrifuged at 7500 rpm for 10 minutes to separate the cells from the supernatant (**Gericke and Pinches 2006b**). The supernatants were subsequently transferred to sterile plastic tubes. The nanoparticle-containing supernatants were then further centrifuged at 13,000 rpm for 5 minutes to harvest the synthesized nanoparticles. The harvested nanoparticles were resuspended in sterile distilled water and centrifuged again. This method was repeated three times to separate purified gold nanoparticles (**Barabadi *et al.* 2014**).

Characterization of extracellularly synthesized AuNPs

The synthesized gold nanoparticles were subjected to various characterization techniques. The morphology, size and distribution of the synthesized gold nanoparticles were analyzed using scanning electron microscope (SEM) (JEOL, Model JSM-6390LV) (SEM accelerating voltage: 20kV). The samples were air-dried, fixed on a copper stage, and SEM images of the gold nanoparticles present in the suspension were taken at various magnifications. Consequently, elemental composition of gold nanoparticles in the suspension was analyzed by energy dispersive X-ray (EDX) analysis using Dual beam Helios Nanolab 600i (FEI, USA) (EDX analysis accelerating voltage: 5kV; Beam current: 86 pA) . EDX spectrum was recorded in the area scan mode by focusing the electron beam onto a region of the sample surface. Sample preparation before EDX analysis involved air-drying of the colloidal form of AuNP suspension. Detection of various functional groups involved in nanoparticle reduction and synthesis was done using Fourier Transform Infrared (FTIR) spectroscopy (Shimadzu Prestige, Pike Technologies, USA). Dried samples were analyzed in the region of 4000 – 1500 cm^{-1} at a resolution of 2 cm^{-1} .

Effect of biosynthesized AuNPs on pyocyanin production

Pyocyanin production was assayed according to the protocol described by **Essar *et al.* (1990)** with modifications. Standard pyocyanin-producing *P. aeruginosa* PA01 isolate was grown in 50 mL Luria Bertani (LB) broth at 37°C to an OD$_{600}$ of 0.5. A total of 1 mL of bacterial culture grown in the LB broth was inoculated to 10 mL secondary medium and incubated with the gold nanoparticles (10, 30 and 50 μL) at 37 ± 0.2°C for 72 hours. A secondary medium containing (grams per liter of distilled water) 20 g peptone, 1.5 g anhydrous K$_2$HPO$_4$, 1.5 g MgSO$_4$ ◦ 7H$_2$O and 1 mL glycerol was used. An untreated medium that served as control was made for the spectrophotometric biomass monitoring. Absorbance was measured at 600 nm every 24 hours. The cells were separated by centrifugation at 5000 rpm for 5 min to obtain the supernatant. A 5 mL culture supernatant was extracted with 3 mL of chloroform, re-extracted with 1 mL of acidified water (0.2 M HCl) and absorbance was measured at 520 nm. The pyocyanin concentration was expressed in μg pyocyanin produced per mL culture supernatant (μg/mL). Specifically, the following formula was used: A_{520} x 17.072 as described by **Essar *et al.* (1990)** Furthermore, student's *t*-test was used to analyze the significance of the effect of gold nanoparticles on pyocyanin production. All set-ups were prepared in triplicates.

RESULTS AND DISCUSSION

Isolation and identification of AuNP-synthesizing bacteria

Bacteria were isolated from Poon Bato Spring, a natural alkaline environment in the Philippine Zambales ophiolite. The measured pH was 11 and the temperature was 28˚C. Chemical analysis of the water samples revealed the presence of calcium, magnesium, sulfate, chloride, and iron. Calcium had the highest amount at 50.3 mg/L (Table 2). The high concentration of calcium detected in the site confirms that the spring is typical Ca $^{2+}$ -OH $^{-}$ type water generated by active serpentinization process (**Cardace *et al.,* 2015**). Using three different growth media, a total of twenty five morphologically distinct isolates were obtained and further screened for the synthesis of AuNPs via reduction of chloroauric acid (HAuCl$_4$). The formation of AuNPs by the isolates was initially monitored through visual observation of color change in the solution. Two isolates were able to reduce HAuCl$_4$ to gold nanoparticles after seven days of incubation. Isolate M1-1 was found to be Gram-positive with bacillus morphology while isolate M2-2 was Gram-negative with bacillus morphology. Both isolates gave positive reactions to catalase and oxidase tests.

Table 2 Physicochemical characteristics of water samples obtained from Poon Bato Spring

PARAMETER	MEASUREMENT
pH	11
Temperature (˚C)	28
Conductivity (µS/cm)	781
Dissolved Oxygen (mg/L)	3.6
Calcium (mg/L)	50.3
Chloride (mg/L)	20.3
Sulfate (mg/L)	4.16
Magnesium (mg/L)	1.31
Iron (mg/L)	0.12
Nitrate (mg/L)	<LoD*
Manganese (mg/L)	ND**

*LoD — Limit of Detection (0.43 mg/L for nitrate)
**ND — Not Detected

Analysis of the 16S rRNA gene sequences revealed that the isolates were phylogenetically-affiliated to the Phyla Proteobacteria and Firmicutes. Specifically, isolate M1-1 was closely related to *Lysinibacillus* sp. while isolate M2-2 was affiliated to *Pseudomonas stutzeri* (Figure 1). The analyzed sequences showed high similarities (99%) to 16S rRNA gene sequences of characterized reference strains (Table 3). The phylum Firmicutes consists mostly of Gram-positive bacteria which are phenotypically and physiologically diverse, allowing them to inhabit a wide variety of environments which include hypersaline habitats. Members of this phylum have been reported in bacterial diversity studies of alkaline environments such as the Lonar Lake in India (**Joshi *et al.,* 2008**; **Kanekar *et al.,* 2007**), Cabeco de Vide aquifer in Portugal (**Tiago *et al.,* 2004**), the Cedars in California (**Morrill *et al.,* 2013**) and the Manleluag hyperalkaline spring in the Philippines (**Baculi *et al.,* 2015**) where the Firmicutes similarly made up the large portion of the total isolates in the said sites.
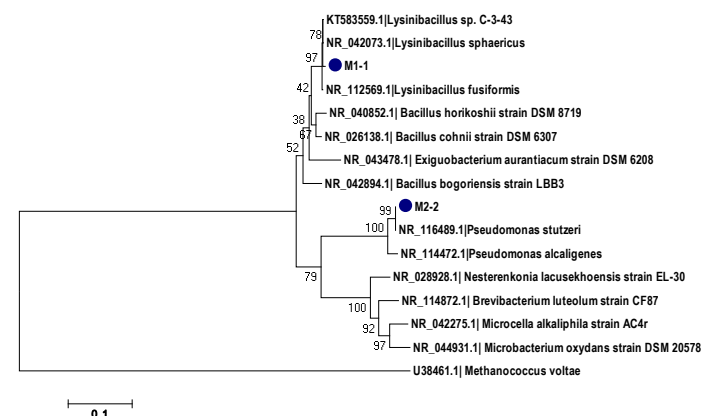

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences highlighting the phylogenetic position of the two isolates relative to other alkaliphilic strains

Methanococcus voltae was used as an outgroup. Sequences were aligned using ClustalW, and phylogenetic inferences were obtained using the neighbor-joining method within Mega 6 software. Bootstrap values are expressed by percentage of 1,000 replicates with a Kimura 2 parameter test and shown at the branching points. The branches of the tree are indicated by the NCBI gene accession numbers followed by the genus and species name of the type strains.

Table 3 AuNP-producing bacteria from Poon Bato Spring and their phylogenetic affiliations.

Isolate	Nearest phylogenetic affiliation	Accesion number of the nearest phylogenetic affiliation	% similarity	Phylogenetic group
M1-1	*Lysinibacillus* sp.	KT583559.1	99%	Firmicutes
M2-2	*Pseudomonas stutzeri*	NR116489	99%	Proteobacteria

The presence of *Lysinibacillus* strains in various alkaline habitats has been previously documented (**Anwar *et al.,* 2015**). This alkaliphilic bacterium has been isolated from the water, sediment and matt samples of the Lonar Lake, an Indian soda lake (**Tambekar *et al.,* 2016**). Additionally, two species have been documented from saline-alkaline soils namely *Lysinibacillus halotolerans* isolated from soils in Shandong Province, China (**Kong *et al.,* 2014**) and *Lysinibacillus alkalisoli* obtained from Hangjin Banner, Inner Mongolia (**Sun *et al.,* 2017**). Biosynthesis of nanoparticles such as silver by *L. sphaericus* MR-1 and *L. varians* has recently been reported (**Gou *et al.,* 2015**; **Bhatia *et al.,* 2016**). Since *Lysinibacillus* species are capable of synthesizing silver nanoparticles that are stable and spherical in shape, the capability of *Lysinibacillus* to synthesize other metallic nanoparticles such as gold nanoparticles may be plausible. Recently, a *Lysinibacillus* sp. isolated from Philippine soil and capable of extracellular biogenic synthesis of AuNPs was characterized (**Cariño *et al.,* 2017**).

The phylum Proteobacteria encompasses a major proportion of Gram-negative bacteria that are known to occur in a broad spectrum of habitats including marine, hypersaline, alkaline and acidic habitats due to their extreme metabolic diversity (**Horikoshi *et al.,* 2011**).The physiological flexibility of *P. stutzeri* has imparted the capacity to colonize wide range of habitats including some alkaline environments (**Lalucat *et al.,* 2006**). Its presence has been documented in different alkaline environments such as Lonar lake in India (**Tambekar *et al.,* 2013**), alkaline groundwater (**Fahy *et al.,* 2008**), and lake Bogoria in Kenya (**Vargas *et al.,* 2005**). The ability of *Pseudomonas denitrificans* (**Mewada *et al.,* 2017**) and *Pseudomonas aeruginosa* (**Husseiny *et al.,* 2007**) to synthesize gold nanoparticles has been previously reported. Although *P. stutzeri* has been reported capable of producing silver nanoparticles (**Klaus *et al.,* 1999**), its ability to synthesize gold nanoparticles is a new finding for this study.

Biosynthesis and characterization of AuNPs produced by the isolates

The synthesis of AuNPs was carried out using bacterial biomass separated from components of the growth medium and mixed with 1×10^{-3} M HAuCl$_4$ solution . This is to ensure that reduction of gold ions is mediated by the functional groups present in the bacterial cell wall. The reduction of gold chloride was done at pH 9 to obtain small-sized nanospheres with limited aggregation of the particles. The inverse proportionality between the pH value and size of AuNPs has been previously documented (**Alzoubi *et al.,* 2015**; **Kumari *et al.,* 2016**). An increase in pH value results in shifting of the surface plasmon resonance peak toward the short wavelength region indicating a corresponding decrease in size of AuNPs. Additionally, pH values between 8.4 and 10.2 have been reported to cause less aggregation of AuNPs (**Alzoubi *et al.,* 2015**), and that pH 9 can produce spherical-shaped AuNPs (**Kumari *et al.,* 2016**). Biosynthesis of AuNPs was evident from the color change of individual reaction mixture into pink or purple after seven days of incubation (Figure 2A-2B). The solution containing *Lysinibacillus* sp. M1-1 and *P. stutzeri* M2-2 cultured in aqueous 1×10^{-3} M HAuCl$_4$ solution exhibited pink color and dark purple color, respectively. The presence of color change in the solution instead of biomass confirmed the extracellular mechanism of gold nanoparticle synthesis by the two isolates. No color change was observed in the control solution containing only HAuCl$_4$ that was incubated at the same condition as the experimental set-ups (Figure 2C). Gold nanoparticles are known to exhibit a range of colors due to surface plasmon resonance and can be characterized accordingly by obtaining the characteristic peak in 500-600 nm range (**Kumari *et al.,* 2016**). The resulting colors of the solutions suggest possible difference in the size of the nanoparticles produced. The interaction of gold nanoparticles with light is dictated by their size and physical dimensions. As the particle size increases, the surface plasmon resonance shifts to longer wavelength which results in the absorption of red light while blue light is reflected yielding solutions with pale blue or purple color (**Mingos, 2014**). This observation was corroborated by the characteristic absorption peaks of the solutions obtained by UV-Vis analysis. The UV-Vis spectra exhibited peaks at 519 nm and 561 nm for gold nanoparticles biosynthesized by *Lysinibacillus* sp. M1-1 and *P. stutzeri* M2-2, respectively. No characteristic absorption of AuNPs was observed in the solution containing only HAuCl$_4$ (Figure D). The absence of gold nanoparticles in the solution without bacterial isolate suggests that the bacterial culture mediated the reduction of HAuCl$_4$ to gold nanoparticles. The peaks obtained are within the critical wavelengths corresponding to the surface plasmon resonance peak of gold

nanoparticles. The difference in the recorded peaks indicates possible difference in size of the synthesized nanoparticles since an increase in particle diameter is associated with an increase in maximum wavelength for the surface plasmon band (**Daniel and Astruc, 2004**). However, it is important to note that surface plasmon resonance maximum wavelength is not only affected by size but it is also influenced by the particle shape, medium dielectric constant, and temperature (**Daniel and Astruc, 2004**). The reduction of $HAuCl_4$ by the isolates, however, appeared to be a slow process requiring seven days of incubation. This observation is consistent with the findings of **Ahmad *et al.* (2003a),** wherein extracellular biosynthesis of gold nanoparticles using the alkalothermophilic actinomycete *Thermomonospora* sp. occurred after 120 hours. In similar manner, the reaction was carried out under alkaline condition of pH 9. Studies suggested that extracellular synthesis of AuNPs is mediated by redox enzymes such as NADH-dependent nitrate reductase required for the reduction of aqueous gold ions into gold nanoparticles. The reduction of gold ions may possibly involve electron transfer from NADH by NADH reductase to the electron deficient gold ions (Au^{3+}) that will then be reduced to gold neutral (Au^o) leading to the formation of gold nanoparticles (**Rai *et al.*, 2013**).

higher pH, Cl^- present in $HAuCl_4$ gets substituted by OH^- from biological moieties resulting in repulsion between the two negatively charged moieties. This will limit the possibility of further crystal growth after development of nuclei maintaining them as small spherical particles (**Kumari *et al.*, 2016**). The difference between the sizes of nanoparticles synthesized by the two bacterial isolates was consistent with the difference in the color of the reaction mixtures and resulting wavelengths. Specifically, solutions with tints closer to a strong purple color are characterized by higher particle wavelengths indicating presence of larger particles (**Owen, 1996**).

In the study, the field EDX spectra showed the presence of peaks for Au along with the peaks specific for other elements such as C, N and O (Figure 4). Apparently, the amount of Au synthesized by *P. stutzeri* M2-2 (4.2%) and *Lysinibacillus* sp. M1-1 (13.12%) were lower relative to the other elements detected. Nevertheless, the characteristic optical absorption peak of Au at 2.2 keV confirmed the presence of elemental gold in trace amounts. The presence of peaks specific for C, N and O could be due to cellular components including proteins and carbohydrates that possibly acted as stabilizing or capping agents in the formation of gold nanoparticles (**Li *et al.*, 2016**). This is consistent with the results of the FTIR analysis which revealed the possible presence of functional groups involved in stabilization of synthesized nanoparticles. It was reported that AuNPs can interact with proteins through free amine groups or cysteine residues via electrostatic attraction of negatively charged carboxyl or carbonyl groups forming a coating and leading to stabilization of the AuNPs (**Li *et al.*, 2016**).
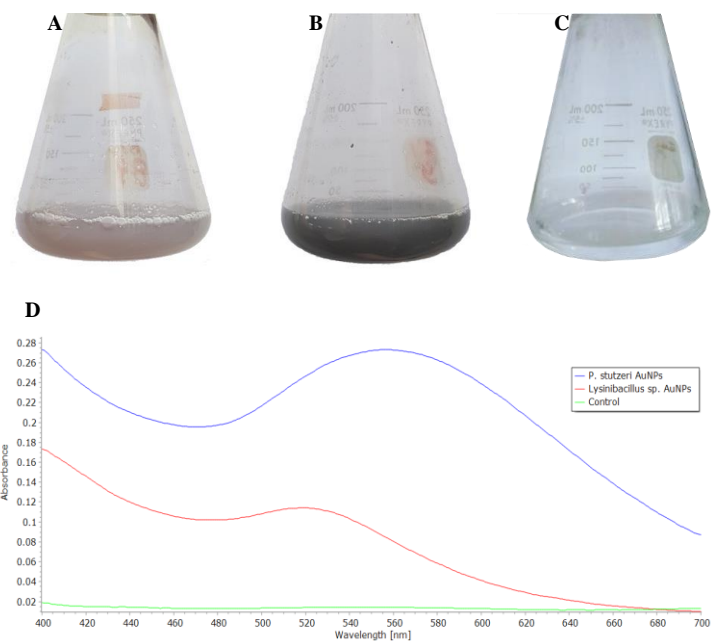

A

B

C

D

Figure 2 Biosynthesis and absorbance spectra of AuNPs synthesized by the isolates. A. Change in the color of the solution containing *Lysinibacillus* sp. M1-1 cultured in aqueous $1x10^{-3}$ M $HAuCl_4$ solution B. Change in the color of the solution containing *P. stutzeri* M2-2 cultured in aqueous $1x10^{-3}$ M $HAuCl_4$ solution C. Appropriate control composed of $HAuCl_4$ solution was measured at the same time for comparison D. Absorbance spectra from 400 nm to 700 nm of AuNPs synthesized by the isolates.

The morphology, size and distribution of the synthesized nanoparticles were analyzed by scanning electron microscopy (SEM). Analysis revealed that gold nanoparticles synthesized by *Lysinibacillus* sp. M1-1 were of spherical and irregular shapes with size ranging from 37.6 to 86.3 nm (Figure 3A). On the other hand, gold nanoparticles synthesized by *P. stutzeri* M2-2 were of the same shapes with size ranging from 32.84 to 88.13 nm (Figure 3B).
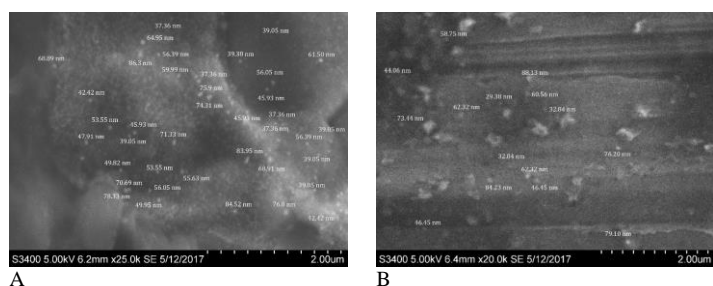

A

B

Figure 3 SEM micrographs of gold nanoparticles synthesized by (A) *Lysinibacillus* sp. M1-1 and (B) *P. stutzeri* M2-2 at 25, 000x.

In this study, the observed particle size and shape were similar to the findings of **Kumari *et al.* (2016)** who demonstrated the synthesis of spherical and irregular-shaped AuNPs of size 50-80 nm at alkaline pH via reduction of $HAuCl_4$. At
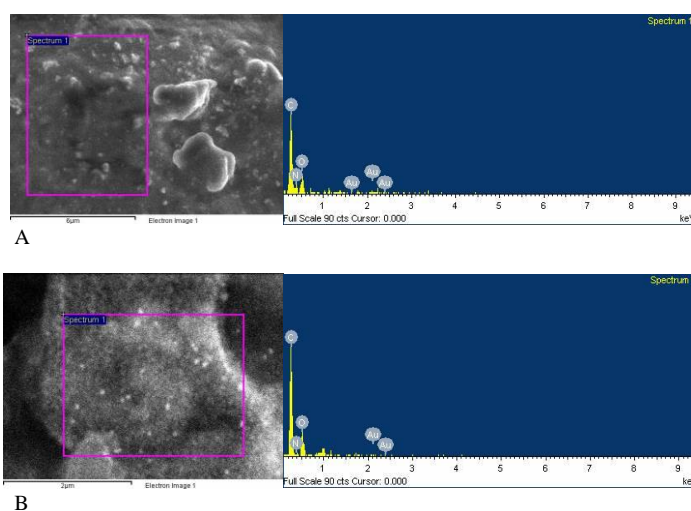

Figure 4 Energy dispersive X-ray (EDX) spectra of gold nanoparticles synthesized by (A) *Lysinibacillus* sp. M1-1 and (B) *P. stutzeri*. M2-2.

FTIR measurement was performed to determine the possible functional groups involved in the reduction of $HAuCl_4$ and stabilization of synthesized gold nanoparticles. The FTIR spectra of the bacterial isolates exhibited peaks at 3000-3400 cm^{-1} (OH-containing groups), 2889 cm^{-1} (C-H stretches) 1684-1685 cm^{-1} (C=O and NH stretches) (Figure 5). After the synthesis of gold particles, the bands shifted in peaks due to possible interaction between gold ions with the functional groups in the bacterial isolates. The FTIR signals of the gold nanoparticles synthesized by *Lysinibacillus* sp. M1-1 showed distinct absorption bands at 3422 cm^{-1}, 2930 cm^{-1}, and 1655 cm^{-1} (Figure 6). The peak at 3422 cm^{-1} corresponds to the OH or NH stretches present in proteins and carbohydrates involved in reduction and capping of Au ions to form gold nanoparticles (**Li *et al.*, 2016**). Moreover, the strong band at 2930 cm^{-1} corresponds to the C-H stretch of alkanes while C=O stretch of amides is assigned at 1655 cm^{-1}. On the other hand, almost similar functional groups were found in the gold nanoparticles synthesized by *P. stutzeri* M2-2 with peak at 3419 cm^{-1} and 2930 cm^{-1} that may be assigned to OH- containing groups in proteins and carbohydrates, and stretching vibrations of C-H of alkanes, respectively. The peak at 1626 cm^{-1} may be assigned to free NH stretches of amides. The detection of peaks specific for amide I and II was consistent with the reports on gold nanoparticles synthesis of *Trichothecium* sp. and *Thermomonospora* sp. (**Ahmad *et al.*, 2003a**; **Ahmad *et al.*, 2015**). The presence of amide I, II, and III bands was also highlighted in the study of gold nanoparticles produced by *Brevibacterium casei*, attributing the presence of these amide bands to that of native polypeptides and proteins (**Kalishwaralal *et al.*, 2009**). This may provide clues on the role of native proteins of the organism in the synthetic process. It was reported that proteins can stabilize gold nanoparticles through free amine groups or cysteine residues to form a coating on the nanoparticles (**Das *et al.*, 2012**). It was reported that the extremophile *Deinococcus radiodurans* contains numerous compounds which are rich in hydroxyl, phospho or amine groups responsible for the reduction and stabilization of the synthesized gold nanoparticles (**Li *et al.*, 2016**). Meanwhile, the OH and NH_2 groups on the cell surface of *Pichia pastoris* (**Das *et al.*, 2012**) and the carbonyl group in protein extract of *Rhizopus oryzae* (**Lin *et al.*, 2013**)

were shown to be involved in the reduction of gold ions and the formation of AuNPs . In this study, the presence of various functional groups in the gold nanoparticles synthesized by the isolates may indicate the likelihood of proteins to stabilize the nanoparticles through a capping mechanism. However, native proteins and the mechanism involved in gold nanoparticle synthesis have yet to be elucidated.
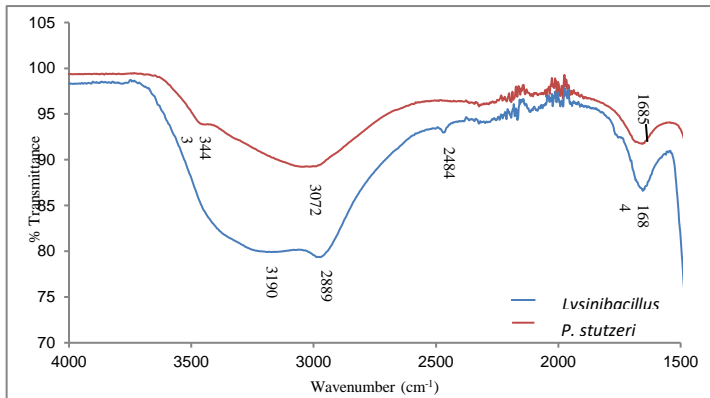

Figure 5 Fourier transform infrared (FT-IR) spectra of the bacterial isolates namely *P. stutzeri* M2-2 and *Lysinibacillus* sp. M1-1
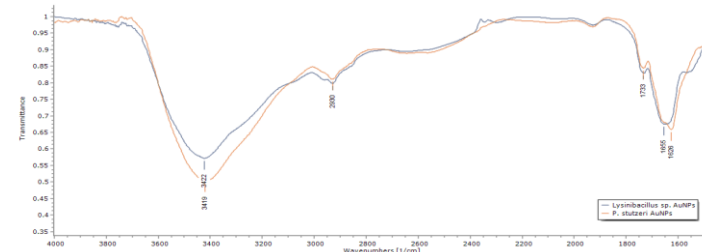

Figure 6 Fourier transform infrared (FT-IR) spectra of gold nanoparticles synthesized by *P. stutzeri* M2-2 and *Lysinibacillus* sp. M1-1
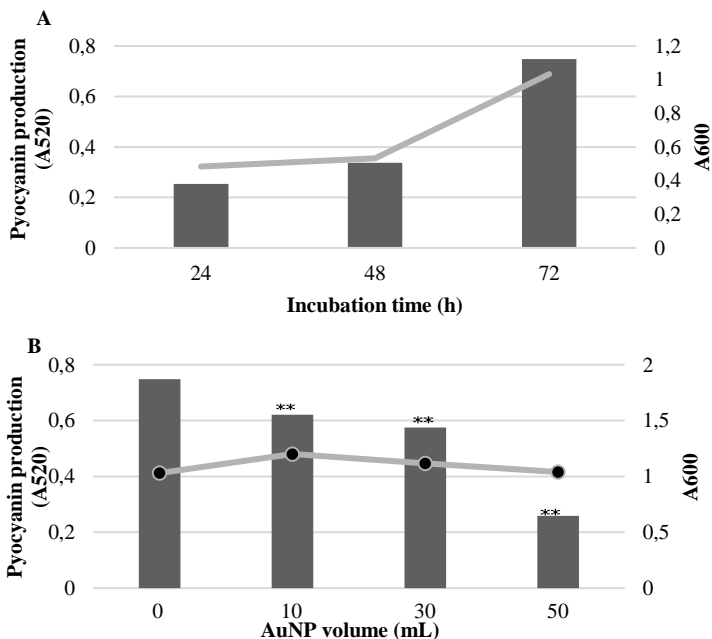
Effect of biosynthesized AuNPs on pyocyanin production

The inhibitory activity of the biosynthesized AuNPs by *Lysinibacillus* sp. M1-1 and *P. stutzeri* M2-2 on the growth and pyocyanin production in *Pseudomonas aeruginosa* PA01 was tested during a 72-hour incubation period. The growth of biomass was monitored as a function of time along with the production of pyocyanin (Figure 7a). Notably, no remarkable inhibition of growth was observed after AuNP treatment (Figure 7b and 7c). The antimicrobial activity of AuNPs has been demonstrated in many microbes and it is attributed to properties on irradiation focusing, strong electrostatic attractions to the cell membrane or conjugation with antimicrobial agents and antibodies. Those properties lead to cell membrane disruption and accumulation of reactive oxygen species leading to cell death (**Huh *et al.*, 2011**). In this study, the absence of growth inhibition possibly suggests a mechanism different from those properties mentioned above.

Figure 7 (a) Growth and pyocyanin production of *P. aeruginosa* PA01 at 24, 48, and 72 h. (b) Effect of AuNPs synthesized by *Lysinibacillus* sp. on pyocyanin production of *P. aeruginosa*. (c) Effect of AuNPs synthesized by *Pseudomonas stutzeri* on pyocyanin production of *P. aeruginosa* PA01. Bars are mean levels (n=3 independent experiments) of pyocyanin production (A_{520}); Lines indicate A_{600} value. Bars of set-ups statistically different from the control group (at $P < 0.05$) are indicated by **

Aside from intrinsic ability of *P. aeruginosa* to develop antibiotic resistance, it is also capable of releasing several virulence factors for host colonization (**Cross, 2008**). Specifically, the synthesis and secretion of the redox-active phenazine exoproduct pyocyanin by *P. aeruginosa* has been the most studied (**Lau *et al.*, 2004**). The production of pyocyanin in *P. aeruginosa* is controlled by the quorum sensing system and the inactivation of this system could provide promising solutions to fight *P. aeruginosa* infections. In the present work, although the synthesized AuNPs showed no toxicity to *P. aeruginosa* PAO1, the biosynthesized AuNPs significantly reduced ($P \leq 0.05$) the quorum-sensing mediated synthesis of pyocyanin (Figure 7b and 7c). Specifically, the gold nanoparticles produced by *Lysinibacillus* sp. M1-1 (3.80×10^{-12} mol/L) demonstrated 17.07 %, 23.10 %, and 65.47 % inhibition of pyocyanin production upon treatment with 10, 30 and 50 μL colloidal AuNPs, respectively. Meanwhile, the gold nanoparticles produced by *P. stutzeri* M2-2 (7.70×10^{-12} mol/L) showed 50.90 %, 62.96 %, 80.81 % inhibition of pyocyanin production using the same volumes of AuNPs (Table 4).

Table 4 Pyocyanin concentration produced by *Pseudomonas aeruginosa* PA01 after 72 hours of incubation in the absence and presence of gold nanoparticles

Volume of AuNPs added	Concentration of pyocyanin (μg/mL) and % inhibition of pyocyanin synthesis in different set-ups				
	Pseudomonas aeruginosa only	*Pseudomonas aeruginosa* + AuNPs synthesized by *Lysinibacillus* sp.	% inhibition of pyocyanin synthesis	*Pseudomonas aeruginosa* + AuNPs synthesized by *Pseudomonas stutzeri*	% inhibition of pyocyanin synthesis
10 μL	12.77	10.59	17.07%	6.27	50.90%
30 μL		9.82	23.10%	4.73	62.96%
50 μL		4.41	65.47%	2.45	80.81%

This observation is consistent with the results of previous investigations on the quorum quenching activity of gold nanoparticles. **Yu *et al.* (2016)** reported that gold nanoparticles synthesized by reduction of HAuCl$_4$ had no significant toxicity to *P. aeruginosa,* but instead inhibited pathogenic biofilm formation. Moreover, the addition of ZnO nanoparticles had no inhibitory effect on the growth of *P. aeruginosa* PAO1 but markedly inhibited virulence factor production (**Lee *et al.*, 2014**; **Garcia-Lara *et al.*, 2015**). Nanoparticles act as quorum quenchers by interfering with bacterial cell-cell communication systems attenuating the expression of several virulence factors without interfering with growth of the pathogen (**Garcia-Lara *et al.*, 2015**). Taken together, the results indicate that the inhibition of the pyocyanin production was related to quorum quenching effect of the gold nanoparticles and not to bacteriostatic or bactericidal activity of the nanoparticles. Recently, the quorum-sensing inhibitory activity of gold nanoparticles synthesized by conventional bioreduction approach using *Clitoria ternatea* methanolic leaf extract has been investigated against pyocyanin production in *P. aeruginosa* (**Vanaraj and Swamiappan, 2016**). However, this current study is the first to examine the inhibitory effect of gold nanoparticles synthesized by bacteria isolated from alkaline environment on virulence factor production in *P. aeruginosa*, such as pyocyanin. Further investigation is required to elucidate the underlying mechanism of inhibition of pyocyanin production by gold nanoparticles.

CONCLUSION

This study demonstrates the ability of microorganisms from highly alkaline environments to synthesize AuNPs. Two alkaliphilic bacteria namely

Lysinibacillus sp. M1-1 and *P. stutzeri* M2-2 were able to biosynthesize AuNPs through reduction of chloroauric acid (HAuCl4) at pH 9. The extracellular synthesis of AuNPs was confirmed by UV-Vis absorbance analysis, SEM, EDX and FTIR analysis. The biosynthesized AuNPs significantly reduced the quorum-sensing regulated process of pyocyanin production in *Pseudomonas aeruginosa* PA01. However, no remarkable growth inhibition on *Pseudomonas aeruginosa* PA01 was observed after biosynthesized AuNP treatments. The exact mechanism of action of AuNPs against pyocyanin production is yet to be determined. The results suggest the potential of AuNPs as therapeutic agents against infection by pathogenic microorganisms while limiting emergence of bacterial resistance.

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