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IDENTIFICATION OF MARINE BACTERIA ISOLATED FROM MARINE SOIL SEDIMENTS AND THEIR ABILITY TO BIOSYNTHESISE AgNPs EXTRACELLULARLY

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Abstract

Over the past few years, nanoparticles synthesis is one of the most active research in the nanotechnology field. The synthesis can be done chemically, physically and biologically. However, some researchers prefer to synthesise it biologically or also known as biosynthesis or 'green synthesis' because it is believed to be safer, environmentally friendly and cost-effective. In this work, we report the extracellular synthesis of 20 isolated marine bacteria from marine soil sediment which were identified and evaluated to synthesise silver nanoparticles (AgNPs). This was done by the addition of silver nitrate ($AgNO_3$) solution with the cell-free supernatant of the isolated marine bacteria at room temperature. The marine bacteria were identified using 16S rRNA identification and neighbour-joining phylogenetic tree were constructed. Identification results showed that the isolated bacteria consist of 19 *Serratia* sp. and 1 *Providencia* sp. The biosynthesised AgNPs colloids were evaluated using morphological and optical analysis. AgNPs were observed for colour change and determined using ultraviolet-visible (UV-Vis) spectrophotometer. The existence of surface plasmon resonance peak at 400 to 450 nm is evidence of AgNPs formation.

INTRODUCTION

Nanotechnology is a branch of science that deals with dimensions and tolerances of 1-100 nm particle size [1, 2]. It is also a field that is rapidly growing as it shows promising applications by creating materials with new properties. Silver nanoparticles (AgNPs) are one of the most used nanoparticles in research and industry due to their high antimicrobial properties and wide applications. AgNPs have been popularly used as antibacterial agents that heal wounds and prevent infections in the health industry. Besides, AgNPs can be applied for food storage, textile coatings, water treatment, cosmetics, nanoscale sensors and catalytic activities [3-10].

AgNPs can be synthesised chemically, physically and biologically. Chemical and physical syntheses produce better AgNPs but it requires high cost, high energy consumption and uses toxic chemicals. Thus, biological synthesis, also known as 'green synthesis', is much preferred as it is environmentally benign. Few researchers have reported that AgNPs can be synthesised using naturally occurring reductants such as bacteria, plant extracts and fungus [11-15]. Among these organisms, the most preferred system is the bacteria as they can be easily handled and manipulated [16]. The first biosynthesis of AgNPs and its distinct size and morphology have been reported by Haefeli et al. [17]. Since then, there is an increase in nanosilver studies using microbes throughout the years.

Although various papers are reporting the use of bacteria as nano-factories, the potential of marine bacteria is least explored. The marine environment, which is the sea, acts as a large reservoir for microorganisms to live in. According to Mazalan et al. [18] the Malaysian marine ecosystem has a vast number of unexploited bacteria. However, some researchers have started to study these marine bacteria using *Ochrobactrum* sp. and its antimicrobial properties towards medically important pathogenic bacteria [19]. Also, a novel strain of Stenotrophomonas isolated from the marine environment was the first to be reported to biosynthesise both AgNPs and AuNPs [20].

In this study, 20 marine bacteria isolated from Port Dickson, Negeri Sembilan, Malaysia were used to biosynthesise AgNPs extracellularly. The synthesis occurred when there were reducing agents that reduce silver ions (Ag^+). The reduction of Ag^+ by the bacteria occurred through the release of reductases in the solution. Thus, this paper proposed that the isolated bacteria can synthesise AgNPs extracellularly.

MATERIALS AND METHODS

Culture and isolation

Soil samples were collected from several beaches at Port Dickson, Negeri Sembilan, Malaysia. Zobell agar plates were made and pure cultures were isolated. The isolated samples were screened for AgNPs synthesis and 20 isolates were cultured in 10 mL Zobell broth at 37℃ on a rotary shaker set at 150 rpm for 24 h.

Identification of isolated bacteria

The identification of the isolated strain began with gram staining procedures. The microscope slides were let to dry and viewed using microscope at 100×magnification. DNA extraction was done using Macherey-Nagel bacterial purification kit by following the protocols given. The extracted DNA was run on agarose gel electrophoresis at 80 V for 45 min. Polymerase chain reaction (PCR) was conducted after the confirmation of the band that appeared upon viewing the gel using Gel Doc. In this PCR, universal forward and reverse primers were used, which were 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3'. It was run using Bio-Rad Thermal Cycler with annealing temperature of 53.5℃ [21]. The PCR products were run on agarose gel electrophoresis at 80 V for 45 min. Then, the PCR products were sent for 16S rRNA sequencing at NextGene. The obtained sequences were used to make phylogenetic trees.

Synthesis of AgNPs

Cultured bacteria in 10 mL Zobell marine broth were centrifuged using an ultracentrifuge at 15000 rpm for 10 min. The supernatant was collected in a 50 mL Falcon tube and the pellet was discarded. One of the supernatants was not added with $AgNO_3$ to be used as a control for the experiment. Then, 10 mL of 0.5 M $AgNO_3$ solution was added into the supernatant and the solution changed its colour from yellow to milky white. The Falcon tube was covered with aluminium foil and left in the dark for 24 h.

Characterisation of AgNPs

Characterization of synthesised AgNPs was done by reading its absorbance using UV-vis spectrophotometer. The formation of AgNPs was monitored using UV-vis spectrophotometer by recording the spectra between 340-500 nm using Jenway 7305 spectrophotometer. The characteristic peaks were monitored simultaneously. Solution spectra were obtained by measuring the absorbance of the AgNPs colloid form in a cuvette with 1 cm optical path.

RESULTS AND DISCUSSION

Sample collection and isolation

Marine soil sediment samples were collected during low tide and brought back to the laboratory for bacterial culture and isolation. All the isolated samples were then screened for extracellular AgNPs synthesis and showed that only 20 isolates that can reduce $AgNO^3$ into AgNPs (**Table 1**). Then, the samples were identified using 16S rRNA identification and gram staining procedures.

Table 1: Sample collection details

Sample	Location	Type of marine sediment
AQ5-NT21	Pantai Purnama, Port Dickson, N. Sembilan, Malaysia 2.4436° N, 101.8559° E	Sandy
AQ5-NT22		
AQ5-NT23		
AQ5-NT24		
AQ5-NT25		
AQ5-NT26		
AQ5-NT27		
AQ5-NT28		
AQ5-NT29	Teluk Kemang, Port Dickson, N. Sembilan, Malaysia 2.4546° N, 101.8599° E	Sandy
AQ5-NT30		
AQ5-NT31		
AQ5-NT32		
AQ5-NT33		
AQ5-NT34	Pantai Cahaya, Port Dickson, N. Sembilan, Malaysia 2.4914° N, 101.8397° E	Sandy
AQ5-NT35		
AQ5-NT36		
AQ5-NT37	Bagan Lalang, Selangor, Malaysia 2.6059° N, 101.6983° E	Muddy
AQ5-NT38		
AQ5-NT39		
AQ5-NT40		

Identification of isolated marine bacteria

Table 2 shows the gram and genus of the 20 isolates based on the 16S rRNA identification results obtained from NextGene Sequencing and BLAST analysis. From the results, 19 bacteria were identified as *Serratia* sp. and 1 was of *Providencia* sp., which were rod-shaped. Besides that, all 20 isolates were Gram-negative. These 20 isolates were named and submitted into the GenBank database since all of them were new and there was no 100% homology with any submitted strains. Strain AQ5-NT37 of *Providencia* sp. is a novel biosynthesis of AgNPs since there is no reported papers on it through our knowledge. Unlike the other 19 isolates, there have been few reports using *S. marcescens* and *Serratia* sp. as silver nano factories [22-26].

Table 2: Gram and genus of the isolated samples.

Sample	Gram	Shape	Genus	GenBank
AQ5-NT21	Negative	Rod	*Serratia* sp.	MN720572
AQ5-NT22	Negative	Rod	*Serratia* sp.	MN720573
AQ5-NT23	Negative	Rod	*Serratia* sp.	MN720574
AQ5-NT24	Negative	Rod	*Serratia* sp.	MN720577
AQ5-NT25	Negative	Rod	*Serratia* sp.	MN720576
AQ5-NT26	Negative	Rod	*Serratia* sp.	MN720579
AQ5-NT27	Negative	Rod	*Serratia* sp.	MN720580
AQ5-NT28	Negative	Rod	*Serratia* sp.	MN720582
AQ5-NT29	Negative	Rod	*Serratia* sp.	MN720595
AQ5-NT30	Negative	Rod	*Serratia* sp.	MN720596
AQ5-NT31	Negative	Rod	*Serratia* sp.	MN720597
AQ5-NT32	Negative	Rod	*Serratia* sp.	MN720598
AQ5-NT33	Negative	Rod	*Serratia* sp.	MN749521
AQ5-NT34	Negative	Rod	*Serratia* sp.	MN720609
AQ5-NT35	Negative	Rod	*Serratia* sp.	MN720610
AQ5-NT36	Negative	Rod	*Serratia* sp.	MN720611
AQ5-NT37	Negative	Straight rods with cell envelope	*Providencia* sp.	MN720613
AQ5-NT38	Negative	Rod	*Serratia* sp.	MN720612
AQ5-NT39	Negative	Rod	*Serratia* sp.	MN720614
AQ5-NT40	Negative	Rod	*Serratia* sp.	MN720615

Phylogenetic tree was constructed to predict the position of the isolates with GenBank data sequence using BLAST, NCBI **(Figure 1)**. A total of 29 DNA sequences were aligned together with *Providencia* spp. as outgroup. The phylogenetic tree portrayed high bootstrap value to support each of the clade forms. All strains except AQ5-NT37 showed a close relation with *Serratia* sp. while AQ5-NT37 was identified as *Providencia* sp. according to its phylogenetic tree, shape, and gram it possessed. The neighbours of AQ5-NT37 are *Providencia rettgeri* strain NCTC 11801 and *Providencia rettgeri* strain DSM 4542, which are opportunistic pathogens and may cause urinary tract infections [27, 28]. Thus, AQ5-NT37 is not suggested to be used as silver nanofactory due to its pathogenicity that may cause harm towards humans. *Providencia vermicola* strain OP1 also clade together with isolate AQ5-NT37 with bootstrap value of 99 which indicates the closest related genus.

The nearest neighbours for the other 19 isolates are *Serratia liquefaciens* strain CIP 103238, *Serratia quinivorans* strain 4364, *Serratia quinivorans* strain LMG 7887, *Hafnia psyhcrotolerans* strain DJC1-1, *Rahnella victoriana* strain FRB 225 and *Rahnella bruchi* strain FRB 226. All of them clade together with all the isolates except AQ5-NT37. It can be said that they are closely related to the genus *Serratia* spp., *Hafnia* sp. and *Rahnella* spp because they share the same ancestors and it shows high bootstrap values of 90 and 99 on the respective nodes.

Synthesis of AgNPs

The biosynthesis of AgNPs by the 20 isolates was determined by the colour change of the supernatant with the presence of 0.5 M of $AgNO_3$ solution (Figure 2). The observations indicated that the extracellular components of the isolates have successfully reduced the Ag^+ into Ag^0, thus causing the change of colour of the solution from milky white into brown within 72 h incubation at room temperature in the dark. According to Dzul-Erosa et al. [29] the colour change is due to the excitation of surface plasmon resonance of the AgNPs. It is also considered as evidence of the formation of AgNPs.

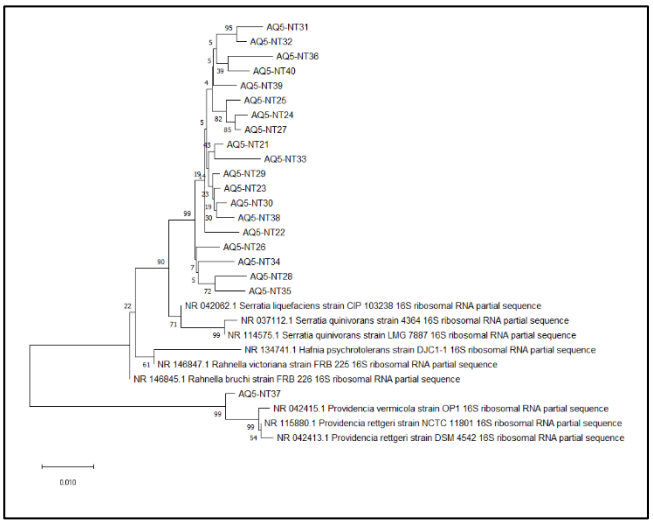


Figure 1: Neighbour-joining tree of 20 isolated marine bacteria from Port Dickson, N. Sembilan, Malaysia and Bagan Lalang, Selangor, Malaysia.

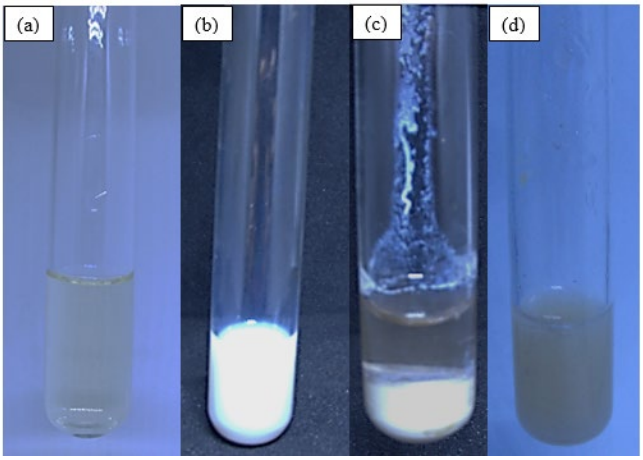


Figure 2: Colour of the solution of a) cell-free supernatant of isolated marine bacteria, b) addition of AgNO3 into the supernatant, c) 72 h incubation in the dark and d) AgNPs colloid at 72 h.

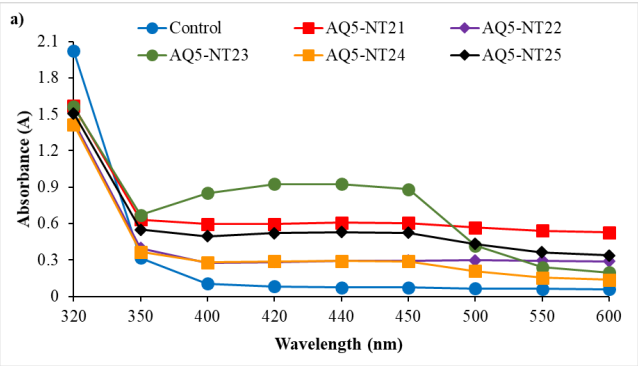
In **Figure 2**, there were two layers observed after 72 h of incubation in the dark. The top layer was liquid and is known as the AgNPs colloid while the bottom layer was sediment formed by the reaction of the cell-free supernatant of marine bacteria and the $AgNO_3$ solution. However, researchers usually use the colloid form of AgNPs for further tests as it is monodispersed and stable compared to the sediment, which contains polydispersed AgNPs. It was also noted that the brown colour of the colloid will intensify and become darker when being kept over time because of the surface plasmon resonance.

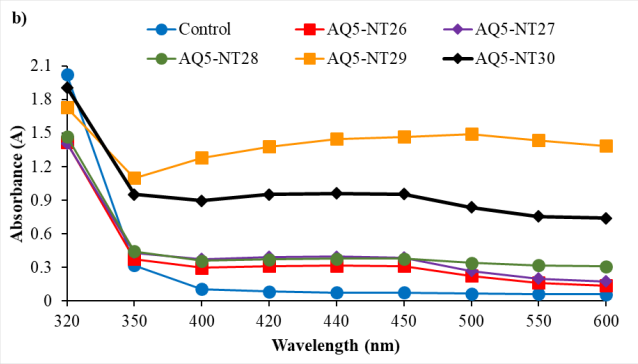
Some researchers reported that the formation of AgNPs by the presence of nitrate reductase enzyme in the bacteria. This is

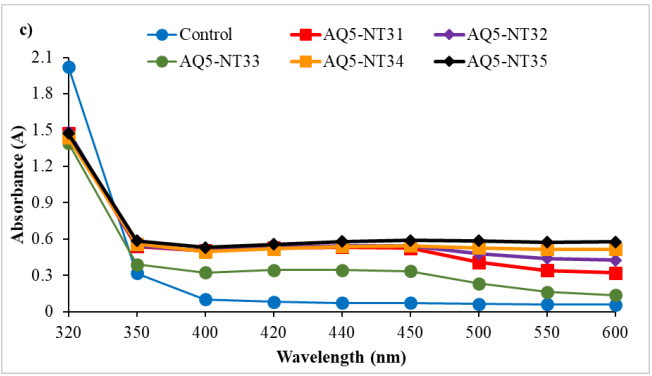
because some bacteria metabolic pathways require nitrate as a major source of nitrogen. The nitrate will then be converted into nitrite by nitrate reductase enzymes [30, 31].

Characterization of AgNPs

Based on **Figure 3**, AQ5-NT23, AQ5-NT29, AQ5-NT35 and AQ5-NT39 showed the highest peak, respectively. However, all the isolates have peak within the range of 400 to 500 nm as none of it was aligned with the control. AQ5-NT21 and AQ5-NT22 only showed their peak at 440 nm with the absorbance of 0.606 and 0.291, respectively. This shows that the production of AgNPs by AQ5-NT22 was much lower compared to AQ5-NT21. On the other hand, AQ5-NT24, AQ5-NT25, AQ5-NT26, AQ5-NT27, AQ5-NT28, AQ5-NT30, AQ5-NT31, AQ5-NT32, AQ5-NT33, AQ5-NT34, AQ5-NT35, AQ5-NT36, AQ5-NT37 and AQ5-NT38 showed their peaks between 420 to 440 nm, which indicate that the AgNPs produced may have an intermediate size of 1 to 100 nm. Meanwhile, AQ5-NT29 may produce various sizes of AgNPs because of the peaks shown from 400 to 500 nm. Similar peaks were observed at 420 to 500 nm by AQ5-NT39. Thus, this UV-Vis spectra results confirmed the biosynthesis of AgNPs by the 20 isolates as according to Dzul-Erosa et al. [29] AgNPs' characteristic peak is specific at 400 to 450 nm. Besides that, it is related to the excitation of surface plasmon resonance that was mentioned earlier. From these UV-Vis results, it can be inferred that the surface plasmon resonance position of synthesised AgNPs exhibits exceptional size-dependence [32]. This statement also supported by Prakoso et al. [33] who stated that the position and shape of the surface plasmon absorption bands are dependent on the shape, dispersity and size of the nanoparticles.







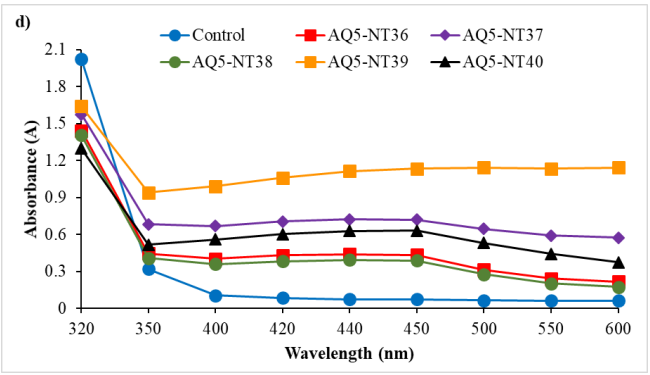


Figure 3: UV-Vis Spectra of isolated marine samples with control for a) AQ5-NT21 to AQ5-NT25, b) AQ5-NT26 to AQ5-NT30, c) AQ5-NT31 to AQ5-NT35 and d) AQ5-NT36 to AQ5-NT40.

CONCLUSION

In the current study, we have reported that the 20 isolated marine bacteria from marine soil sediments which consist of *Serratia* sp. and *Providencia* sp. can biosynthesise AgNPs extracellularly at room temperature. The molecules or proteins in the cell-free culture supernatant present acted as a stabilizing and reducing agents to convert $AgNO_3$ solution into AgNPs. It is also noted that there might be the presence of nitrate reductase enzymes as stated by some papers. However, extended analysis for the biosynthesised AgNPs should be done by using High-Resolution Transmission Electron Microscopy (HR-TEM), Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD) and Dynamic Light Scattering (DLS)which will provide the deeper insights on the size and shape of AgNPs, and determine the functional groups that help in converting Ag^+ to Ag^0.

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REFERENCES

1. Williams D. (2008). The relationship between biomaterials and nanotechnology. *Biomaterials,* 29: 1737-1738.

2. Ehrlich, H. (2019). *Biomaterials and biological materials.* In: Marine Biological Materials of Invertebrate Origin. Springer, Cham.
3. Zhong, L. S., Hu, J. S., Cui, Z. M., Wan, L. J. and Song, W. G. (2007). In-situ loading of noble metal nanoparticles on hydroxyl-group-rich titania precursor and their catalytic applications. *Chemistry Materials,* 19: 4557-4562.
4. Duran, N., Marcato, P. D., De Souza, G. I. H., Alves, O. L. and Esposito, E. (2007). Antibacterial effect of silver nanoparticles produced by fungal process in textile fabrics and their effluent treatment. *Journal of Biomedical Nanotechnology,* 3: 203-208.
5. Bhattacharya, R. and Mukherjee, P. (2008). Biological properties of "naked" metal nanoparticles. *Advanced Drug Delivery Reviews,* 60: 1289-1306.
6. Li, Q., Mahendra, S., Lyon, D. Y., Brunet, L., Liga, M. V., Li, D. and Alvarez, P. J. J. (2008). Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. *Water Research,* 42: 4591-4602.
7. Manno, D., Filippo, E., Di Giulio, M. and Serra, A. (2008). Synthesis and characterization of starch-stabilised Ag nanostructures for sensors applications. *Journal of Non-Crystalline Solids,* 354: 5515-5520.
8. Liu, P. and Zhao, M. (2009). Silver nanoparticle supported on halloysite nanotubes catalysed reduction of 4-nitrophenol (4-NP). *Applied Surface Science,* 255: 3989-3993.
9. Smith, B. R. and Gambhir, S. S. (2017). Nanomaterials for in vivo imaging. *Chemical Reviews,* 117: 901-986.
10. Lee, S. H. and Jun, B. H. (2019). Silver nanoparticles: synthesis and application for nanomedicine. *International Journal of Molecular Sciences,* 20: doi:10.3390/ijms20040865.
11. Iravani, S. (2011). Green synthesis of metal nanoparticles using plants. *Green Chemistry,* 13: 2638-2650.
12. Mittal, A. K., Chisti, Y. and Banerjee, U. C. (2013). Synthesis of metallic nanoparticles using plant extracts. *Biotechnology Advances,* 31: 346-356.
13. Akhtar, M. S., Panwar, J. and Yun, Y. S. (2013). Biogenic synthesis of metallic nanoparticles by plant extracts. *ACS Sustainable Chemistry and Engineering,* 1: 591-602.
14. Mittal, J., Batra, A., Singh, A. and Sharma, M. M. (2014). Phytofabrication of nanoparticles through plant as nanofactories. *Advances in Natural Sciences: Nanoscience and Nanotechnology,* 5: 1-11.
15. Tran, Q. H., Nguyen, V. Q. and Le, A. T. (2018). Silver nanoparticles: synthesis, properties, toxicology, applications and perspectives. *Advances in Natural Sciences: Nanoscience and Nanotechnology,* 9: 2-23.
16. Vaidyanathan, R., Gopalram, S., Kalishwaralal, K., Deepak, V., Pandian, S. R. and Gurunathan, S. (2010). Enhanced silver nanoparticle synthesis by optimization of nitrate reductase activity. *Colloids and Surfaces B: Biointerfaces,* 75: 335-341.
17. Haefeli, C., Franklin, C. and Hardy, K. (1984). Plasmid-determined silver resistance in Pseudomonas stutzeri isolated from silver mine. *Journal of Bacteriology,* 158: 389-392.
18. Mazalan, N., Zain, M. M. and Hamzah, A.S. (2012). Antimicrobial activity of marine bacteria from Malaysian coastal area. *2012 IEEE Symposium on Humanities, Science and Engineering Research,* 32: 1273-1277.
19. Thomas, R., Janardhanan, A., Varghese, R. T., Soniya, E. V., Mathew, J. and Radhakrishnan, E. K. (2014). Antibacterial properties of silver nanoparticles synthesised by marine Ochrobactrum sp. *Brazilian Journal Microbiology,* 45: 1221-1227.
20. Malhotra, A., Dolma, K., Kaur, N., Rathore, Y. S., Ashish., Mayilraj, S. and Choudhury, A.R. (2013). Biosynthesis of gold and silver nanoparticles using a novel marine strain of Stenotrophomonas. *Bioresource Technology,* 142: 727-731.
21. Subramaniam, K., Mazuki, T. A. T., Shukor, M. Y. and Ahmad, S. A. (2019). Isolation and optimisation of phenol degradation by Antarctic isolate using one factor at time. *Malaysian Journal of Biochemistry and Molecular Biology,* 22: 79-86.
22. Khalaf, K. J. (2009). In vitro and In vivo effects of silver nanoparticles biosynthesized by Serratia marcescens. *Journal of Global Pharma Technology,* 10: 409-417.
23. Lakshmipathy, M. and Nanda, A. (2013). Biocatalysts of silver nanoparticles synthesized using an environmental isolate, Serratia marcescens S01. *International Journal of ChemTech Research,* 5: 1162-1168.
24. Akilandeswari, K., Karpagam, P. and Amutha, K. (2014). Rapid biosynthesis, characterization and antimicrobial effects of silver nanoparticles from microorganism Serratia marcescens. *International Journal of Molecular Biology and Biochemistry,* 2: 15-24.
25. Ajah, H. A., Khalaf, K. J. and Hasan, A. M. (2018). Extracellular synthesis of silver nanoparticles using Sphingomonas paucimobilis, *Serratia* sp. and *Pseudomonas aeruginosa* and their antimicrobial activity. *Indian Journal of Public Health Research and Development,* 9: 911-915.
26. Adan, M. F. Y., Baybay, Z. K., Lantican, N. B., Fernando, L. M., Paterno, E. S., Villegas, L. C., Ilag, L. L. and Montecillo, A. D. (2018). Silver nanoparticles extracellularly produced by *Serratia* sp. NBL1001 have antibacterial properties. *Philippine Science Letters,* 11: 75-83.
27. Hawkey, P. M. (1984). Providencia stuartii: a review of a multiply antibiotic-resistant bacterium. *Journal of Antimicrobial Chemotherapy,* 13: 209-226.
28. O' Hara, C. M., Brenner, F. W. and Miller, J. M. (2000). Classification, identification, and clinical significance of Proteus, Providencia, and Morganella. *Clinical Microbiology Reviews,* 13: 534-546.
29. Dzul-Erosa, M. S., Cauich-Diaz, M. M., Razo-Lazcano, T. A., Avila-Rodriguez, M., Reyes-Elamawi, R. M., Al-Harbi, R. E. and Hendi, A. A. (2018). Biosynthesis and characterization of silver nanoparticles using Trichoderma longibrachiatum and their effect on phytopathogenic fungi. *Egypt Journal of Biological Pest Control,* 28: doi:10.1186/s41938-018-0028-1.
30. Li, G., He, D., Qian, Y., Guan, B., Gao, S., Cui, Y., Yokoyama, K. and Wang, L. (2012). Fungus-mediated green synthesis of silver nanoparticles using Aspergillus terreus. *International Journal of Molecular Sciences,* 13: 466-476.
31. Guilger-Casagrande, M. and de Lima, R. (2019). Synthesis of silver nanoparticles mediated by fungi: A review. *Frontiers in Bioengineering and Biotechnology,* 7: doi:10.3389/fbioe.2019.00287.
32. Peng, S., McMahon, J. M., Schatz, G. C., Gray, S. K. and Sun, Y. (2010). Reversing the size-dependence of surface plasmon resonances. *Proceedings of the National Academy of Sciences of the United States of America,* 107: 14530-14534.
33. Prakoso, S. P., Taufik, A. and Saleh, R. (2017). On-step microwave-assisted colloidal synthesis of hybrid silver oxide/silver nanoparticles: characterization and catalytic study. *IOP Conference Series: Materials Science and Engineering,* 188: doi:10.1088/1757-899x/188/1/012021.