PRODUCTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES PRODUCED BY *PEDIOCOCCUS ACIDILACTICI*

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In this study antimicrobial potential of silver nanoparticles (AgNPs) synthesised by *Pediococcus acidilactici* against non bacteriocin producing *Bacillus subtilis* and its antimicrobial potential on human pathogens were evaluated. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) confirmed that both AgNPs preparations were consistent in their spherical shape, with average diameters of 16 nm and 12 nm derived from culture free supernatants of *P. acidilactici* and *B. subtilis*, respectively. The presence of functional groups over the surface of nanoparticles was confirmed by Fourier transform infrared spectroscopy (FTIR) and Energy dispersive X-ray (EDX) analysis. Minimum inhibitory concentration (MIC) of AgNPs displayed no significant difference ($p>0.05$) in antibacterial activity. Antifungal activities of AgNPs preparations were statistically significant ($p<0.05$) in the case of *C. albicans* and *C. krusei*. These results highlights that sunlight irradiation, a relatively unexplored primary energy source was able to photo-catalyse nanoparticle formation completely and AgNPs synthesised by *Pediococcus acidilactici* and *B. subtilis* extracts with potential for application as broad-spectrum antimicrobial agents.

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1. Introduction

Microbial development of drug resistance is a complex phenomenon driven by various intricate factors, of which the foremost is through the improper use of antibiotics [1, 2]. This is due to the inherent ability of microbes to rapidly develop resistance mechanisms when a subset of the microbial population survives exposure to the drug [3]. The antimicrobial effects of silver salts although not well understood has been in use since ancient times [4]. As stated, with the increasing prevalence of microbial drug-resistance, the application of this metal using modern technologies to generate its nano-particulate form has resurfaced as a promising antimicrobial candidate [4, 5].

In recent years, ongoing advancements in nanotechnology have led to the development of greener alternatives for AgNP synthesis wherein environmentally inert capping agents are utilised in the reduction of metal ions [6, 7]. To this end, a variety of capping substrates from diverse biological sources have been explored for AgNP synthesis including plants, algae, fungi, yeast, bacteria, mammalian cells and viruses [8-10]. Incidentally, laboratory based nanoparticle synthesis from extracts of a biological resource is favored over their biosynthesis in living organisms due to

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several distinct advantages including: short reaction times; ease of handling extracts compared to whole organisms; high yield of nanoparticles and importantly, the lack of costly downstream processing required to obtain to the produced particles [11].

The use of bacterial extracts as reducing agents in the production of AgNPs have been reported with optimised reaction times that are comparable to chemical approaches for AgNP synthesis [12]. Furthermore, the abundance and rapid growth rate of bacteria coupled to the possibility of their genetic modification for increased production of a well-defined capping substrate marks these organisms as promising candidates for AgNP production [9]. Accordingly, the present study focused to evaluate the bio efficacies of AgNPs generated using sunlight as energy source to drive nanoparticle formation from an antimicrobial peptide producing bacterium *Pediococcus acidilactici* PAC1.0. to their counterparts generated from a non-producing strain. The data presented in this study seemingly suggest that AgNPs produced are interesting candidates for further development towards controlling drug-resistant microbial infections.

2. Experimental section

2.1 Materials

All chemicals, solvents and media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa, unless stated otherwise. Antibiotics were purchased from Sigma Aldrich, Germany.

2.2 Microbial Strains

The bacteriocin producing strain *P. acidilactici* PAC1.0.was kindly provided Professor L.M.T Dicks from the Department of Microbiology, Stellenbosch University. A non-producing *Bacillus subtilis* strain was obtained from the Department of Biochemistry, University of Kwa-Zulu Natal. Strains used in bioactivity assays were from the American Type Culture Collection (ATCC): *Staphylococcus aureus* (43300); *Enterococcus faecalis* (5129) (Gram-positive); *Escherichia coli* (35218); *Pseudomonas aeruginosa* (27853); *Klebsiella pneumoniae* (700603) (Gram-negative); *Candida albicans* (10231); *Candida krusei* (6258) and *Candida parapsilosis* (22019).

2.3 Preparation of Culture free supernatants (CFSs)

Preparation of CFSs was conducted by the method of Gonzalez and Kunka [13]. Briefly, 100 ml of de Man, Rogosa and Sharpe (MRS) broth was individually inoculated (1%) with overnight cultures of *P. acidilactici* and *B. subtilis* grown in the same medium. Cultures were incubated statically at 35°C for 24 h and centrifuged at 4000 rpm for 10 min. The CFSs were filter sterilised (0.45 µm filters, (GVS, USA) and subsequently stored at 4°C . The spot on lawn method was used to confirm bacteriocin production by *P. acidilactici* and non-production by *B. subtilis* [14]. A clear zone of inhibition around spotted inoculum areas indicated a positive result for the presence of bacteriocins in the CFS. The total protein content of CFSs was determined using the microplate procedure of the BCA Protein Assay (Pierce 23225, Thermo Scientific, USA) according to the manufacturer's specifications. Absorbance of test wells at 562 nm were determined using a multimodal plate reader (Biotek Synergy HT, USA) with Gen 5 software (Biotek Synergy HT, USA Ver 2.01.14).

2.4 Synthesis of silver nanoparticles

Preparation of reaction solutions for AgNP synthesis was adapted from Shah Verdi *et al.* [12]. An aliquot (5 ml) of *P. acidilactici* CFS extract was added to 50 ml of aqueous $1x10^{-3}$ M silver nitrate ($AgNO_3$) contained in a 100 ml clear glass reaction vessel. The aforementioned reaction was also performed using 5 ml of *B. subtilis* CFS extract. To drive formation of AgNPs, the reaction mixtures were exposed to direct sunlight. Colour change of the reaction mixtures were monitored to determine nanoparticle formation which is indicated by a dark brown colour. Once colour intensities of the solutions reached a maximum, the vessels were removed from sunlight and stored in darkness at room temperature to prevent agglomeration of the nanoparticles. A 50 ml

aliquot of $AgNO_3$ containing 5 ml distilled water was processed as described above and used as a negative control [15]. Preliminary characterisation of synthesised AgNPs was carried out by UV-Vis spectral analysis at room temperature as function of time using Specord 210 Analytikjena, Germany.

2.5 Purification and concentration of silver nanoparticles

The purification and concentration of AgNPs from the final reaction mixture was adopted from the method available in literature, [12]. Reaction mixtures were split into two equal parts and transferred to pre-weighed sterile 50 ml centrifuge tubes (United scientific, South Africa). The preparations were then centrifuged at 4000 rpm for 2 h (Eppendorf centrifuge 5810 R, Germany), at 4°C. Supernatants were discarded and the pellet washed in 10 ml of sterile distilled water before centrifugation for 1 h as per the conditions described above. This wash step was repeated twice to remove water soluble biomolecules such as proteins and cellular metabolites. One half of each replicate was then dried in an oven at 37°C for 24 h to determine the dry mass of the AgNPs (difference between mass of tube + nanoparticles, and mass of tube), whilst the other half of each replicate was reconstituted in 1 ml of distilled water. The mass of each dried pellet was applied as the equivalent mass of its corresponding reconstituted pellet since each replicate was equally split. Thus, the concentration of AgNPs was determined on a mg/ml basis. Dry AgNP samples were kept at room temperature whilst reconstituted samples were stored at 4°C prior to use.

2.6 Characterisation and analysis of AgNPs

2.6.1 UV-Visible spectral analysis

Characterisation techniques were adopted from Gannimani *et al*.[16]. The production of pure Ag ions was determined by analysing each solution, after 24 h, using a SHIMADZU UV-2600 UV–VIS Spectrophotometer (Japan) at a range of 190–900 nm at a medium scan speed. One mM AgNO3 solution was used as a blank.

2.6.2 Scanning Electron Microscopy (SEM) Measurements

Morphological characteristics of AgNPs synthesised were carried out using scanning electron microscopy (FEGSEM Zeiss Ultraplus). Samples prepared by adding a drop of nanoparticle solution over the carbon tape glued over metal stub were then coated with gold and observed at 10000 X magnification followed by EDX analysis at 20 kV to determine the elemental composition of the particles. AZTEC software (Oxford Instruments NanoAnalysis, Ver 1.2), was used for the analysis.

2.6.3 Transmission Electron Microscopy (TEM) Measurements

Reconstituted AgNPs solutions were first sonicated for 15 min to disrupt any possible aggregates prior to TEM analysis. A small amount of this sonicated suspension was placed onto a carbon-coated copper grid which was then dried under a lamp. Sample images were obtained using a JEOL TEM (1010) at an accelerating voltage of 100 kV. The sizes of the nanoparticles and data analysis of the sizes was accomplished using ITEM (Soft imaging system, Germany Ver 5.0).

2.6.4 Transform Infrared (FTIR-ATR) Spectroscopy Measurements

The nanoparticle colloidal solution was centrifuged at 4000 rpm for 20 min and washed with distilled water to remove any unreacted materials. The nanoparticle pellet thus obtained was dried in a desiccators containing calcium chloride and thereafter used for FTIR analysis using Perkin Elmer Spectrum One, FTIR-ATR to identify biological functional groups capping the surface of the nanoparticles. The respective spectra were obtained using Spectrum Analysis Software.

2.7 Antimicrobial bioassays

2.7.1 Antibacterial assay

Minimum Inhibitory Concentration (MIC) defined as the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after overnight incubation, was

determined by monitoring the growth of bacteria in a microplate reader (Synergy HT, BioTek Instruments) at 630 nm by micro dilution method as per NCCLS guidelines [17]. The bacterial test cultures used in this study were *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 43300), *Enterococcus faecalis* (ATCC 5129) and *Pseudomonas aeruginosa* (ATCC 27853). Serial twofold dilutions of AgNPs solutions were prepared in sterile 96-well plates over the range of 200-1.25 µg/ml. The wells were then inoculated with diluted overnight broth culture initially adjusted to 0.5 McFarland turbidity standards and incubated at 35°C for 24 hours. Neomycin (Sigma) served as a positive control; MIC was recorded as the lowest concentration at which no growth was observed. All experiments were carried out in triplicates.

2.7.2 Antifungal assay

The MIC values were determined using the standard broth microdilution method according to M27-A2 (for yeast) as per Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. Briefly, yeast strains, viz. *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were grown aerobically overnight at 35 °C on Sabouraud dextrose agar (Merck, South Africa) plates. Yeasts were harvested and suspended in 1% sterile saline solution and the turbidity of the supernatants measured spectrophotometrically at 625 nm with an absorbance of 0.08–0.1 equivalents to the No. 0.5 McFarland standard following the NCCLS M27-A2 guidelines. The working suspension was diluted 1:20 in a mixture containing RPMI 1640 medium and 0.165 M morpholinepropanesulfonic acid (Sigma-Aldrich, South Africa) buffered to pH 7.0. The working suspension was further diluted with the medium (1:50) to obtain the final test inocula ($1–5 \times 10^3$ CFU ml^{-1}). The microtitre plates were allowed to thaw and equilibrate to room temperature under aseptic conditions, which contained serial two-fold dilutions of Ag nanoparticles, which was prepared in Sabouraud dextrose broth. One hundred-microliter aliquots of working inoculum suspensions were dispensed into each well, and the plates were then incubated in an aerobic environment at 35 °C for 24 h. After incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5- (3- carboxymethoxyphenyl)-2-(4-18sulfophenyl)-2H-tetrazolium salt (Promega Corporation, USA) was added directly to each well, incubated at 37 °C for 4 h, and the absorbance was recorded at 490 nm on a 96-well plate reader (BioTek Synergy Instruments Inc., USA) using the GEN 5 Ver. 2.01.14 software (USA). Amphotericin B was used as a standard for the comparison of antifungal activity. IC50 values for MIC assay against different fungal pathogens were analyzed. All experiments were carried out in triplicates.

2.8 Statistical analysis

All data were statistically analysed using SPSS version 21. All percentage data obtained from the biological assays were arcsine transformed and analysed for normality ($p>0.05$) using a Kolmogorov-Smirnov test. Parametric data (which in some cases the consequence of transformation) were subjected to an analysis of variance (*ANOVA*). Differences were considered significant at the 0.05 level and where possible, means were separated by a Tukey post-hoc test. Nanoparticle size was analysed for normality as above and subjected to an independent sample t-test.

3. Results and discussions

3.1 Production of AgNPs from CFS

The reaction mixtures for AgNP synthesis from CFS of both bacterial strains initially displayed a faint yellow colour (Fig. 1a & Fig. 1c). Upon exposure of reaction vessels to direct sunlight, a brown colour immediately started to develop which signified the reduction of Ag^+ to the resulting AgNPs [19]. High brown colouration intensity was observed at 1-hour indicating completion of AgNP synthesis (Fig. 1b & Fig. 1d). No colour change was observed in the negative control solution. This finding is in agreement with similar approach for AgNP production and reported a reduction time of 80 minutes using the CFS of *Bacillus amyloliquefaciens* [20]. Our

data suggests that sunlight irradiation may provide a viable alternative to non-renewable energy to drive AgNP formation at acceptable reaction times.

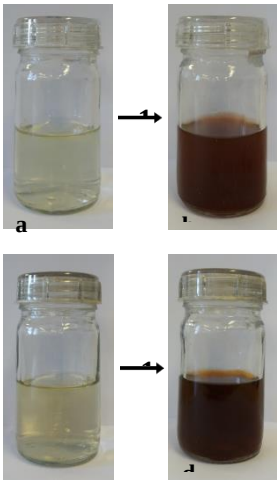

Fig. 1. Colour change of reaction solutions containing CFSs of P. acidilactici (a & b) and B. subtilis (c & d) with $AgNO_3$ at 0 h and 1 h, respectively

3.2 UV -Visible studies

Surface plasmon resonance a unique optical phenomenon of metal nanoparticles, in the UV region enables their easy detection. In this study, AgNP formation was noted at 1 hour by the observed colour change of reaction solutions, irrespective of the bacterial CFS used. Fig. 2 illustrates the UV-visible absorbance spectra of reaction mixtures of both AgNP solutions displaying broad peaks in the range of 340-530 nm. Absorbance associated at wavelengths in this range are typical of spherical AgNPs due to their surface plasmon [21]. Furthermore, broadened peaks indicated that the AgNPs are polydispersed. This finding is in accordance with [20] who used a similar approach for AgNP production and reported a reduction time of 80 minutes using the CFS of *Bacillus amyloliquefaciens*. There were no peaks observed for the negative control. This confirms that the CFS of each bacterium reduced Ag^+ for the subsequent generation of AgNPs.

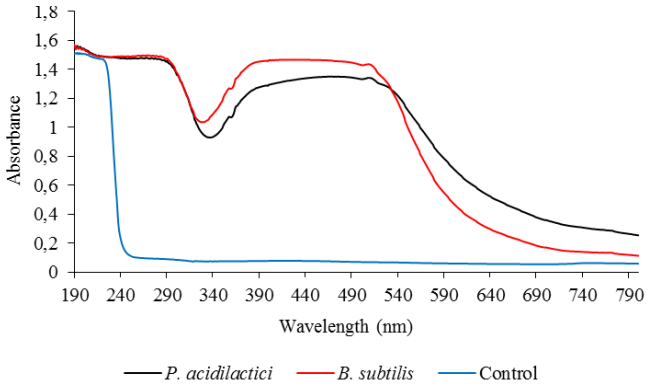

Fig. 2. UV-Vis spectral profiles of AgNP containing solutions derived from CFSs of P. acidilactici and B. subtilis and control solution after 1 h of reaction

3.3 SEM and TEM Analysis

SEM analysis confirmed that particles are of a nano-size. The spherical shape of the nanoparticles is shown in the SEM images (Fig. 3a & Fig. 3c). Furthermore, the particles appear to be agglomerated, possibly because of the nature of sample preparation comprising of several

centrifugation steps and their subsequent dehydration. As such, conclusive morphological characteristics could not be determined with SEM analysis. EDX spectral analysis of both AgNP preparations revealed a strong well defined silver signal at approximately 3 keV, typical for metallic silver. In addition, weaker signals corresponding to carbon, oxygen and nitrogen were observed and they possibly originate from biomolecule capping structures bound to the AgNP surface (Fig. 3b & Fig. 3d).

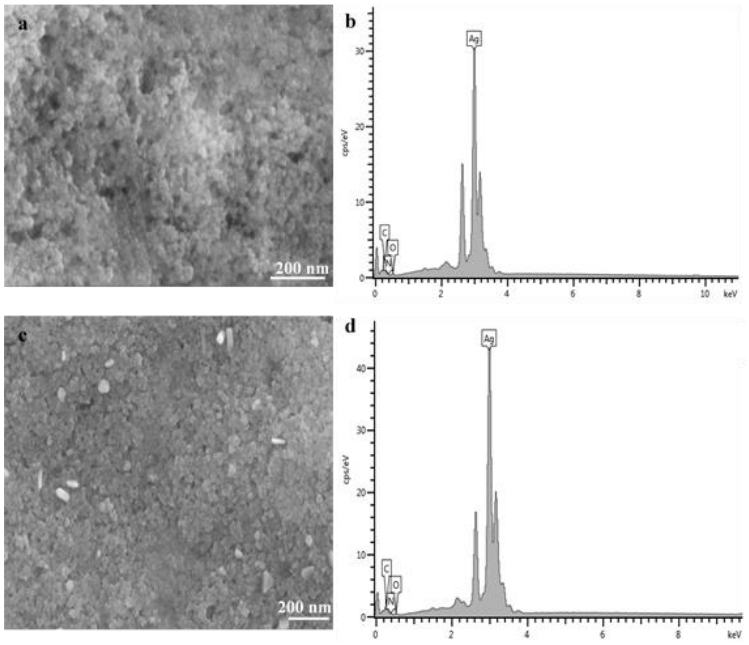

Fig. 3. SEM images and EDX spectra of AgNPs derived from CFSs of P. acidilactici (a and b) and B. subtilis (c and d), respectively

TEM analysis of AgNPs from both preparations confirmed that nanoparticles are spherical in shape and appear well dispersed (Fig. 4a & Fig. 4b). In addition, the analysis did not require samples to be subjected to physical dehydration, which as previously described in SEM analysis, could potentially lead to nanoparticle agglomeration thereby limiting their precise characterisation. Very interestingly, TEM analysis also revealed that the prepared AgNPs from both bacterial CFSs seem to have a surface coating. It can be tentatively suggested that the surface coating seems important in preventing their agglomeration as observed from the high incidence of mono-nanoparticles. This seemingly suggests that biomolecule capping structures present in both CFS extracts co-function as reducing and stabilising agents. These finding are in agreement with previous studies using biological substrates [22].

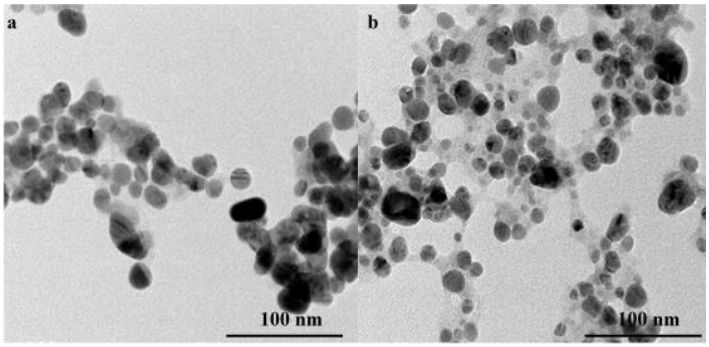

Fig. 4. TEM images of AgNPs derived from CFS of (a) P. acidilactici and (b) B. subtilis

Size and size class distribution studies indicated narrow size distributions (Fig. 5a & Fig. 5b) with average diameters of 16±3.9 nm and 12±3.1 nm for AgNPs prepared from *P. acidilactici* and *B. subtilis* CFS (p>0.05, t-test), respectively.

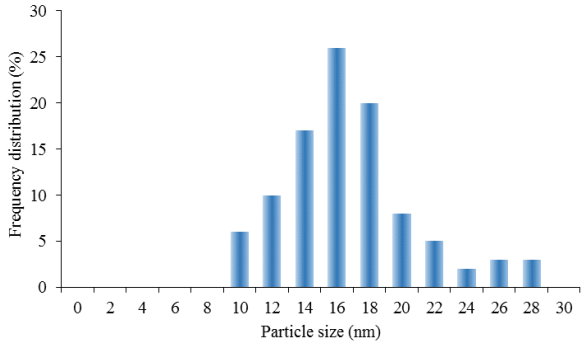

Fig. 5a. Size class distribution of AgNPs derived from P. acidilactici CFS

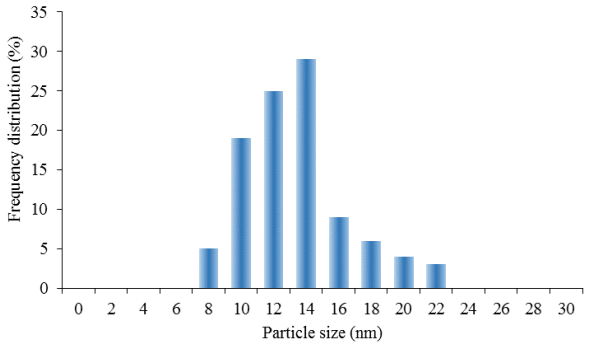

Fig. 5b. Size class distribution of AgNPs derived from B. subtilis CFS

3.4 FTIR-ATR studies

FTIR analysis was conducted to determine the biomolecules responsible for capping and subsequent synthesis of AgNPs. For the analysis, dry preparations of purified AgNPs were used. AgNPs prepared from CFS of *P. acidilactici* indicated major absorbance peaks at 3095; 1564 and 1400 cm^{-1} (Fig. 6a). These peaks are understood to arise from N-H stretching vibrations of primary amines (NH_2) and secondary amines (NH) and carbonyl vibrations of amide I ($CONH_2$) and amide II (CONH) groups. Likewise, analysis of AgNPs from CFS of *B. subtilis* indicated major peaks at 3448; 3146; 2875; 1574 and 1400 cm^{-1} which also correspond to amine and amide functional groups (Fig. 6b).

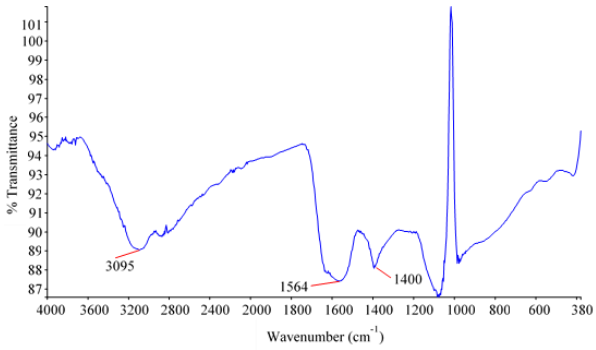

Fig. 6a. FTIR spectrum of AgNPs derived from P. acidilactici CFS

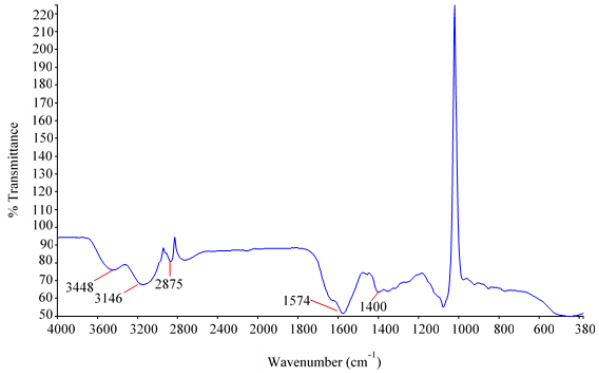

Fig. 6b. FTIR spectrum of AgNPs derived from B. subtilis CFS

3.5 Bioactivity of AgNPs

The antimicrobial effects of Ag and its counterparts are known since antiquity [23]. However, these agents have limited effectiveness in a biological setting due to the interfering effects of salts and the discontinuous release of inadequate amounts of Ag^+ from the metal. In contrast, AgNP formulations have been shown to overcome these limitations and are therefore promising candidates in the development of novel antimicrobial agents [24]. The antimicrobial activities of AgNPs derived from bacterial CFS extracts are relatively unexplored in terms of their MIC values. The AgNP preparations of this study displayed strong antimicrobial activities irrespective of the bacterial extract used and are comparable to AgNPs derived from other biological extracts. In the present study, the antimicrobial effect of varying concentrations (6.25-200 µg ml^{-1}) of bacterial-derived AgNPs was quantitatively assessed based on the growth of cells treated with AgNPs in comparison to the growth of non-treated cells.

3.5.1 Antibacterial studies

AgNP preparations from both bacterial CFSs displayed inhibition against Gram-positive and Gram-negative bacteria **(Table 1)**. The highest MIC values in this study were noted for *K. pneumoniae* and *S. aureus* (25 µg ml^{-1}) whilst *E. coli* displayed most sensitivity (6. 25 µg ml^{-1}) to both AgNP preparations. These findings are in accordance with previous AgNP studies in which activities for *K. pneumoniae* and *S. aureus* were either low or absent [25-26]. Interestingly, *E. faecalis* displayed sensitivity to AgNPs prepared from *P. acidilactici* CFS at a two-fold decreased concentration when compared to AgNPs prepared from *B. subtilis* CFS ($p<0.05$). Since *E. faecalis* initially displayed sensitivity to the pediocin PA-1 containing CFS, this may tentatively suggest that pediocin PA-1 probably participated in Ag^+ reduction and in so doing enhanced the bioactivity of its nanoparticle formulation. Furthermore, the data seems to suggest that in certain instances, AgNPs prepared in this study are not only more effective at lower concentrations but also display a broader susceptible bacterial spectrum range.

Table 1 MIC_{80} of AgNPs synthesised from CFSs against bacterial strains

Organism	AgNPs from *P. acidilactici* CFS	AgNPs from *B. subtilis* CFS	Neomycin
E. coli	6.25±0.02[a]	6.25±0.00[a]	6.25±0.04
E. faecalis	6.25±0.04[a]	12.5±0.02[b]	NI*
K. pneumoniae	25±0.02[a]	25±0.00[a]	25±0.04
P. aeruginosa	12.5±0.02[a]	12.5±0.03[a]	NI*
S. aureus	25±0.02[a]	25±0.02[a]	NI*

Data are reported as the mean ± SD. Values followed by a or b superscript alphabets are significantly different. ($p<0.05$, for AgNP preparations within each microorganism tested, *ANOVA*) NI = No inhibition.

*Commercial antibiotics showed no inhibition in terms of effecting 80 % kill at their evaluated concentrations.

3.5.2 Antifungal studies

Three *Candida* reference strains were employed to evaluate the antifungal potency of the AgNP preparations (Table 2). Both AgNP preparations displayed good antimicrobial activities against fungal strains with MIC values of 6. 25 µg ml^{-1} for AgNPs from *P. acidilactici* CFS and 6.25-12.5 µg ml^{-1} for AgNPs from *B. subtilis* CFS. The difference in MIC's is at present unclear and further studies need to be conducted to determine if capping structures present in the CFS constitute to the marked difference in bioactivities. These findings are in contrast to what has been suggested by Nabikhan *et al*. [27] that AgNPs are better antibacterial agents rather than antifungal agents. This notion was supported by the finding that AgNPs are able to freely adhere to and penetrate bacterial cells, whilst they are unable to enter fungal cells at low concentrations [28]. The AgNPs produced in this study show no alteration in bioactivity between bacterial and fungal organisms, suggesting that their mode of action remains unaffected by the difference in the cell wall structures of these organisms. Importantly, AgNPs produced in this study display bioactivities at concentration doses that are generally associated with limited or no cytotoxic potential [29] and therefore provide promising broad-spectrum antimicrobial alternatives to orthodox antibiotics which may provide much needed relief to the health care delivery system.

Table 2 MIC_{80} of AgNPs synthesised from CFS against fungal strains

	MIC (µg ml^{-1})		
Organism	AgNPs from *P. acidilactici* CFS	AgNPs from *B. subtilis* CFS	Amphotericin B
C. albicans	6.25±0.03[a]	12.5±0.01[b]	0.02±0.00
C. krusei	6.25±0.02[a]	12.5±0.04[b]	0.02±0.00
C. parapsilosis	6.25±0.02[a]	6.25±0.01[a]	0.02±0.00

Data are reported as the mean ± SD. Values followed by a or b superscript alphabets are significantly different. (p<0.05, for AgNP preparations within each microorganism tested, *ANOVA*)

4. Conclusions

Nanotechnology provides a much needed platform for the development of new or improved antimicrobial agents. Physical characteristics of AgNPs such as shape and size are critical in the development of low concentration dose bioactive pharmaceuticals. The use of sunlight irradiation for AgNP production from *P. acidilactici* and *B. subtilis* CFSs produced nanoparticles with desired physical attributes at favourable reaction times. Furthermore, capping structures present in the CFSs reduced Ag^{+} and stabilised the resultant AgNPs. Significantly, AgNPs displayed excellent antimicrobial activities and thus represent as promising novel Ag-based antimicrobial agents as alternatives to current antibiotics.

Acknowledgements

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References

[1] D. T. King, S. Sobhanifar, N. C. Strynadka, "The Mechanisms of Resistance to β-Lactam Antibiotics*".* Handbook of Antimicrobial Resistance, Springer, New York , p. 177, 2017.

[2] A.W. Fothergill, " Antifungal susceptibility testing: clinical laboratory and standards institute (CLSI) methods, in Interactions of Yeasts, Moulds, and Antifungal Agents", Springer,

New York p. 65, 2012.
[3] J. T. Seil, T.J. Webster, Int J Nanomedicine **7,** 2767 (2012).
[4] J.S. Kim, E. Kuk, K.N. Yu, J.H. Kim, S.J. Park, H.J.Lee, S.H. Kim, Y.K Park, Y.H. Park, C. Y. Hwang, Nanomedicine: Nanotechnology, Biology and Medicine **3**(1), 95 (2007).
[5] M.A. Rai, A. Yadav, A. Gade, Biotechnology advances **27**(1), 76 (2009).
[6] P. Dauthal, M. Mukhopadhyay, Industrial & Engineering Chemistry Research **55**(36), 9557 (2016).
[7] S. Rajput, R. Werezuk, M.L. Ralph, M.T. McDermott, Langmuir **32**(34), 8688 (2016).
[8] V. Makarov, A. J. Love, O. V. Sinitsyna, S. S. Makarova, I. V. Yaminsky, M. E. Taliansky, N.O. Kalinina, Acta Naturae **6**(1), 20 (2014).
[9] N. Pantidos, L.E. Horsfall, Journal of Nanomedicine & Nanotechnology **5**(5), 1 (2014)
[10] K. N. Thakkar, S. S. Mhatre, R. Y. Parikh, Nanomedicine: Nanotechnology, Biology and Medicine **6**(2), 257 (2010).
[11] D. Preethi, M. Mukhopadhay, Industrial & Engineering Chemistry Research **55**(36), 9557 (2016).
[12] A. R. Shahverdi, S. Minaeian, H. R. Shahverdi, H. Jamlifar, A.S. Nohi, Process Biochemistry, **42**(5), 919 (2007).
[13] C. F. Gonzalez, B. S. Kunka, Applied and Environmental Microbiology **53**(10), 2534 (1987)
[14] M. J. Pucci, E. R. Vedamuthu, B. S. Kunka, P. A. Vandenbergh, Applied and Environmental Microbiology **54**(10), 2349 (1988).
[15] M. Veshara, B. Izel, S. B. N. Krishna, J. N. Mbatha, Saudi Journal of Biological Sciences **24**(6), 1294 (2017).
[16] R. Gannimani, A. Perumal, S.B.N. Krishna, N. Sershen, K.P. Muthusamy, A. Mishra, P. Govender, Digest Journal of Nanotechnology and Biostructures **9**(4), 1669 (2014).
[17] P. Villanova, *Performance standards for antimicrobial disk susceptibility tests.* National Committee for Clinical Laboratory Standards. Approved Standard. 7th ed. National Committee for Clinical Laboratory Standards, **20**, M2-A7 (2000)
[18] NCCLS, *Reference Methods for Broth Dilution Antifungal Susceptibility Testing of Yeast: Approved Standard-Second Edition M27-A2.* CLSI Document. Clinical and Laboratory Standards Institute, Villanova, PA, **22**(15, 2nd ed) (2002).
[19] R. Veerasamy, T. Z. Xin, G. Subhasini, T. F. W. Xiang, E. F. C. Yang, N. Jaykumar, S. A. Dhanraj, Journal of Saudi Chemical Society **15**(2), 113 (2011).
[20] X. Wei, M. Luo, W. Li, L. Yang, X. Liang, L. Xu, P. Kong, H. Liu, Bioresource Technology,. **103**(1), 273 (2012).
[21] T. R. Jensen, G. C. Schatz, R. P. Van Duyne, Journal of Physical Chemistry B **103**(13), 2394 (1999).
[22] M. Sigamoney, S. Shaik, N. Sershen, P Govender, S.B.N. Krishna, South African Journal of Botany **103**, 230 (2016).
[23] D. Maity, M. Kanti Bain, B. Bhowmik, J. Sarkar, S. Saha, K. Acharya, M. Chakraboty, D. Chattapadhay, Journal of Applied Polymer Science **122**(4), 2189 (2011).
[24] K. Jyoti, M. Baunthiyal, A. Singh, Journal of Radiation Research and Applied Sciences **9**(3), 217 (2016).
[25] T. Prasad, E. Elumalai, Asian Pacific Journal of Tropical Biomedicine, **1**(6), 439 (2011).
[26] C. Dipankar, S. Murugan, Colloids and Surfaces B: Biointerfaces **98**, 112(2012).
[27] A. Nabikhan, K. Kandasamy, A. Raj, N.M. Alikunhi, Colloids and Surfaces B: Biointerfaces **79**(2), 488 (2010).
[28] P. Velusamy, G.V. Kumar, V. Jeyanthi, J. Das, R. Pachiayapan, Toxicological research **32**(2), 95 (2016).
[29] A.M. Awwad, N.M. Salem, A.O. Abdeen. International Journal of Industrial Chemistry **4**(1), 29 (2013).