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Formulation and Evaluation of Bacteriocin Fused Silver Nanoparticles

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ABSTRACT
Bacteriocins are the natural antibiotics produced from the probiotic microorganisms, predominantly the Lactobacillus family and are proteinaceous in nature. The commercial exploitation of bacteriocin is facing from some drawbacks such as short stability period and low yield. So, to develop new strategies to overcome such hurdles is the need of the hour. One such approach is to incorporate bacteriocins in metallic nanoparticles. Nanotechnology proves to be a boon in the field of medical and engineering science. The present research focuses on the fusion of bacteriocins, produced and isolated from probiotic microorganisms with the silver nanoparticles. UV is used to characterize bacteriocin-fused nanoparticles. Effects of various temperature and pH conditions on the formation of nanoparticles are studied using visible spectroscopy. The disc- diffusion method is used to study the antimicrobial activity of bacteriocins and bacteriocin fused silver nanoparticles. As an indicator strain, Propniobacterium acnes was employed. Clindamycin is administered as a positive control and sterile normal saline is utilized as a negative control. Scanning electron microscopic methods were used to conduct morphological studies. Bacteriocins were obtained from the strains isolated from probiotic curd culture. Silver nitrate is procured from CDH and sodium borohydride is obtained from Qualigence.During UV-visible spectroscopic study, a wide range was seen between 24 and 48 hours at 400 and 600 nm, respectively.All three varying temperatures shows, a 600 nm and a 300 nm SPR peak. A pH of 4 was found to be the best as a robust SPR peak was observed 500 to 700 nanometers. Zone of inhibition was recorded as 32±0.0 mm and 37.5±0.3mm of bacteriocins and bacteriocin fused silver nanoparticles respectively. Nanoparticles fused with bacteriocins shows greater antimicrobial potential than bacteriocins or nanoparticles alone. Additionally they are even more stable.
Keywords: *Bacteriocins, Nanoparticles, Antimicrobial, Spectroscopy, Scanning electron microscopy*.

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INTRODUCTION

Nanotechnology is a scientific technology in which particles are synthesized at nano-scale, such as in agricultural processing, food processing, compound fabric manufacturing and engineering, also in bio medicinal applications. In recent decade, applications of nanomaterials as effective antimicrobials draw maximum attention of many researchers. Scientific substantiation of the antimicrobial activity of many metal oxide nanoparticles such as ZnO, CuO, MgO,SiO_2 and NiO is already reported. These metal oxide nanoparticles can successfully kill many microorganisms. Factors that may affect the effectiveness of nanomaterials in lowering the bacterial cell count, the surface charge of the metallic nanoparticle, shape, metal type, concentration of nanoparticles, dispersion, time of contact of nanoparticle with the microorganism, presence of oxygen, pH of the medium and its components, specific surface-area-to-volume ratios, bacterial cell wall composition and growth rate.

Use of antibiotics as antimicrobials is associated with a common side effect of multidrug resistance due to a lengthy cycle of production and consumption. This ultimately reduces the efficacy. Secondly, Usage of subpar materials and misbranded, bogus medications in emerging and underdeveloped nations further discourage the use of antibiotics. Use of metallic NPs as bactericidal agents proves to be an emergent approach against this challenge. This give rise to emergence of an efficient approach to design nanostructures loaded with antimicrobial agents for targeting the community of pathogenic microorganisms efficiently. Additionally, the problem of multidrug resistance is resolved by this

approach. At effective doses employed to destroy microbial cells, the metal oxide NPs have little toxicity toward humans, which makes their application on a large scale advantageous. But several investigations have indicated that NPs are effective antibacterial agents and can efficiently be used in topical formulations [1].

Nanoparticles as effective antimicrobials:

Synthesis of Metal-based nanoparticles is not altogether a new concept. It is generally known that some microbes produce metallic nanoparticles as a method of detoxifying heavy metals. However, over the last decades the versatility of nano technology has only been more pronounced, with metallic nanoparticles being widely used in textile and cosmetic industry [2].The research community's interest in metal-based nanoparticle flexibility led to an ongoing search for novel nanoparticle compositions, synthesis techniques, and applications. Silver, gold, iron, zinc and also copper are the most often employed the metals in creation of metal-based nanoparticles [3-5].

Transition metals show being the ideal choices because of their partially filled d-orbitals, which make them more redox active for the creation of metal-based nanoparticles. This facilitates the aggregation of nanoparticles.Physical, chemical, and biological processes can all be used to create nanoparticles [4].

The positively charged nanoparticles are attracted to the Negatively charged bacterial cell walls due to electrostatic forces of attractions. The breakdown of cell walls caused by alternatively positively charged metallic nanoparticles interacting strongly with biomembranes results in enhanced permeability. In addition to this, the metal ions released from the metal based nanoparticles enter the extracellular space, can interfere with biological functions. Reactive oxygen species (ROS) are produced inside the cell as a result the oxidation of glutathione, leads to generation of oxidative stress, thus conquering the bacteria' antioxidant defence system against Reactive Oxygen Species. The ions of metal interact with the genetic material, cellular structures and, disrupt cell functions [6].

Atoms like N, S or O are abundantly found in the both biomolecules and organic substances, form strong coordination bonds with the metal atoms. The bond is nonspecific in nature and thus metallic nanoparticles exhibit a broad spectrum of activity [7].

MATERIAL AND METHODS

Culture collection:

Bacteriocin producing strains are obtained from the homemade curd culture. According to Sharma et al., 2021, the isolated strains were grown on Man, Rogosa, and Sharp agar media and incubated for 24 hours at 35 °C [8].The pathogenic strain used in this study i.e. *Propniobacterium acnes(MTCC 1975)* was obtained from Rapture biotech Noida. $AgNO_3$, $NaBH_4$were obtained from CDH and Qualigence respectively.

Identification of the probiotic strain:

With the aid of a straightforward compound microscope, the probiotic strains were identified morphologically. Phenotypic identification was carried out using techniques including the indole test, the coagulase test, the catalase test, the whole cell protein analysis, and others.

Bacteriocins' antimicrobial action against harmful bacteria:

The antibacterial activity of the bacteriocins isolated from probiotic cultures is evaluated using the disc diffusion method. A total of seven isolates of probiotics are isolated and are sub cultured for bacteriocin production. Bacteriocins obtained from isolate 6 shows maximum zone of inhibition of about 32 mm as reported by Sharma *et al*., 2021 [8].

Synthesis of silver nanoparticles using bacteriocins obtained from probiotic strains:

Bacteriocin isolated from probiotic culture is fused with silver nanoparticles which are reported to have greater antimicrobial potential than bacteriocin alone. Two distinct sets were used in this experiment: one for the creation of bacteriocin-fused silver nanoparticles (BF SNPs), other for the creation of naked silver nanoparticles (bare-SNPs). Drop by drop, 20 ml of a 1 mM $AgNO_3$ solution was added to a 60 ml, ice-cold, 2 mM NaBH4 solution.These steps were carried out utilising a magnetic stirrer to vigorously stir continuously and are made with deionized water. Effervescence was seen as a result of the reaction's heat creation and hydrogen's release (H_2). In the presence of borohydride, the Ag? ions transform into Ag0 states. The reaction mixture transforms from being colourless to pale yellow. Continuous stirring under ice-cold condition was maintained till the end of effervescence. In one of the set no capping material was added and left for stirring. So, the Ag0 seeds agglomerate through Ostwald's ripening method and this leads to formation ofunstable bare-SNPs which only lasts for 2 months. For the formation of bacteriocin fused silver nanoparticles (BF SNPs) under steady stirring, drops of bacteriocin solution were applied to the exposed Ag0 seeds until there was a minor change in colour. The block diagram for the sequential production of silver nanoparticles is displayed **Fig 1.** (both bare-SNPs and BF SNPs) [9,10].

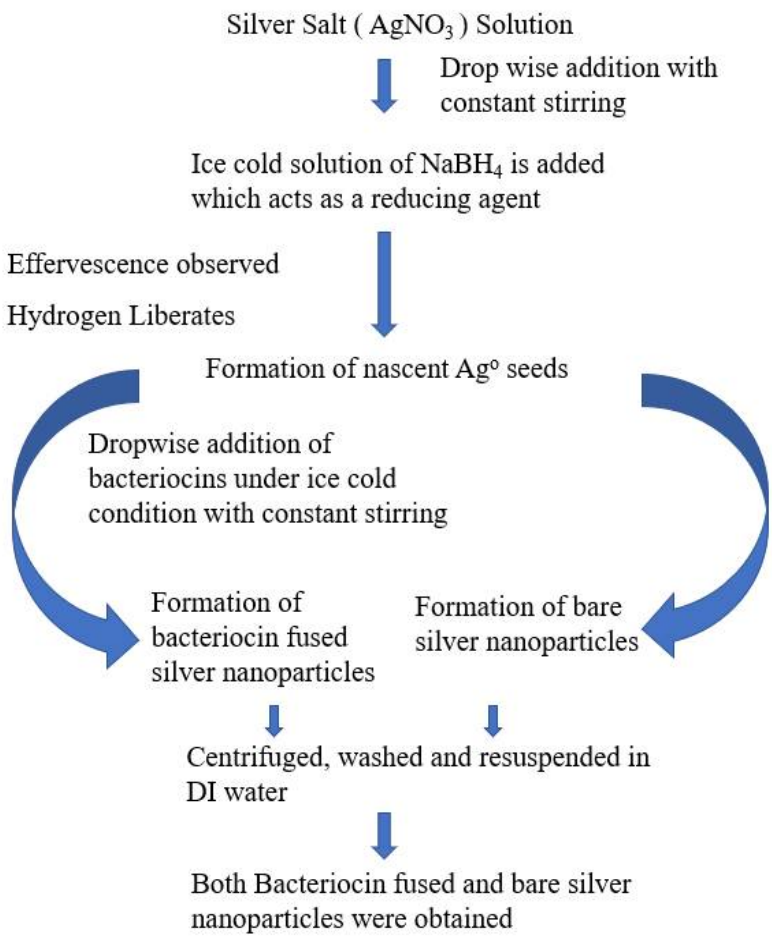

Fig 1. Flowchart showing step by step procedure for synthesis of bacteriocin fused silver nanoparticles

Characterization of synthesized bacteriocin fused silver nanoparticles

Silver nanoparticle analysis using scanning electron microscopy (SEM), UV visible spectrophotometry, and visual detection.

When compared to a control, the colour of the SNPs biosynthesized utilizing the bacteriocin isolated from probiotic culture changes from colorless to yellowish brown. The appearance of silver nanoparticles is indicated by this shift in colour. Morphological examination of the bacteriocin fused research on silver nanoparticles was done by utilizing SEM technique. The dried aqueous sample of sample is subjected to SEM analysis. The UV-Visible spectrophotometer was used for the spectrophotometric analysis. Spectrophotometric methods with a resolution of 0.5 nm and a wavelength range of 200–800 nm were used to measure the reduction of silver ion (Ag^+) to silver nanoparticles (AgO) [11].

Antibacterial potential of the biosynthesized nanoparticles:

The disc diffusion method is utilized to assess the antimicrobial activity of the bacteriocin fused silver nanoparticles. Discs impregnated in bacteriocin are placed on the plate containing MRS agar media. Clindamycin is administered as a positive control, and sterile normal saline is utilized as a negative control. Gram positive bacteria i.e. *Propniobacterium acnes* was taken as indicator strains [12].

Effect of some parameters on silver nanoparticles synthesis:

pH, temperature, and silver nitrate concentration impacts on the production of bacteriocin fused SNPs were assessed. On the biosynthesis of silver nanoparticles, the effects of temperature (25°C, 30°C, 35°C, and 40°C) and pH (3, 4, 5, 6, 7, and 8 and 9) were investigated. The biosynthesized SNPs were analyzed after the reaction mixture including the bacteriocin fused silver nanoparticles was incubated in a BOD incubator for around 48 hours [13].

RESULTS AND DISCUSSION

Sharma *et al*., 2021 report on the morphological and phenotypic traits of the bacteria isolated from probiotic curd culture. In the isolates, non-sporing,Microbe-positive rods. All the isolates shows positive carbohydrate fermentation test whereas negative results are reported in catalase and indole test. This

observation for the isolated strains refers to Bergey's Manual of Systematic Bacteriology's description of the Lactobacillus family. Prabhu *et al*. [12] reported the isolation of LAB from Yoghurt. Adebayo-Tayo and Onilude reported a similar work, whose research extracted LAB from fermented foods [13] Olasupo *et al.* [14]bacteria belonging to the *Lactobacillus* family have good ability to withstand the acidic environment and has good bile tolerance. The pathogenic microorganisms i.e. *Propniobacterium acnes* is susceptible to the metabolites of probiotics isolated from the curd culture.

Characterization of silver nanoparticles using bacteriocins isolated from probiotic curd culture

UV- Visible spectrophotometric analysis of the silver nanoparticles:

Using a UV-visible spectrophotometer, bacteriocins fused SNPs were characterized. **Fig 2** displays the UV-visible spectra of SNPs coupled with bacteriocin. At 400 and 600 nm, respectively, a broad band was seen after 24 and 48 hours. At 500 nm, the resonance (SPR) peak was seen. Kanmani and Lim. [9] reported similar work with parallel peaks. The maximal wavelength of silver nanoparticles, which is between 400 and 500 nm, is visible [12]. Additionally, Prabhu et al. [12] found that their SNPs had an SPR peak at 446 nm. Shivashankar et al. [15] observed that the metallic nanoparticles they manufactured displayed a distinctive surface plasmon absorption band at 386 nm in their UV-Visible spectra.

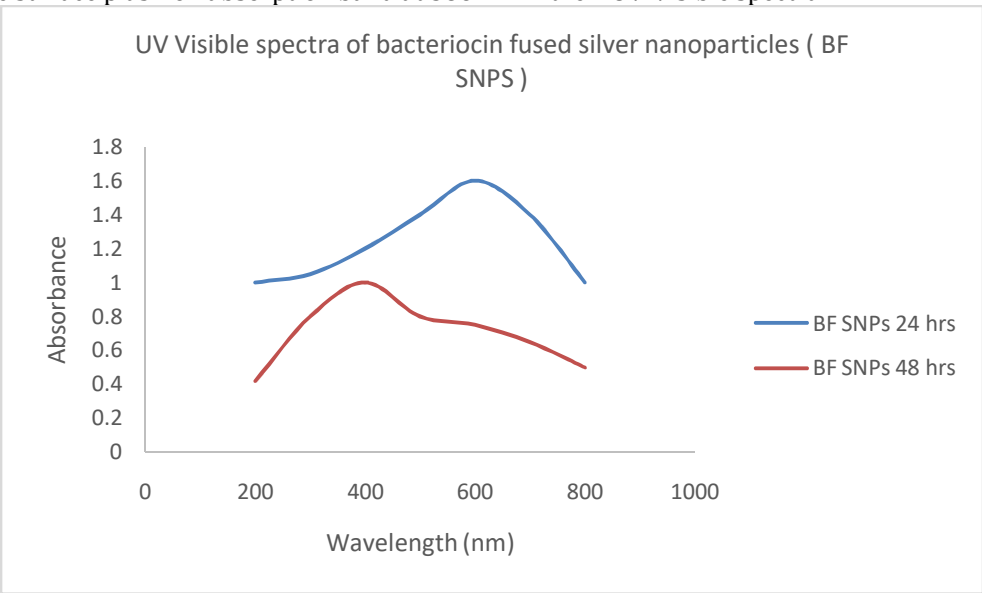

Fig2. UV Visible spectra of bacteriocin fused silver nanoparticles (BF SNPs)

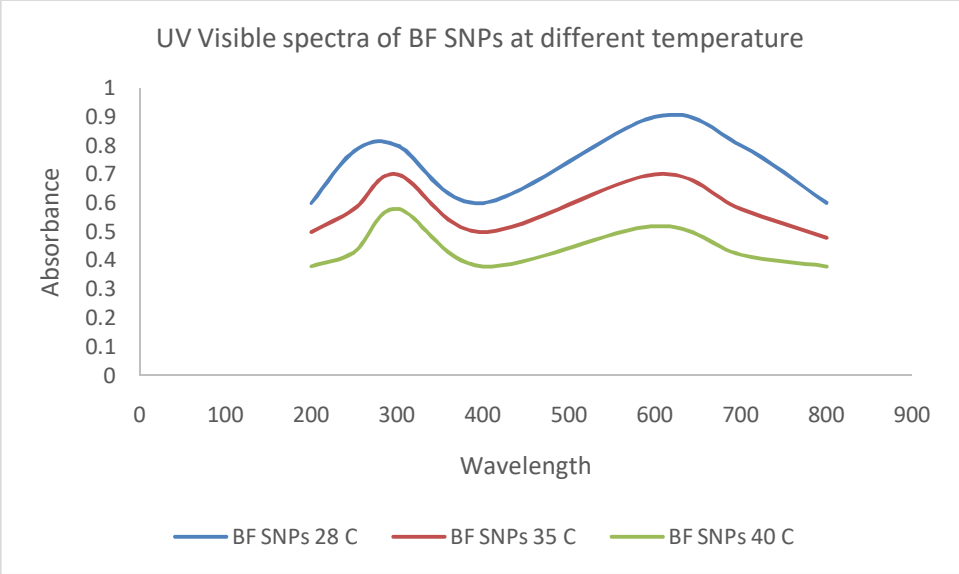

Fig3. UV Visible spectra of BF SNPs at different temperatures

Effect of different temperatures regarding the biosynthesis of Bacteriocin fused SNPs is shown in **Fig 3**.Spectra showed that there are 300 and 600 nm each have an SPR peak across all three temperature ranges. 28°C was the temperature at which the maximum peak was seen, then at 35 and 40 degrees. One

important physical factor that has an impact regarding the creation of BF SNPs is temperature. Temperature's impact on the synthesis of BF SNPs assessed, and also 28°C was shown to be the best temperature for BF SNP generation in this study. The outcomes are consistent with research by Prabhu et al. demonstrate the production of metallic nanoparticles at room temperature. The synthesis of silver nanoparticles, however, is directly proportional to the reaction temperature, according to a related work published by Annadurai *et al.* [16]. Using *Coleus aromaticus* plant extract raised the reaction's temperature. The study went on to say that the ideal temperature for the creation of nanoparticles is 70°C.

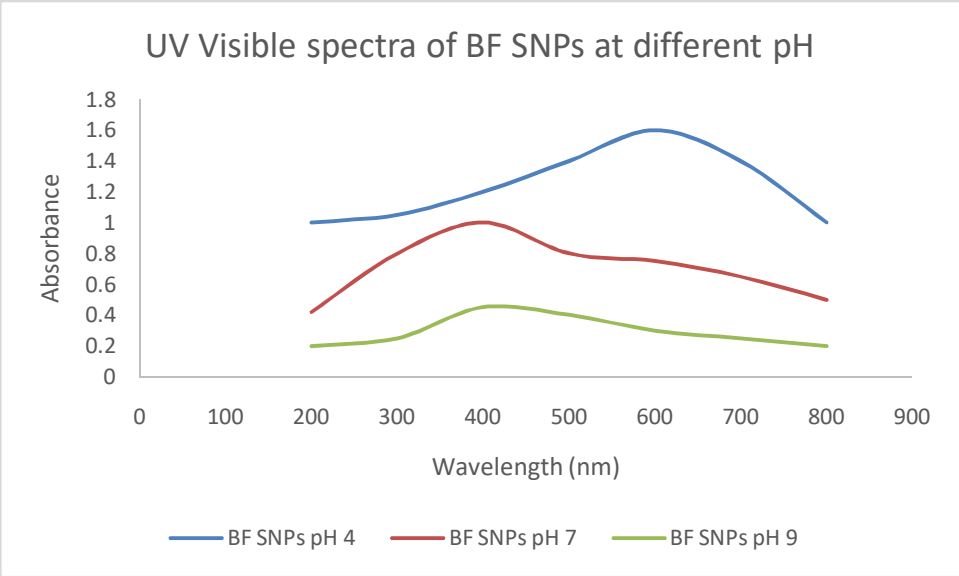

Fig4. UV Visible spectra of BF SNPs at different pH

Fig 4, depicts how pH affects the production of BF SNPs. Since a prominent SPR peak was seen between 500 nm and 700 nm, a pH of 4 was found to be the best. From 200 nm to 600 nm, the absorbance first rose before further falling. At pH levels of 7 and 9, a prominent 400 nm was the peak distance. A prominent the SPR peak was 400 nm. also seen with a pH of 4. Between 500 and 700 nm, a wide band was discovered to exist at pH 9. At pH 4, the most nanoparticle production was seen. This outcome conflicts with research by Muhammad Amin et al. [17], which shown that a higher pH causes silver ions to be reduced.

UV- Visible spectra of BF SNPs at different concentrations of $AgNO_3$:

The impact of various silver nitrate ($AgNO_3$) solution concentrations can be seen in the UV-visible spectrum. i.e., 2 – 10 mM on the synthesis of BFSNPs is shown in **Fig 5**. SPR maximum of the synthesized BF SNPs produced with different concentration of $AgNO_3$ (2 – 10 mM) shows absorbance peaks between 400 and 600 nm, and 10 mM with an SPR peak at 500 nm, $AgNO_3$ supported the maximum SNPs formation. The SPR peak intensity rose as $AgNO_3$ concentration increased (1 to 10 mM), indicating a faster reduction rate as precursor salt concentration increased.

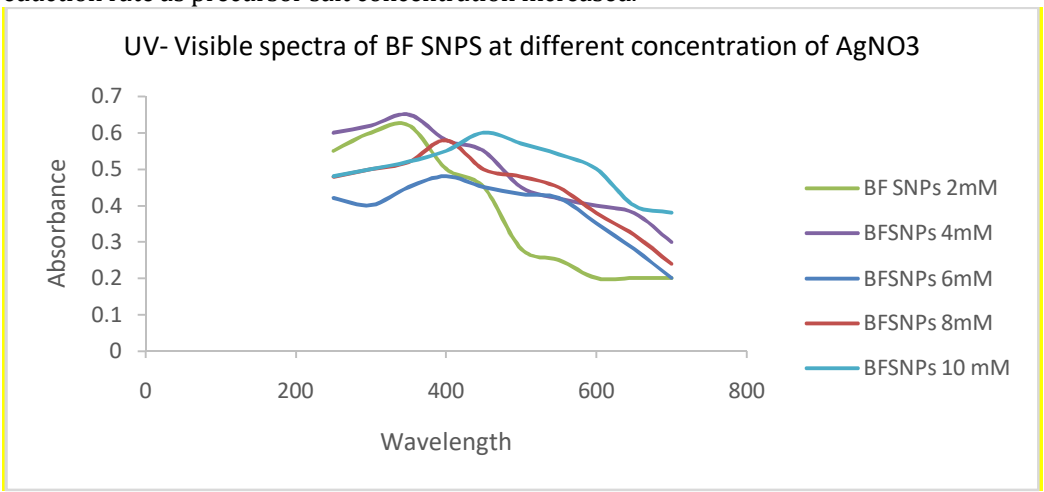

Fig5. UV- Visible spectra of BFSNPs at different concentration of $AgNO_3$

Determination of the antibacterial activity titer

To assess the antibacterial activity titer, the disc diffusion method was employed [18]. A 100 L solution of an adjusted indicator strain was used to inoculate the Muller Hilton agar plates (10^6). The free AgNB solution, an extracted bacteriocin, and silver nanoparticles loaded with bacteriocin were synthesised in two-fold serial dilutions. The muller Hilton agar plates were covered with discs that had been impregnated with the suspension of the aforementioned substances. After 24 hours of incubation at 37 °C, the plates were measured for the zone of inhibition.

The greatest concentration of bacteriocin that exhibits inhibitory action was used to define the antibacterial activity of the probiotic. Using the formula ab 20, where "a" is equal to 2, and "b" is the number of discs placed on the Muller Hilton agar plate to determine the zone of inhibition, the inhibitory activity was represented as arbitrary units per millilitre (AU/mL).

Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) is calculated using the broth macro-dilution method [19]. In this procedure, the recovered bacteriocin, bacteriocin-loaded silver nanoparticles, and free silver nanoparticles were used to create stock solutions in various quantities (AgNP's). The test sample was taken in a concentration of 0.5mg/ml. Then, in 1 mL of MH broth, a series of two-fold dilutions were made 1 mL of each preparation is added to 1 mL of broth. To finish the serial dilution, this was done again. Each dilution was then infected with 100 L of the indicator strain, resulting in a final concentration of roughly 5 105 CFU/mL in each tube, and incubated for 24 h at 37 °C (CLSI recommends using a 0.5 Mc farland equivalent, then diluting this 1:20 to yield 5* 10^6 cfu, and inoculating at a 10 fold dilution to yield 5* 10^5.Additionally, a negative control tube containing only media and a positive control tube with only 1 mL of twofold diluted medium and 100 L of the indicator strain were created. MIC was the final concentration of a studied bacteriocin that could prevent the indicator strain from growing visibly, and it was also stated as mg/mL. [20].

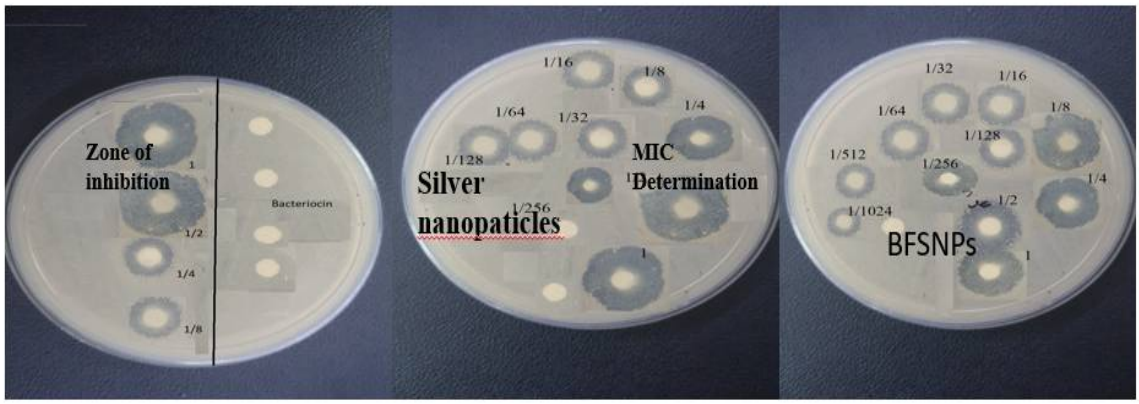

Fig 6. The arbitrary unit detection of bacteriocin using disc diffusion method, against P.acnes a) Extracted bacteriocin b) Free AgNP's solution c) Bacteriocin-incorporated silver nanoparticles

Table 1.MIC, MBC, and stability of isolated bacteriocin and nano-silver bacteriocin over a 60-day period against *P.acnes*

Tested Types	Total antibacterial activity	MIC mg/ml	MBC mg/ml
Bacteriocin	180	10	16
BFSNPs	20860	0.00048	0.0039
SNPs	6200	0.00195	0.0156

Scanning Electron Microscopic analysis of the silver nanoparticles:

By utilizing a scanning electron microscope, bacteriocin fused SNPs were further described. SEM is a method for examining what the morphological features of SNPs, such as size and shape of them. **Fig 7** (a), (b) and (c) shows the SEM micrograph of the bacteriocin fused SNPs. The SNPs were spherical and ranged in size from 2 m. Sadowski et al. [18]study on the manufacture of bacteriocin fused SNPs utilizing Penicillium strains obtained from soil showed a similar condition. The physical processes, such as the drying method used to prepare SNPs for SEM analysis, can have an impact on the SNPs' size and form.

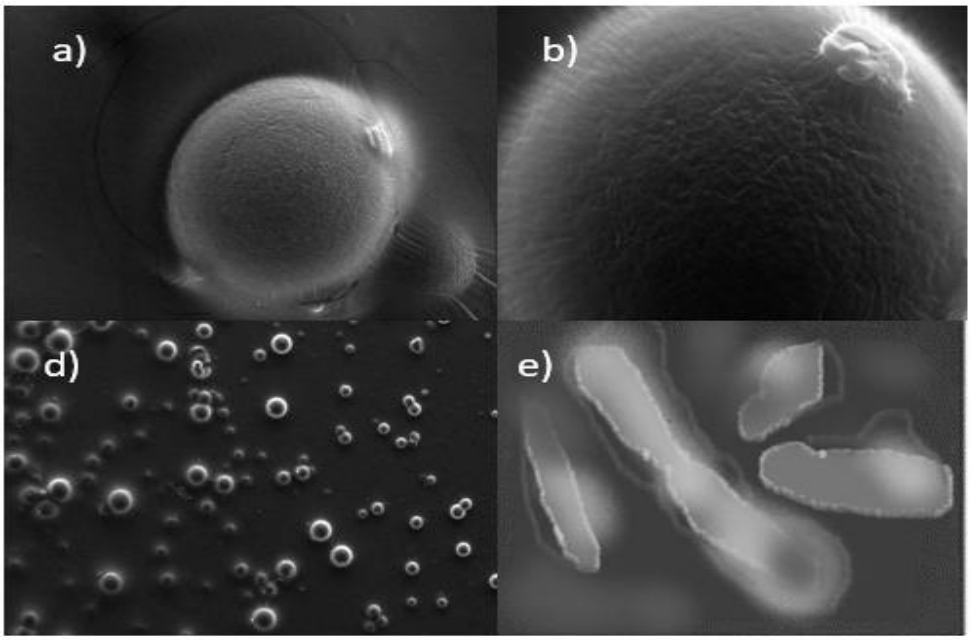

Fig7. Scanning Electron Microscopic images of a) Bare AgNPs b),c) Bacteriocin fused AgNPs d) Effect of bacteriocin fused silver nanoparticles on P. acnes cells

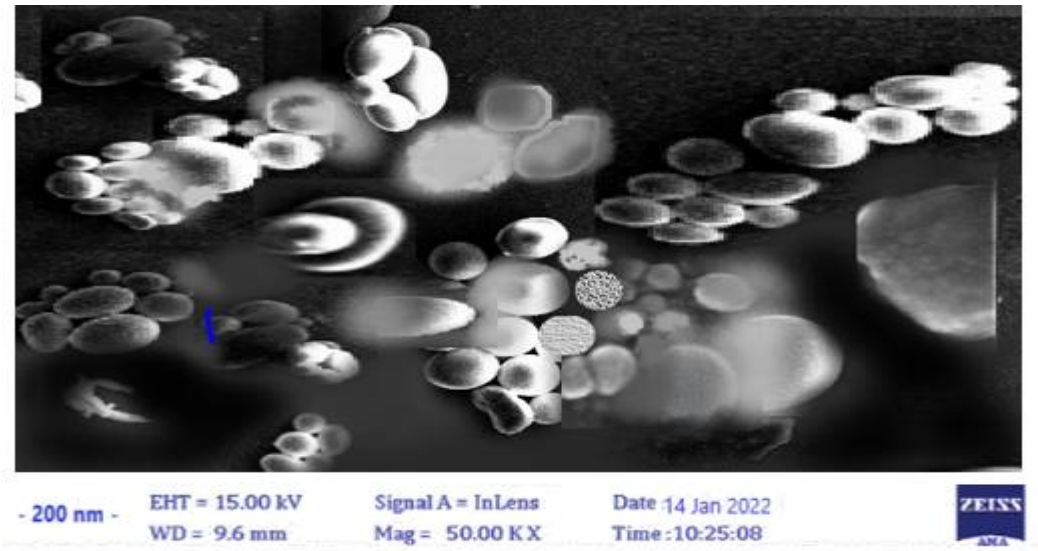

Fig8. TEM image of bacteriocin fused silver nanoparticles

Antibacterial potential of silver nanoparticles

The biosynthesized bacteriocin fused SNPs in mm are evaluated for their antibacterial activity using the disc diffusion method's area of inhibition the bacteriocin isolated from probiotic curd culture and bacteriocin fused silver nanoparticles is measured as 32 mm and 37.5 mm respectively. Clindamycin is taken as positive control. Several mechanisms have been proposed which explains the antimicrobial effect of metallic nanoparticles. Main reason associated with the toxicity of SNP's is the passage through the biomembranes due to small size. Nanoparticles can interact with the genetic material i.e.,DNA thus loses its capacity to multiply, which ultimately causes cell death [18]. Additionally, according to Prabhu et al. [12], Gram positive bacteria, like *Staphylococcus aureus* was the most susceptible to their SNPs**.**

CONCLUSION

The bacteriocin fused silver nanoparticles shows greater efficacy as compared to the bacteriocin alone. The nanoparticles are efficiently used in topical formulation used in the treatment of dermatological disorders like acne, eczema, skin inflammation etc.Bacteriocin is obtained from the probiotic strain isolated from the curd culture. Bacteriocin fused silver nanoparticles were made and effect of parameters

such as different temperature, pH etc is studied with the help of UV- Visible spectroscopy. Morphological studies of the nanoparticles is carried out with the help of SEM (Scanning Electron Microscopy). Antimicrobial potential of the bare SNPs and the BFSNPs is observed with the help of disc- diffusion method.

ABBREVIATIONS:

BFSNPs – Bacteriocin fused silver nanoparticles
SNPs – Silver nanoparticles
MRS – Maan Rogosa and Sharp media

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