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SYNTHESIS AND ANTIMICROBIAL EFFICACY OF SILVER NANOPARTICLES AND BACTERIOCIN FROM MILK AND CURD BACTERIA

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Abstract – Bacteriocins are one of the natural secondary metabolite of bacteria that inhibit the growth of other bacteria. Silver nanoparticle (AgNP) also has such activity which is produced from bacteriocin. In this study we have synthesised bacteriocin and AgNP from milk and curd bacteria. Antimicrobial activity of bacteriocin and AgNP are discussed herein.

INTRODUCTION

NPs exhibit size and shape-dependent properties which are of interest for applications ranging from bio sensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes. These particles also have many applications in different fields. The development of antimicrobial resistance has become a serious problem in public health; therefore there is a strong incentive to develop new microbicides (Shahverdi *et al.,* 2007). Lactic acid bacteria can produce a wide range of antimicrobial substances with the capacity to inhibit the growth of pathogenic and spoilage micro organisms. Organic acids, hydrogen peroxides, and bacteriocins are included among these compounds. Bacteriocin are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland *et al.,* 2001). Bacteriocin from lactic acid bacteria are natural antimicrobial peptides or proteins with interesting potential application in health care. NISIN is the best known lactic acid bacteria bacteriocin, with safe and effective application (Janes *et al.,* 1999). The bacteriocin produced by gram positive bacteria like lactic acid bacteria are small peptides, 3-6 kDa, in size (Nes *et al.,* 1996). The present study is an attempt at the extracellular synthesis of silver nanoparticles using bacteria isolated from milk and curd. This work also explores the antimicrobial efficacy of these AgNPs as well as bacteriocin present in the culture supernatant of the milk and curd bacteria

MATERIALS AND METHODS

Sample collection and Isolation of pure culture

Bacteria were isolated from raw milk & curd samples. 1 mL of the milk was taken & serially diluted upto 10^{-7}.100 µL was taken from each of three dilutions ($10^{-3,}$ 10^{-5}, and 10^{-7}) and a nutrient agar spread plate was prepared for each. The plates were incubated at room temperature for 24 hours. From each plate one colony was chosen and sub-cultured on nutrient agar by quadrant streak method to get pure culture isolates. The three pure isolates obtained from each plate were named as M1, M2 and M3 indicating that they are isolated from Milk. The same procedure was performed with curd. Three isolates were obtained and they were named C1, C2 and C3.

Storage and maintenance of pure culture

The isolates were maintained in nutrient agar slants at 4°celcius and sub-cultured periodically throughout the experimental period.

Identification of the isolates were done by series of Biochemical assays like Colony Morphology, Gram staining, Motility test, IMViC Tests, Indole test, Catalase test, and Oxidase test.

Synthesis of silver nanoparticles

24 hour broth culture of all 6 isolates were prepared. The culture was filtered using Whatmann no.1 filter paper. 10 mL of culture filtrate was mixed with 100 mL of 10-3 M silver nitrate solution in Erlenmayer flask and incubated at room temperature in a shaker until visible colour change was observed.

Characterization of silver nanoparticles

Silver nanoparticle synthesized by lactic acid bacteria was confirmed and characterized by following methods; colour change, UV-visible spectroscopy, SEM-EDAX analysis.

Antimicrobial assay

The antimicrobial activity both bacteriocin and silver nanoparticles synthesized extracellularly were tested against selected microorganisms using Agar well diffusion method on Muller- Hinton agar plates. The organisms tested *were Pseudomonas sp, Bacillus sp and Eschericia coli.*).100 µL of each test culture was spread onto separate Muller- Hinton agar plates. Wells of About 10mm diameter were cut into the medium using a well cutter. 100 µL of each silver nanoparticle samples produced from the culture supernatant of all 6 isolates, C1, C2, C3, M1, M2, M3 were added into the wells. Antimicrobial assay of leaf extracts and spice extracts were performed as separate experiments. The culture supernatant of 24 hour broth culture of all 6 isolates were used for testing antimicrobial efficacy of bacteriocin using the same method.

RESULTS AND DISCUSSION

Pure cultures of bacterial colonies were isolated from milk and curd. The three isolates from milk were named as M1, M2 and M3. The three isolates from curd were named as C1, C2 and C3. All six isolates were screened for their ability to produce silver nanoparticles and bacteriocin. The 24 hour culture supernatants were used as a source of bacteriocin. It was tested for bactericidal effect on three known bacterial cultures viz. *Pseudomonas sp, E.Coli and Bacillus sp.* using agar well diffusion method. Bacteriocin produced by all 6 isolates were able to produce moderate to good levels of inhibition on *Pseudomonas sp.* Of the six isolates C2 and M3 showed highest zones of inhibition (17 mm) followed by C3 and M2 (16 mm). C1 and M1 showed only marginal zones of inhibition measuring 12 mm each (Table 3 and Figure 2).

With the exception of C2 (16 mm zone of inhibition) all the other isolates showed only very little inhibitory activity against *Bacillus sp.* M1nad

Table 1. Morphology and Biochemical characterization of the Isolates

Organism	Name of the Test									
	Indole test	Methyl red test	Voges –proskauer test	Citrate utilization test	Motility test	Macconkey agar	Oxidase test	Catalase test	Gramsmorphology (Gram staining)	Colony morphology
C1	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies
C2	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies
C3	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies
M1	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies
M2	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies
M3	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+), rod	White, round, elevated, Shining colonies

M2 did not show any bactericidal effect on the tested *Bacillus* species. M3 showed marginal zone of inhibition measuring at 10 mm, barely bigger than the diameter of the well (Table 3 and Figure 2).

In case of *E.coli* , M1 isolate's Bacteriocin showed excellent inhibitory effect recorded at 25mm. C1 showed a marginal inhibitory activity measuring clear zone of 13mm. All other isolates did not show any inhibitory activity on *E. coli* (Table 3 and Fig. 2).

The 24 hour culture supernatants were used for the extracellular synthesis of silver nanoparticles. After 10 days of incubation in a shaker the colour of the supernant changed from translucent white to various shades of brown. This was taken as a visible indicator for the formation of silver nanoparticles (AgNPs).

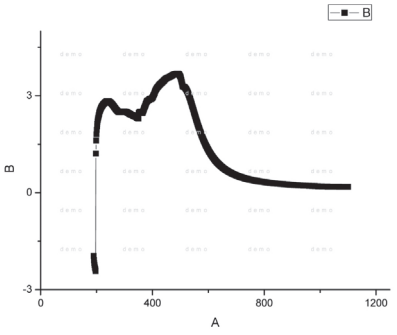
These nanoparticles were then characterized with UV-Visible spectrophotometer and peaks were obtained for all 6 isolates in the range of 300-400nm. SEM and EDAX characterization was also done on C3 isolate since C3 isolate produced the highest yield of AgNPs. SEM analysis showed the shape of the nanoparticles which were roughly square shaped. EDAX spectrum showed the presence of silver atoms.

The silver nanoparticles (AgNps) synthesized using the culture supernatant of all the 6 isolates were tested for their antimicrobial efficacy against the same three test organisms by well diffusion method (Plate).

The silver nanoparticles synthesized by all the 6 isolates exhibited better inhibitory activity and greater zones of inhibition than their bacteriocin counterparts. The zones of inhibition against pseudomonas was recorded as 14mm for C1, 17mm for C2, 18mm for C3, 16mm for M1,17mm for M2 and 18 mm for M3. These results are greater than the zones observed in case of bacteriocin activity against the same organism AgNPs of the isolates show only slightly moderate antibacterial activity against *Bacillus sp. and Pseudomonas sp.* with zones of inhibition in the range of 11 -13 mm. This is much less than the expected results. However this activity is greater than the inhibitory effect of bacteriocin counterparts (Figure 1 and 2).

SEM Analysis of C3 synthesized Silver Nanoparticles

EDAX Analysis of C3 synthesized Silver Nanoparticles Spectrum: sample 8590.spx



C1 OD- 3.63 500

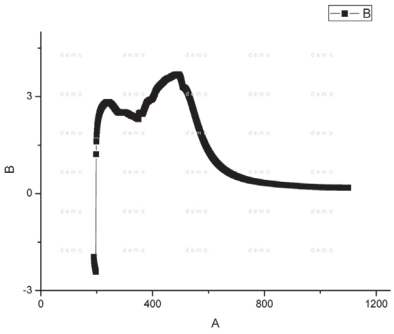
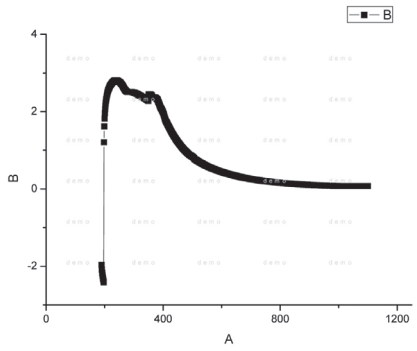


Table 2. Antimicrobial activity of agnps against *Pseudomonas sp, Bacillus sp, E.coli sp.*

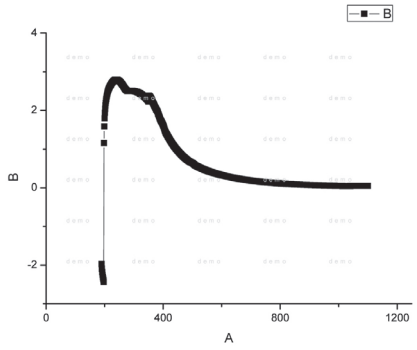
Organism	Zone of inhibition (mm) –AgnPs					
	C1	C2	C3	M1	M2	M3
Pseudomonas sp	14	17	18	16	17	18
Bacillus sp	11	12	12	13	10	12
E.coli sp	12	12	11	10	11	13

Table 3. Antimicrobial activity of Bacteriocin AGAINST *Pseudomonas sp, Bacillus sp, E.coli sp.*

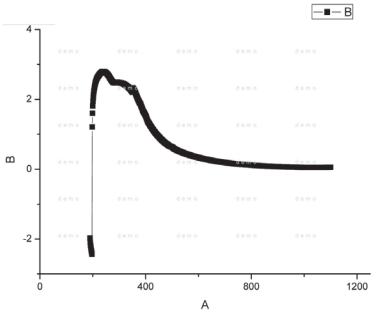
Organism	Zone of inhibition (mm) – Bacteriocin					
	C1	C2	C3	M1	M2	M3
Pseudomonas sp	12	17	16	12	16	17
Bacillus sp	12	16	11	-	-	10
E.coli sp	13	-	-	25	-	-



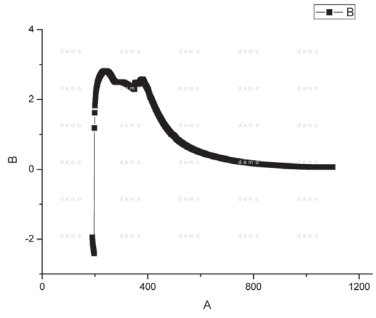
C3 OD- 0.83



M1 OD- 2.45



M2 OD-2.45 350 nm



M3 OD- 2.42 350nm

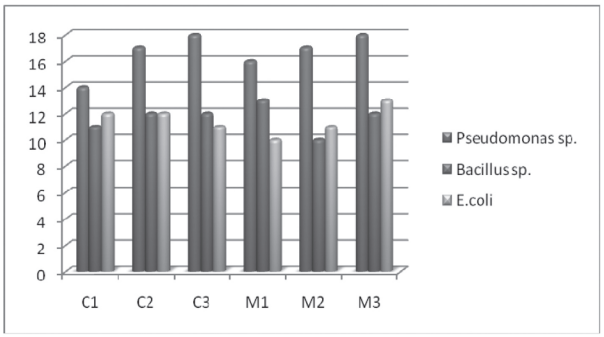


Fig. 1. Antimicrobial activity of Agnps against *Pseudomonas sp, Bacillus sp, E.coli sp.*

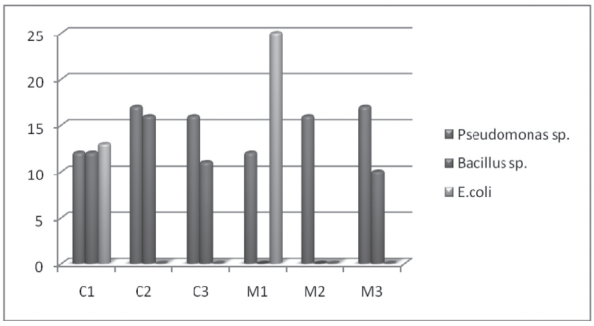
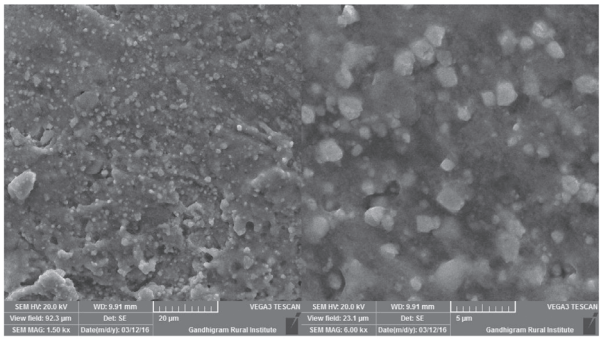
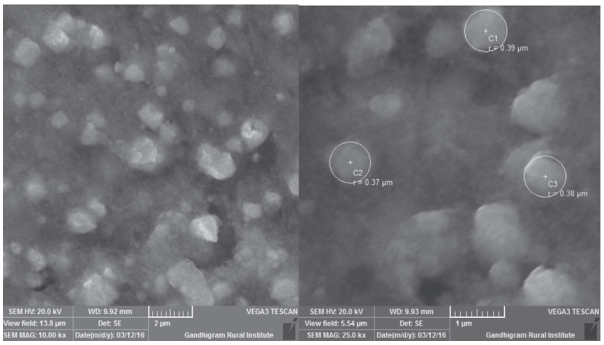
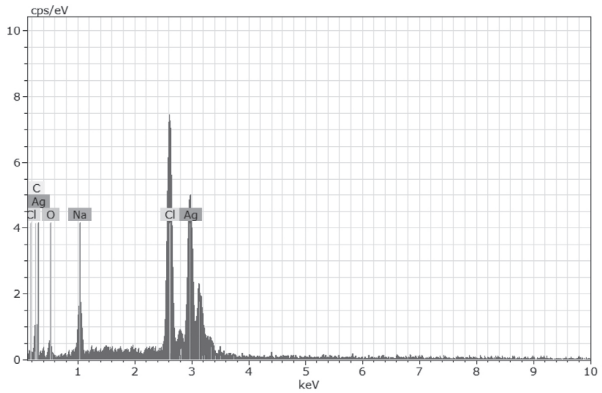


Fig. 2. Antimicrobial activity of bacteriocin against *Pseudomonas sp, Bacillus sp, E.coli sp.*





In case of silver nanoparticles synthesized extracellularly using the culture supernatant the overall bactericidal efficiency is much higher compared to that of bacteriocin alone. The AgNPs of all 6 isolates are able to show moderate to good inhibitory effect. Of the three organisms tested *Pseudomonas* sp. appears to be most sensitive to AgNPs. Both *Bacillus* and *E. coli* sp. are only moderately inhibited by AgNPs. However their average zones of inhibition is better than that of their bacteriocin counterparts. The case of M1 isolate is contrary to all other isolates in that its bacteriocin shows very good inhibition (25 mm) against *E. coli*. Furthermore the AgNPs produced by M1 show only marginal zone of inhibition (10mm) against the same organism *E. coli.* This is in stark contrast to the activity of AgNP recorded in case of the other 5 isolates.



El AN Series unn. C norm. C Atom. C Error (1 Sigma)

	[wt.%]	[wt.%]	[at.%]	[wt.%]
Cl 17 K-series	19.35	24.84	29.64	0.80
O 8 K-series	8.69	11.16	29.49	3.69
Ag 47 L-series	41.35	53.10	20.82	1.54
Na 11 K-series	8.49	10.90	20.05	0.80

Pseudomonas is found to be sensitive to both bacteriocin and AgNP of all 6 isolates. This is in contrast to the resistance of *E.coli* seen in case of bacteriocin of C2, C3, M2 and M3. Also only marginal antibacterial activity (10-13 mm) is recorded in case of AgNP against *E. coli.* Since both *E.coli* and *Pseudomonas* are Gram Negative species this difference in sensitivity cannot be attributed to the Gram's staining reaction of these bacteria.

This preliminary investigation into the antimicrobial efficacy of bacteriocins and AgNPs synthesized by *Bacillus* species has shown that both bacteriocins and AgNPs show good bactericidal activity. This calls for further exploration into the bactericidal effect of *Bacillus* sp. based bacteriocins and AgNPs.

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