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Production, Characterization and Biosynthesis of Silver Nanoparticles Bacteriocin of *Lactobacillus sakei* Isolated from Fish Samples

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ABSTRACT

Lactic acid bacteria (LAB) represent a bacterial group widely spread in niches of fermented food and gastrointestinal tracts of humans and many animals. They are especially known for their ability to produce a wide variety of bioactive metabolites. Thus, the current study aimed to isolate and characterize bacteriocin producing LAB from fish samples. The selected isolate was identified based on morphological, biochemical and molecular as *Lactobacillus sakei* (GenBank, Accession No. MG654786). The bacteriocin was purified by salt precipitation, and molecular mass was determined by SDS-PAGE as 5 kDa. The potential of nanotechnology in enhancing the antimicrobial activity of *Lactobacillus sakei* bacteriocin was investigated. The characterization of silver nanoparticles was done by UV–Vis spectroscopy, scanning electron microscopy (SEM). The biosynthesized silver nanoparticles showed an extreme inhibitory action against tested bacterial pathogens. The present investigation would provide an eco-friendly approach to medicine, future drug development and may have applications on food biopreservative.

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria that can be isolated naturally from several fermented foods such as vegetables, fish, meat, and dairy products (**Freire *et al.*, 2017**). *Lactobacilli* genera are classified as probiotics that have beneficial effects on human health such as assist in lactose metabolism, a controlling factor for blood cholesterol, decreasing cancer incidence, and reduction of the risk of various infectious diseases (**Jabbari *et al.*, 2017).** Also, they inhibit the growth of pathogenic bacteria in distinctive eco-systems through the production of a variety of antimicrobial substances such as ethanol, formic acid, acetic acid, organic acids, diacetyl, hydrogen peroxide, and bacteriocins **(Aruna *et al.*, 2016).** The development of antimicrobial resistance has

become a serious problem that affects the whole world. Probiotic induces no drug resistance or drug residues **(Idoui, 2014).** Therefore there is a robust incentive to develop new microbicides **(Shahverdi *et al.*, 2007).** One of the alternatives eco-friendly antimicrobials as natural food preservatives to achieve this demand is bacteriocin (**Henning *et al.*, 2015**).

Bacteriocins are proteinaceous compounds composed of heterogeneous groups of bacterial antagonists that vary considerably in biochemical properties, molecular weight, mode of action, and range of sensitive hosts **(Marwa *et al.*, 2018).** LAB-bacteriocins are often active across a wide range of pH values, thermostable and showed either bacteriostatic or bactericidal activity toward various food-borne pathogenic microbes (**Juliana *et al.*, 2017 and Chikindas *et al.*, 2018**). Bacteriocins are sensitive to digestive proteases such as pancreatin complex, trypsin and chymotrypsin, and thus do not impact negatively on the gut microflora (**Egan *et al.*, 2016**). Nisin and pediocin are the well-known LAB-bacteriocins, with the secure and effective application (**Chi and Holo, 2018**).

Nanotechnology is an auspicious interdisciplinary approach of science that suggests novel nanoscale materials with anticipated applications in different structural areas (**Vaseghi *et al.*, 2017**). Due to their conventional small size (1–100 nm), nanoparticles have been shown quite challenging unique phenomena of biological functional and physicochemical properties when compared to bulk materials and single atoms or molecules. Culture supernatants of LAB may act both as reducing and capping agents for AgNPs biosynthesis. They contain eco-friendly benign chemical complexes such as polysaccharides, proteins, enzymes, amino acids, and vitamins which are responsible for the reduction of Ag+ ions (**Collera-Zuniga *et al.*, 2005**). AgNPs have been evaluated for their antimicrobial properties against a wide range of pathogenic organisms (**Seetharaman *et al.*, 2018; Youssef *et al.*, 2019**).

The current work aimed to isolate and identifie of bacteriocin producing LAB from fish samples. The produced bacteriocin was purified and characterized. Also, the potential of nanotechnology was investigated for enhancement of bacteriocin activity.

MATERIALS AND METHODS

Isolation, identification, and probiotic characterization of isolated bacteriocin producing LAB

The bacteriocin producing LAB strains were isolated from fish marine samples (*Mugil Seheli* and *Tilapia zillii*). Aseptically, one g of the intestinal content of each fish sample was homogenized. Serial dilutions were prepared and about 0.1 ml from each dilution was subculture in duplicate into sterilized De Man's Rogosa and Sharpe (MRS) agar medium supplemented with 0.1% (w/v) nystatin to inhibit fungal growth (**Elyass *et al.*,**

2017). The plates were incubated at 37±2°C for 48-72hr. Isolated colonies with typical characteristics of LAB were picked and purified. The selected colonies were initially tested for Gram-reaction and catalase test (**Tambekar e*t al*., 2009**).

Preparation of crude bacteriocin

One ml of each bacterial sample (optical density (O.D), 1.0) was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of sterilized MRS broth medium and incubated at 37 ^{o}C on a rotary shaking incubator (160 rpm) for 72 h. The grown cultures were centrifuged at 6000×g for 10 min at 4^{o}C. 5-ml of the supernatant was mixed with catalase enzyme (Sigma) at pH 7.0 to eliminate the effect of hydrogen peroxide produced by the strain and incubated at 37±2°C for 2 hr and the pH was adjusted to 6.5 using 1N NaOH or 1N HCl. After that the supernatant was heated at 80 °C and filter sterilized, to obtain the crude bacteriocin (**Liao and Nyachoti, 2017**)

Assay for antibacterial activity by agar well diffusion method

Crude bacteriocins of the recovered isolates were tested for antibacterial activity by agar well diffusion assay **(Shivashankar *et al.*, 2013).** Different pathogenic bacterial strains were used as indicator strains: *Pseudomonas aeruginosa* (ATCC 8739), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* as Gram-negative, *Bacillus cereus, and Staphylococcus aureus* (ATCC 6538) as Gram-positive bacteria (kindly provided by Prof. Dr. Aida Farag, National Institute of Oceanography and Fisheris, Alexandria, Egypt). Mueller-Hinton agar plates were swabbed with 100 µl of each indicator strain using a sterile swab. The wells were made with the help of sterile gel puncture and 50 µl of each crude bacteriocin was loaded on each well. The plates were incubated at 37±2ºC for 24 hr. The antibacterial activity was evaluated as the diameter of inhibition zones (mm) around the well; assays were done in triplicate.

Identification of the most antagonistic potential of a bacteriocinogenic isolate

Morphological and biochemical characterization

It was done according to method described by **Logan and De Vos (2009)**. The following tests were performed: cell morphology, Gram reaction, catalase test, growth at 10°C for 5 days and 45°C for 48 hr, in MRS- broth, salt tolerance (4% and 6.5% NaCl in MRS-broth). Sugar fermentation tests were applied using glucose, lactose, mannitol, galactose, D- cellobiose, raffinose, D-trehalose and sucrose.

Molecular identification

Identification was made by the sequencing of 16S rRNA gene (Sigma Scientific Services Company). Chromosomal DNA of the most potent LAB strain was extracted as follows: 2 ml of cell suspension was centrifuged at 6000 rpm for 20 min. After the centrifugation, the cell pellets were resuspended in the proteinase K buffer (0.01 M EDTA; 0.1 M Tris-HCl; pH 8.0), incubated at 55 ^{o}C in the presence of sodium dodecyl sulfate, proteinase K

and lysozyme. Then, the partial 16S rRNA gene was amplified by using the primers. Forward primer AGA GTT TGA TCC TGG CTC AG, and reverse primer, GGT TAC CTT GTT ACG ACT T. The PCR amplification was done using IU of Taq DNA polymerase. The fragments of 16S rRNA sequencing were detected using a Perkin ABI PRISM377 DNA sequencer (Perkin Elmer, Foster City, CA, U.S.A.). The 16S rRNA gene sequences were compared to that database in GenBank (by the NCBI Blast software). The sequence was estimated by applying BioEdit sequence alignment editor program (version 7.2.5) (**Altschul *et al*., 1990**). The neighbor-joining approach (with the BioEdit sequence alignment editor) of obtained sequences of the 16S rRNA gene was estimated.

Partial purification of crude bacteriocin

One liter of MRS broth seeded with 10 ml of an overnight culture of bacterial strain under aseptic conditions was incubated at 37±2°C for 48 hr. After incubation, centrifugation (6000 xg for 15 min at 4°C) was done and the clear supernatant was filtered through sterilized 0.45 mm membranes. Catalase enzyme was added, and pH was adjusted to 6.5. Bacteriocin was precipitated by salt saturation method with different fractions of ammonium sulfate (**Farag et al., 2018**).

Characterization of partially purified bacteriocin

Molecular weight determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) was performed to determine the molecular weight of the partially purified bacteriocin according to the method described by **Schägger and von Jagow, 1987**.

Stability of partially purified bacteriocin at different temperatures and its sensitivity to proteolytic enzymes

Stability of bacteriocin was detected by heating the bacteriocin sample (5 ml) at various temperatures ranged from 60 to100 ℃ for 30 min and at 121 ℃ for 15 min in an autoclave. The untreated bacteriocin was used as a control. One ml of partially purified bacteriocin was added to 1mg/ml of α-amylase, proteinase K, catalase and lipase, respectively, to indicate its sensitivity to proteolytic enzymes**.** Samples were incubated at 30 ^{o}C for 30 min and then heated at 95-97 ^{o}C for 5 min **(Oh *et al.*, 2000)**. Both treated and untreated samples were assayed for antimicrobial activity by using agar well diffusion method.

Synthesis of bacteriocin-incorporated silver nanoparticles (LB-AgNPs)

Synthesis of Sa-AgNPs was carried out according to the method described by **Amer et al. (2021)**. It was prepared at room temperature by addition of one ml extracted bacteriocin solution to freshly prepared 15 µl of silver nitrate (3 mM,) and 100 µl $NaBH_4$ (0.6 mM). A free AgNB solution (without bacteriocin) was prepared. The obtained mixture was

exposed to natural visible light. After 30 min, the degree of the mixture color turned from colorless to brown, which reflected the synthesis of AgNPs.

Characterization of silver nanoparticle

UV-visible spectroscopy analysis

The optical absorption spectra of the Sa-AgNPs suspension were obtained with a Model T70 Split-Beam UV/VIS Spectrophotometer, England. The reduction of pure Ag+ ions was monitored by measuring the UV–Vis spectrum of the reaction mixture at the range of 200 to 800 nm (**Philip and Unni, 2011**).

Scanning electron microscopy (SEM) analysis

SEM (JOEL, Japan, Model- 6360) was employed to image the sample surface by scanning it with a high energy beam of an electron. This scanning was engaged to provide further investigation insight into the surface morphology of Sa-AgNPs and analyze their structure and size **(Awwadi and Nida, 2012**).

Antibacterial efficiency of the biosynthesized Sa-AgNPs

The antibacterial activity of Sa-AgNPs was investigated using agar well diffusion method against the pathogenic indicator strains: *S. aureus*, *E.coli* and *K. pneumonia* as previously discussed. The incubated plates were examined for zone of inhibition around the individual wells. The diameter of the inhibition zone around the well was measured.

Statistical analysis:

All of the experiments were performed in triplicate, and the data were calculated as the Mean ± SD with MS-Excel..

RESULTS

Isolation of bacteriocin producing LAB

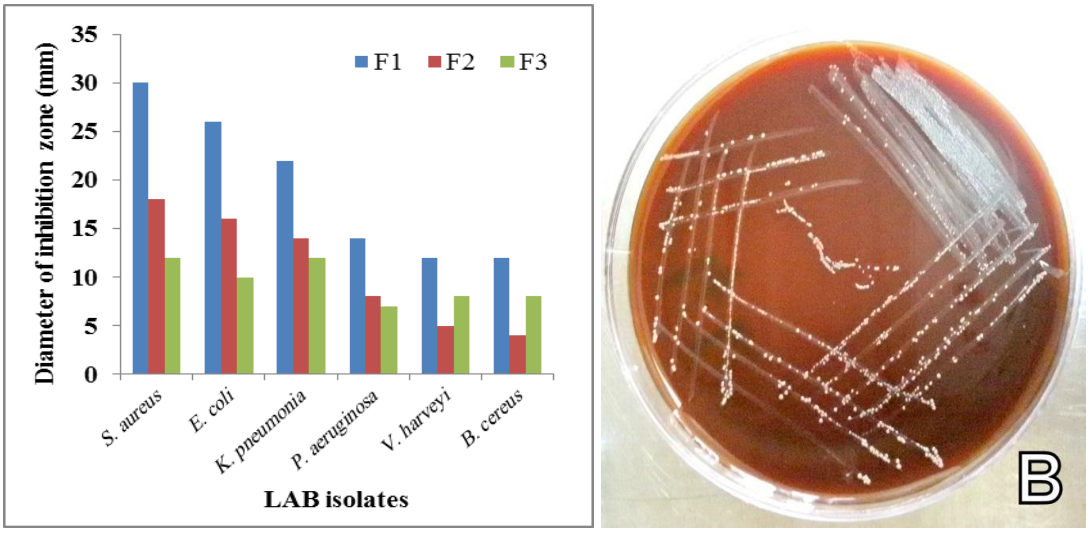
The bacteriocin producing LAB strains were isolated from fish marine samples (*Mugil Seheli* and *Tilapia zillii*). Three LAB isolates (F1, F2 and F3) were identified morphologically, microscopically, and biochemically. The strains are Gram-positive rods and cocci, catalase, oxidase negative and non-spore forming. The bacterial isolates were identified as *Lactobacillus* spp.

Table 1. Morphological, microscopic and biochemical characteristics of LAB isolates

Characteristics	**Observation**		
	LAB isolate of *Mugil Seheli*	**LAB isolate of *Tilapia zillii***	
	(F1)	**(F2)**	**(F3)**
Colony appearance	Creamy-white, elevated	Creamy-white	Creamy, white and little sticky
Gram reaction	Gram- positive long rods	Gram- positive Cocci	Gram- positive long rods
Endospore–staining	Non-endospore	Non-endospore	Non-endospore
Oxidase test	Negative	Negative	Negative
Catalase test	Negative	Negative	Negative
Indole	Negative	Negative	Negative
Methyl red	Positive	Positive	Positive
Vogus proskauer test	Negative	Negative	Negative
Possible microorganism	*Lactobacillus* sp.	*Lactobacillus* sp.	*Lactobacillus* sp.

Screening of bacteriocin production by agar well diffusion assay

The antibacterial potency was investigated against various indicator species. The results (**Fig. 1A**) revealed that all the three LAB isolates were able to produce bacteriocin with different levels. The bacterial isolate F1 (isolated from *Mugil Seheli*) was the highest bacteriocin producer and showed the highest diameter of inhibition zone 30, 26, 22, 14, 12, and 12 mm, respectively, against *S. aureus*, *E. coli*, *K. pneumonia*, *P. auroginosa*, *V.harveyi* and *B. cereus*, respectively. According to the previous results, bacterial F1 (**Fig. 1B**) isolate was selected for molecular identification and current investigation.

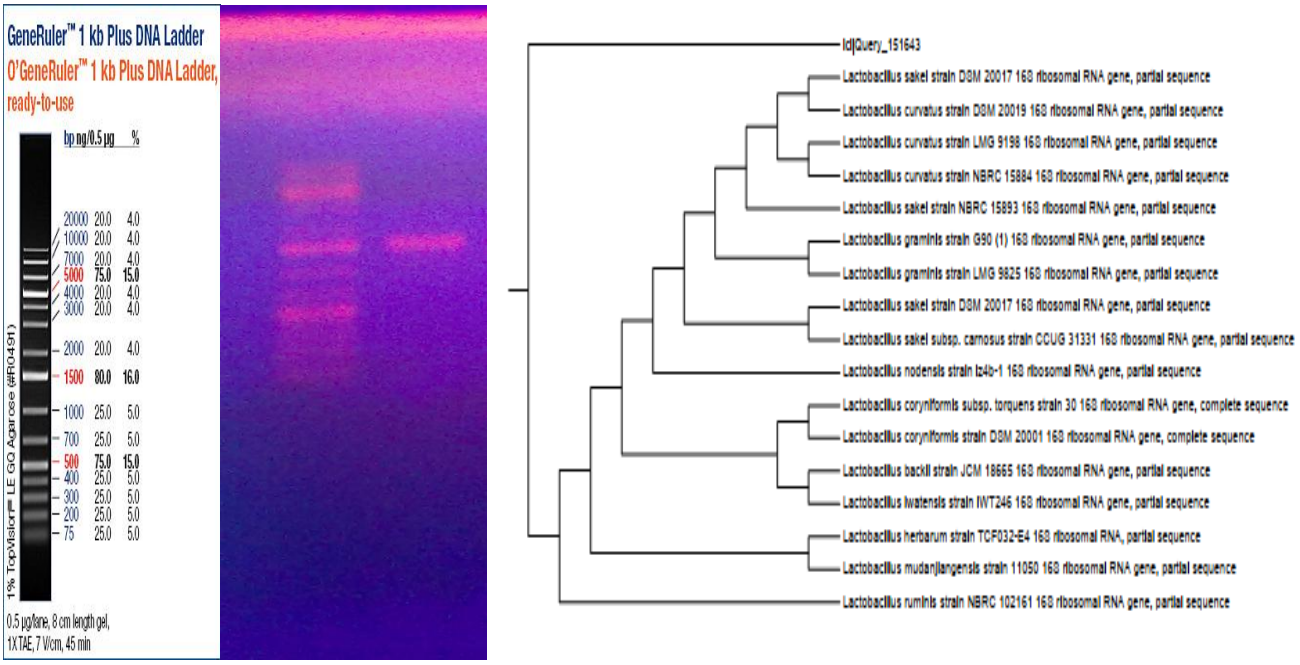


(A) **(B)**

Figure 1:(A); Antagonistic effect of different isolates against different pathogenic bacteria; (B) small creamy colonies of F1 isolate on MRS agar**.**

Molecular identification of the bacteriocin producer isolate

The identification of the selected strain was made on the bases of 16s rRNA gene sequences. The nucleotide sequence analysis of the sequence was performed at BlastN site at NCBI server. The selected isolate has 400 bp (**Fig. 2A**) and showed 100 % similarity to a member of genus *Lactobacillus*. The sequence was deposited in GenBank, maintained by NCBI, USA (Accession No: MG654786), and the organism was identified as *Lactobacillus sakei*. The sequence was also subjected to phylogenetic analysis using the clustal X2. Phylogenetic tree based on 16S rRNA sequence analysis was constructed using TREEVIEW X to demonstrate the multi-sequence alignments between the obtained sequences and the nearest strains (**Fig. 2**B).



(A) **(B)**

Figure 2. (A); Agarose gel electrophoresis of the amplified region of *Lactobacillus sakei* (B): Phylogenetic tree based on 16S rRNA gene sequence analysis and reference sequences extracted from the Database of GenBank (***Lactobacillus sakei*** and constructed by ClustralX2).

Partial purification of *L. sakei* bacteriocin

Among all obtained fractions, 65% ammonium sulphate fraction showed the highest diameter of inhibition zone (41mm) and gave about 1.37- fold activity higher than that obtained from crude bacteriocin (30mm) (data not showed). All other fractions showed a relatively lower bacteriocin production. Also, there was no any activity in higher (85%) ammonium sulphate fraction.

Characterization of partially purified bacteriocin

Molecular weight determination

Molecular weight of the partially purified *L. sakei* bacteriocin (was achieved with 65% saturation of ammonium sulfate) was estimated as 5 kDa by SDS-PAGE gel electrophoresis (**Fig. 3**).

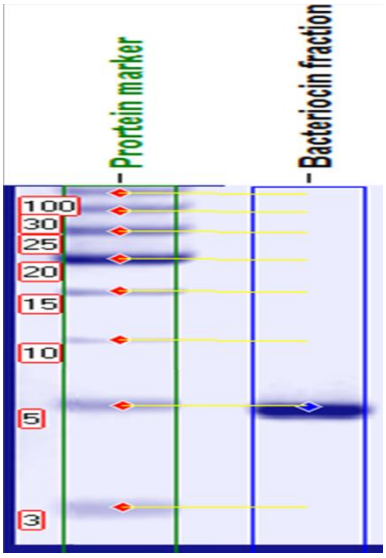


Figure 3. Molecular weight of partially-purified *L. sakei* bacteriocin

Stability of partially purified bacteriocin at different temperatures

The results (**Table 2**) revealed that partially purified *L. sakei* bacteriocin was stable over a broad range of temperatures. It retained more than 81.0, 77.4 and 66.9% of its original activity even at 100 °C for *S. aureus*, *K. pneumonia*, and *E. coli*, respectively. The residual activity of bacteriocin against *S. aureus* was 72.03% remaining even at 121°C for 15 min. So, the obtained results indicate that it could be used as biopreservative with thermal food processing.

Table 2. Effect of heat treatment on the activity of partially purified *L. sakei* bacteriocin

Temperature/time treatment (^{o}C)/ (min)	**Bacetriocin relative activity (%)**		
	S. aureus	***E. coli***	***K. pneumonia***
Control	100	100	100
60 / (30)	96.69	91.12	100
80/(30)	87.11	90.22	84.64
100/(30)	81.00	66.94	77.44
121/(15)	72.03	51.89	63.56

Control: Untreated partially purified bacteriocin**.**

Sensitivity to proteolytic enzymes

The bacteriocin activity was entirely destroyed by treatment with proteinase K as there was no inhibition zone appeared (**Table 3**) compared with control (bacteriocin without

enzyme treatment) which confirming its proteinaceous nature. Treatment of the bacteriocin with catalase and α-amylase did not result in any evidenced changes indicating that the potential of inhibition was not by hydrogen peroxide**.**

Table 3. Effect of different enzymes on the sensitivity of partially purified bacteriocin

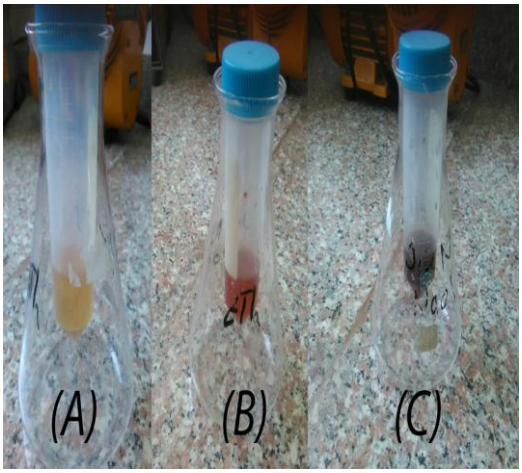
Enzyme	Diameter of inhibition zone (mm)		
	S. aureus	***E. coli***	***K. pneumonia***
(Control)	41	38	28
Proteinase K	ND	ND	ND
Catalase	38	38	28
Amylase	36	31	28
Lipase	31	29	25

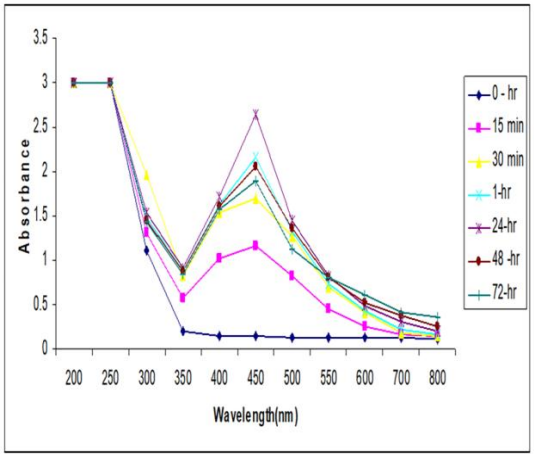
Control: Partially purified bacteriocin without enzyme treatment, ND: not detected.

Biosynthesis and Characterization of synthesized LB-AgNPs

Color change and UV-vis spectroscopy analysis

LB-AgNPs formation was confirmed by the appearance of brown color in the solution containing the biomass confirmed the formation of silver nanoparticles (**Fig. 4 a**). A sharper peak and strong surface plasmon resonance (SPR) was observed at a range of 400–450 nm (**Fig. 4 b**). The results indicated that the intensity of the peak increased with increasing time reaching its maximum absorption with a sharper peak at 430 nm after 24 h of incubation**.**





(A) **(B)**

Figure 4. (A): colour intensity: at zero time(A) , 20min (B) and 24h (C); (B) UV-visible spectra of synthesized AgNPs of different incubation periods .

Scanning electron microscope of LB-AgNPs

The scanning electron micrographs of LB-AgNPs showed high density and spherical to polyhedral forms nanoparticles. The particle size ranged from 20.20-60.61 nm (**Fig. 5A**) and from 28.14-51.41 nm (**Fig. 5B**).

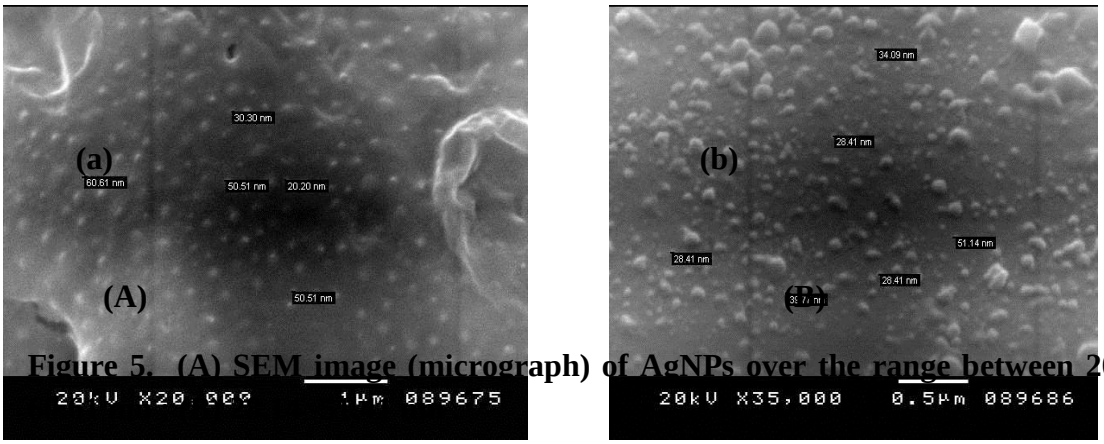


Figure 5. (A) SEM image (micrograph) of AgNPs over the range between 20.20 - 60.61 nm (a) and from 28.14-51.41 nm (b).

Antibacterial activity of LB-AgNPs

The susceptibilities of Gram-positive and Gram-negative bacteria to the growth inhibition by crude, partially purified and LB-AgNPs were detected (**Fig. 6**). The maximum activity was observed against *S. aureus* while *K. pneumoniae* was the most resistant**.**

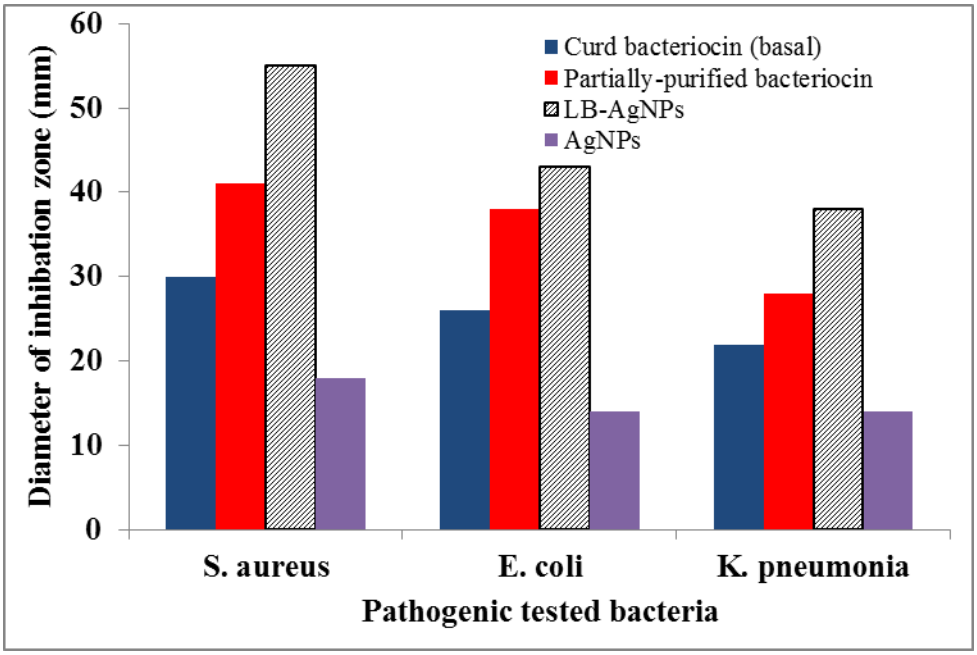


Figure 6. Antibacterial activity of the AgNPs, crude, partially purified and LB-AgNPs

DISCUSSION

Bacteriocins are ribosomally assembled peptides that show antimicrobial activity to a wide range of bacteria (**Ng *et al*., 2020**). They are either bacteriostatic or bacteriocidal to obliterate or repress the development of different microorganisms as a way of competition and survival in the microbial community (**Darbandi *et al*., 2021**). Bacteriocins produced by LAB species are significantly dissimilar from one another in terms of the mode of action, inhibitory spectrum, molecular weight, biochemical properties and genetic origin (**Klaenhammer, 1993**).

In the current research, LAB were isolated from different fish samples as producers of bacteriocin on the MRS media were purified and identified. MRS medium is the chemically defined medium which was most commonly used for isolation of LAB. The identification of the most potent selected isolate F1 (isolated from fish, ***Mugil Seheli***) was done on the bases of 16s rDNA gene sequences. The sequence was deposited in GenBank, (Accession No: MG654786), and the bacterial strain was identified as *Lactobacillus sakei*. The genus *Lactobacillus* used to comprise over 200 species, making it the largest and most diverse genus of LAB. The lactobacilli group occupies a variety of niches, including plant and milk surfaces, as well as the gastrointestinal tract of animals and human (**Hill *et al*., 2018**). *Lactobacillus* spp. was also studied extensively as fermentation starter culture and as probiotics, of which bacteriocin production has been considered an important trait (**Zhang *et al*., 2020; Dai *et al*., 2021; Leslie *et al*., 2021 and Younas *et al*., 2022**).

Precipitation is a first step of purification for many proteins such as (bacteriocin derived from LAB). Bacteriocins must be obtained in purified form to be studied and characterized. So, partial purification of the crude bacteriocin produced by *L. saki* cultures was fulfilled by fractional precipitation with different fractions of ammonium sulphate. Among all the obtained fractions, the 65% ammonium sulphate fraction showed the highest inhibition zone diameter (41 mm) and about of 1.37-fold purification. Ammonium sulfate is considered as the most proper salt for initial purification step for proteins due to its high yield and solubility (**Chen *et al*., 2018 and Amer *et al*., 2021)**). Additionally, the protein precipitate formed is stable against proteolysis and bacterial degradation.

The purity of bacteriocin was analyzed by SDS-PAGE electrophoresis. Many investigators have used SDS-PAGE for determination of molecular weight of the bacteriocin. The molecular weight of the partially purified bacteriocin found in the present study as 5 kDa. Our results are in agreement with many investigators (**Todorov and Dick, 2005; Barbosa *et al*., 2014).** On the other hands, different molecular mass of bacteriocin were reported (**Powell *et al*., 2007; Marwa *et al*., 2018)**.

Different heat treatments of the bacteriocin was determined to examine its stability.

Thermostability of bacteriocin at high temperature makes it feasible to used foods which needs to be pasteurised before consumption. It retained about 87.11% of its activity when heated at 80° C for 30 min which revealed that it is able to withstand pasteurization which is related to its molecular structure composed of small peptides with no tertiary structure (**Parada *et al*., 2007)**.The results are in confirmed with other investigators (**Barbosa *et al.* 2014**; **Izuchukwu, 2017**). The treatment with proteinase K, catalase and α-amylase was confirming its proteinaceous nature (**Bromberg *et al*., 2005**). The results are in agreement of **Elayaraja *et al*. (2014)** who found that antimicrobial activity does not require carbohydrate moieties.

Recently, the most bacteriocin nanoconjugated types were with metallic nanoparticles, especially with silver. Silver nanoparticles proved to be more appropriate for conjugation with bacteriocins, because it provide higher antimicrobial potential with broad spectrum against pathogenic microorganisms, more active surface, and greater chemical stability of the nanoparticles which led to increase in released free bacteriocin when exposed to the human gut system without being affected by the digestive enzymes.

The synthesized silver nanoparticles (LB-AgNP) were characterized by UV-vis spectroscopy analysis which revealed that a sharper peak and strong surface plasmon resonance (SPR) was detected a sharper peak at 430 nm after 24 hr of incubation. Also, **Bankura *et al*. (2012**) found that the absorption maxima of dextran-stabilized AgNPs shifted to 425 nm after 30 days of storage.

The SEM examination of nanoparticles showed that the particle size of AgNPs ranged 20.20-60.61 nm and from 28.14-51.41 nm. The difference in the particles size in the sample indicates that it was formed in different times (**Geethalakshmi and Sarada, 2012**). **Wu *et al.* (2014**) reported that AgNPs activity was strongly dependent on the size in which the bactericidal activity of AgNPs of smaller dimensions (<30 nm) was found to be optimal against *S. aureus* and *K. pneumonia.*

Assessment of the *L. sakei* bacteriocin mediated silver nanoparticles showed that *S. aureus* was the most susceptible, while *K. pneumoniae* was the most resistant to the AgNPs. This might be due to differential susceptibility of tested organisms against nanoparticles. The current results are in agreement with **Prabhu *et al*. (2014)** who reported that *S. aureus* was the most susceptible to AgNPs. Also, the results proved that the combination of bacteriocin with metal nanoparticles found to be more productive and with higher bactericidal effect than AgNPs and free bacteriocin due to their small size and high surface area of the positively charged metal nanoparticles, which accelerate their binding power to the negatively charged bacterial membrane (**Dhanalakshmi and Rajendran, 2014**).

CONCLUSION

Lactic acid bacteria and their bacteriocins have been presented as viable eco-friendly alternatives to chemicals and antibiotics in the fields of food technology and enhance the synergistic efficacy of the antibacterial mechanism against clinical bacteria. *L. sakei* bacteriocin was characterized by high stability at different heat treatments, and completely inactivation by proteinase K enzyme. *L. sakei* bacteriocin mediated silver nanoparticles in the size of 20.20 to 60.61 nm was showing an auspicious synergistic activity against various microbial pathogens compared with the free bacteriocin, confirming its potential in therapeutics and pharmaceutical applications. Nanotechnologies are estimated to be commercially available in the near future. Further investigations are also required to assess the *in vivo* efficiency of LAB bacteriocin for food safety, preservation, and future scope for clinical application.

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