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ORIGINAL RESEARCH ARTICLE

Enhancing the Antimicrobial Activity of Nisin by Encapsulating on Silver Nanoparticle Synthesized by *Bacillus* sp.

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ABSTRACT

Bacteriocins are antimicrobial proteins that are generally inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria. Nisin is the most extensively characterized bacteriocin, mass-produced by *Lactococcus lactis* and purified using silicic acid, yielded 1 mg / ml of medium. Molecular weight of the nisin was determined as 3. 5 kDa. In order to improve the nisin activity, it was encapsulated on silver nanoparticle synthesised by *Bacillus* sp., and assayed for antimicrobial activity against multi-drug resistant microbial pathogens of *Escherichia coli*, *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Bacillus* sp., *Enterococci* sp., and *Candida albicans*. About 22% increase in antimicrobial activity was recorded with encapsulated nisin than non-encapsulated nisin, as well as concentration required to inhibit the growth of the pathogens were reduced from 125 µg/ml to 62. 5 µg/ml. So, the study recommends the use of nisin encapsulated silver nanoparticle as preservative to control the establishment of food and water borne pathogens in packed food.

Key words: Nisin; Silver nanoparticle; Encapsulation; Antimicrobial activity; Nanoparticle synthesis.

1. INTRODUCTION

Intake of food not only provides nutritional value to human but also serves as a vehicle for disease transmission. Major food and water borne pathogens that causes infections in human are *Salmonella* sp., *Campylobacter jejuni, Listeria monocytogenes, Escherichia coli, Staphylocccus aureus, Clostridium botulinum, C. perfringens, Bacillus cereus, Vibrio cholerae, Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., and *Shigella* sp.,[1,2]. An U. S., report on food borne illness estimated to affect 76 million people every year. It is due to the unsanitary storage of foods [3,4]. Another study on food borne illnesses in the year 2000 revealed that about 2.1 million people died from diarrhoeal diseases.

Nowadays food and water borne pathogens have acquired multi drug resistance (MDR) due to widespread and excessive usage of antibiotics [5,6]. Pathogens like methicillin resistant *Staphylococcus* (MRSA), vancomycin resistant *Enterococci* (VRE) and extended spectrum β-lactamase producing Gram-negative bacilli harbours genetic determinants, which render them resistance to the most of the available antimicrobial drugs [7]. Infections caused by MDR strains are difficult to treat and become life threatening. So the demand for more natural, safe and minimally processed drug is prerequisite of the day. As a result, there has been great interest and research on naturally produced antimicrobial drugs, such as bacteriocins as a drug to control the pathogens transmitted through food and water [8].

Microorganisms produce variety of compounds, which exhibit antimicrobial properties. Among them self-defending bacteriocins are produced by lactic acid bacteria that plays major role in antibacterial activity. Wide spread occurrence of bacteriocins in intestinal tract, oral and other epithelial surfaces have suggested that it has regulatory role in terms of population dynamics with in bacterial ecosystem.

Among bacteriocin, nisin is an antimicrobial polypeptide containing 34 amino acids and is remarkably heat and acid stable [9]. Nisin is produced by strains of *Lactococcus lactis* subsp. lactis. It has a narrow spectrum of antimicrobial

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activity and helps to control intestinal flora [10-13]. The World Health Organization has recognized nisin as safe food additive [14,15]. Currently excessive quantities of nisin, more than the recommended dosages are required to guarantee effective growth control of MDR pathogens. A slow and steady release system is required to ensure nisin activity at lower concentrations.

Nanoparticles are most promising for generation of newer applications in existing medicine because they have better or improved properties than bulk materials of same element. Nisin–loaded polymeric micro-and nanoparticles seem to be promising formulation to achieve long–lasting antimicrobial activity. The present work evaluates the antimicrobial activity of nisin encapsulated on silver nanoparticle produced by *Bacillus* sp., against MDR microbial pathogens.

2. EXPERIMENTAL PROCEDURE

Isolation and identification of *Lactococcus lactis*

Fermented milk product, curd sample was collected aseptically, diluted and inoculum was uniformly spread on De Man Rogosa Sharpe (MRS) agar by spread-plate technique. After 48 h of incubation, the isolated organism was sub-cultured and maintained on MRS agar slant at 4°C. Biochemical tests were performed for genus and species level identification of isolate[16].

Mass culture and purification of Nisin

One liter of MRS broth was inoculated with 10 ml of seed culture of *Lactococcus lactis* and incubated at 37ºC under anaerobic condition for 48 h. The cell free culture supernatant was collected by centrifugation at 1000 rpm for 20 min. Then nisin was purified using silicic acid [17].

Characterization of nisin

Collected bacteriocin was subjected to protein estimation by Biuret assay using bovine serum albumin as standard. For further confirmation of presence of protein, purified nisin sample was run in SDS-PAGE along with commercial nisin and protein marker [18].

Biological synthesis of silver nanoparticle

Two flasks of 50 ml of nutrient broth were prepared and each was supplemented with 3.5 mM $AgNO_3$. One flask was inoculated with *Bacillus* sp., and another uninoculated medium was maintained as control. Flasks were incubated for 7 days in dark. After incubation the broth was centrifuged at 10,000 rpm at 28º C. To the culture supernatant, 3.5 mM $AgNO_3$ solution was added and the colour change was recorded [19]. Absorbance spectra of the supernatant were recorded from 400 nm to 700 nm and absorbance curve was plotted.

Encapsulation of nisin on silver nanoparticle

One ml of purified nisin and 1 ml of synthesized silver nanoparticle were kept under vacuum for 24 h. Then sample was filtered through 0. 45-µm membrane filter paper and used for further assay.

Assay of antimicrobial activity of purified Nisin

Overnight broth cultures of MDR strains, MRSA *Staphylococcus* sp., *Escherichia coli* CIP^R, CZ^R, OX^R, *Pseudomonas* sp., CZ^R, VA^R, OX^R, *Bacillus* sp., CIP^R, GEN^R, VA^R, OX^R, P^R, TB^R, CZ^R, *Salmonella* sp., OX^R, VA^R, CZ^R, *Candida* sp., CIP^R, OX^R, VA^R, CZ^R, C^R, P^R, and *Enterococci* sp., S^R, OX^R, VA^R, CZ^R,C^R, P^R, RIF^R were swabbed on separate Muller-Hinton agar plates and two wells were made on agar plates at uniform distance (CZ = Cefazolin; P = Penicillin; TB = Tobramycin; CIP = Ciprofloxacin; OX = Oxacillin; S = Streptomycin; GEN = Gentamycin; VA = Vancomycin; C = Chloramphenicol; RIF = Rifampicin). Twenty microlitres of the nisin, and nisin-encapsulated nanoparticle were added to the wells of each plate. Plates were incubated at 37°C for 24 h. After incubation zone of inhibitions were measured. Minimal inhibitory concentration of nisin and encapsulated nisin required to control the growth of selected pathogens were assayed [20].

3. RESULTS AND DISCUSSION

Isolation of *Lactococcus lactis* from curd

After incubation, on MRS agar plates isolate produced pinhead size, creamy white, circular colonies similar to the reports of Cheig *et al*. in 2002 [21]. On nutrient agar plates, isolate produced bright orange coloured colonies. Biochemical tests have confirmed the isolate as *Lactococcus lactis.*

Extraction and concentration of Nisin

Silicic acid method of purification of nisin yielded 0.1 g of bacteriocin from 100 ml of culture supernatant. The extracted nisin appeared as pure white powder after lyophilization. The yield of the nisin indicated that bacteriocin purification by silicic acid method is rapid, simple and yielded high [22].

Estimation of total protein

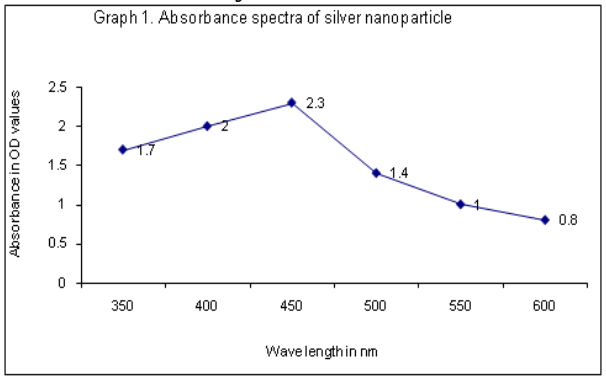
Biuret assay confirmed the presence of 1 mg of protein/ml of fraction collected by silicic acid method of purification. Adam and Moss in 1995 proposed that nisin is a 34 amino acids containing polypeptide and is remarkably heat stable and acid stable. So, presence of protein confirmed the nisin production. SDS – PAGE analysis of purified nisin samples has further substantiated that

produced protein is 3.5 kDa in molecular weight which was similar to the bands formed by the standard nisin.

Synthesis of silver nanoparticles

Nisin is used in combination with surfactants, chelators and adjuvant to improve its activity at lower concentrations. In the present study, an experiment on encapsulating nisin on silver nanoparticle has been attempted. The work was focused towards the biosynthesis of silver nanoparticle because antibacterial activity of silver species has been well known since the ancient times. Moreover, at lower concentrations silver is non-toxic to human cells[23]. Nano-silver particles are generated by several methods from metallic silver and are generally used in food, consumer products and medical products as an antibacterial agent. Because of its small size, nanoparticles can potentially pass through biological membranes and reach more and different organs and tissues in the body where the silver can exert its antibacterial effects [24].

In the present study *Bacillus* sp., was used for synthesis of silver nanoparticle. Production of silver nanoparticle by *Bacillus* sp., in nutrient broth medium was confirmed by visual observation of change in the colour of the media from yellow to brown. The colour change was due to the reduction of silver nitrate to elemental silver by the reductive enzymes produced by the *Bacillus* sp., and excitation of surface plasmon vibrations in silver nanoparticles [25]. Obtaining broad absorbance spectrum peak at 450 nm for culture filtrate from *Bacillus* sp., inoculated broth (treated with silver nitrate) has further confirmed nanoparticle synthesis. Jain *et al.*, in 2010 [25] proposed that broad absorbance spectrum for silver nanoparticles at 450 nm indicative of poly-dispersed nanoparticle. The present study resulted in a simple, rapid, safe and economical route to biosynthesize silver nanoparticles using *Bacillus* sp. Nisin loaded nanoparticles seem to be promising formulation to achieve long–lasting antimicrobial activity.



Antimicrobial activity of Nisin against pathogens

The inhibitory spectrum of Nisin against several Gram-positive and Gram-negative pathogens was determined in order to evaluate the possibility of using the nisin as an additional barrier against spoilage by food borne microorganisms. Common pathogens that contaminate food and water and leading to food and water borne illness are *Salmonella* sp., *Enterococcus* sp., *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Enterococcus* sp., *Candida* sp., and *E. coli.* Preservatives used in foodstuffs must strictly control the growth of these pathogens. In view of this concept, highly drug resistant pathogens were collected and antimicrobial activity of Nisin and Nisin encapsulated silver nanoparticle were evaluated. The purified bacteriocin of *L. lactis* exhibits wide spectrum of antimicrobial activity against pathogenic microorganisms. Purified Nisin has restricted the growth of pathogens *Salmonella* sp., *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *E. coli* to a diameter of 17 – 30 mm in agar well diffusion assay. Results obtained were similar to results published by Gupta and Batish [26]. But, MDR strains of *Enterococcus* sp., and *Candida* sp., exhibited resistance towards purified Nisin. Ec *et al*. [27] also obtained similar results in 2001 against these pathogens. Compared to nisin and silver nanoparticles, the encapsulated nisin inhibited pathogens by producing zone of inhibition between to 23 mm and 37 mm. Growth of MDR pathogens, *Candida* sp., and *Enterococci* sp., also were restricted by nisin encapsulated nanoparticles (**Table 1**).

Table 1: Measurement of zone of inhibition by antimicrobial agents

Test organisms	Zone of inhibition (in mm)		
	Nisin	Silver nanoparticle	Encapsulated Nisin
Salmonella sp.,	27	8	34
Staphylococcus sp.,	13	10	25
Bacillus sp.,	22	10	37
Pseudomonas sp.,	20	13	30
Escherichia coli	16	9	32
Enterococcus sp.,	-	9	30
Candida albicans	-	9	23

Assay of Minimal Inhibitory Concentration (MIC)

In minimal inhibition concentration (MIC) assay, absence of turbidity indicates the control of pathogens by the antimicrobial agent. The culture tubes having different concentrations of nisin showed turbidity for *Bacillus* sp., and *Staphylococcus* sp., from nisin concentrations of 62. 5 µg/ml (4th dilution tube), for *E. coli*,

Pseudomonas sp., and *Salmonella* sp., turbidity was recorded from 125 µg of nisin/ml (third tube) till 31. 25 µg of nisin / ml (fifth dilution). The turbidity was comparatively high in fifth dilution. Rest of the test tubes showed absence of growth. In tubes inoculated with *Candida* sp., and *Enterococci* sp., turbidity was observed from 500 µg of nisin /ml (first dilution). So the MIC of purified nisin for *Bacillus* sp., and *Staphylococcus* sp., were 125 µg/ml, for *Pseudomonas* sp., *E. coli*, and *Salmonella* sp., were recorded as 250 µg/ml. *Candida* sp., and *Enterococci* sp., were found resistant even at 500 µg/ml of nisin.

The mode of action of nisin of *L. lactis* was proven as bacteriostatic. So, in practical food preservation, the level of nisin treatment is unlikely to exceed 0.25 mg/ml of food. Because higher concentration could be necessary in order to achieve a bactericidical effect. Also addition of excess amount of nisin is required to guarantee the effective growth inhibition of pathogens. But in food, addition of excess of nisin may change its nature.

Rai, *et al*. in 2009 [28] reported that silver nanoparticles exhibit antibacterial activity against multi drug resistant (MDR) human pathogens, *E. coli*, *P. aeruginosa* and *Staphylococcus* sp., as it showed a clear inhibition zone in antimicrobial sensitivity test. The current work assays the MIC nisin encapsulated silver nanoparticles for MDR pathogens. For *Staphylococcus* sp., and *Bacillus* sp., MIC was recorded as 62. 25 µg/ml of nisin, for *Pseudomonas* sp., *E. coli* and *Salmonella* sp., was above 125 µg/ml and for *Enterococcus* sp., and *Candida* sp., were recorded as 250 µg/ml. The study concludes that silver nanoparticles encapsulated nisin showed antimicrobial activities against the isolated MDR pathogens at lower concentrations. Because, encapsulated nanoparticles offers significant potential as carriers with stability, protection and controlled release properties [29,30]. Controlled release system provides the benefits of protection from rapid degradation, targeting delivery, controlled release rate and prolonged duration of bioactive agents. The encapsulated nisin showed 22% increased activity than the non-encapsulated nisin; this is due to the slow release of polymeric physically stable antimicrobial nisin compound by the silver nanoparticle.

4. CONCLUSION

Biologically safe silver nanoparticles can be produced by *Bacillus* sp., and nisin can be encapsulated on the silver nanoparticle. This efficient and slow release system ensures the long-term effective control of all kind of food and water borne microbial pathogens. So, the study strongly recommends that efficiency of the nisin can be improved by encapsulating on silver nanoparticle; thereby it can be used for long term preserved usage of packed food materials.

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