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SHORT COMMUNICATION

A STUDY ON THE BACTERICIDAL EFFECT OF NISIN PURIFIED FROM *LACTOCOCCUS LACTIS*

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ABSTRACT: Nisin, the best known LAB (Lactic Acid Bacteria) bacteriocin, is a promising alternative to antibiotics, and displays a broad spectrum of activity against a wide range of spoilage and pathogenic Gram positive bacteria. The aim of the study was to determine the bactericidal effect of nisin. The *Lactococcus lactis* MTCC 440 used for the study was obtained from IMTECH, Chandigarh. The nisin was purified by ammonium sulphate precipitation and dialysis. It was found that the addition of nisin showed bactericidal activity against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus cereus* at 11µgm/10µL, 07µgm/10µL and 08µgm/10µL, respectively. The bactericidal effect of nisin against susceptible test strains showed minimum inhibitory concentrations of 50, 80 and 70 µgm/mL against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus cereus*, respectively in MIC test. The molecular weight of nisin was characterized using SDS PAGE and it was found to be 3000 and 7000 Daltons. The study revealed that nisin exhibited bactericidal effect against some of the selected food-borne pathogens and hence can be effectively used in any food product to control either the Gram positive food-borne pathogens or spoilage bacteria.

Key words/phrases: Bactericidal, *Lactococcus lactis*, Nisin, Test strains.

INTRODUCTION

Almost all the preservation procedures describe addition of chemical preservatives to control food-borne pathogens, and to prevent germination of spore-forming bacteria and in combination with the mild heat treatment to kill vegetative microorganisms. Bacteria showing multiple drug resistance towards antibiotics are emerging as a global problem in the food industry which requires new approaches to kill harmful microorganisms. Bacterial resistance to antibiotics has become a major problem in the contemporary treatment of infectious diseases. Pneumococcal resistance to penicllin, staphylococcal and enterococci resistance to vancomycin are widely known examples (Bonev *et al*., 2004). Every consumer demands

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food that contains natural preservative with extended shelf-life. Reported outbreaks of food-borne diseases are rising issue on one hand, and on the other hand, legislation has restricted the use of currently approved preservatives according to the standard recommended level. Biological preservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesirable microorganisms in food (Jeevaratnam *et al*., 2005). The bacteriocin Nisin has been repeatedly shown to be useful in foods over the past 30 years (Rajaram *et al*., 2010). It is a promising alternative for antibiotics and displays a broad spectrum of activity against Gram positive bacteria. Nisin is a natural polypeptide produced by *Lactococcus lactis subsp. lactis* and has been found as probiotic and aids human digestion. Nisin is effective against a wide range of spoilage and pathogenic gram positive bacteria (Han, 2005). The potential application of nisin in food preservation was first demonstrated in 1951 (Hirsch *et al*. 1951). Its use was first established as preservative in cheese products and since then numerous other applications in food and beverages have been identified (Flores *et al*., 2003). It is used to increase the shelf life of sterilized milk for periods as long as sixty days (Wajid and Kalra, 1976). Nisin is considered safe for foods because it is readily destroyed by digestive enzymes. Therefore, it is harmless to man and no maximum permitted quantity is specified.

Considering the above facts, the objective of the present study was to evaluate the bactericidal action of nisin on selected Gram positive test strains including *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*, *Listeria monocytogens* and a Gram negative strain *Salmonella typhimurium*. All these strains are representatives of either food-borne pathogens or common agents of food spoilage.

MATERIALS AND METHODS

Bacterial strains and media

Gram positive *Staphylococcus aureus* MTCC 3160, *Micrococcus luteus* MTCC 106, *Listeria monocytogens* MTCC 1143, *Bacillus cereus* MTCC 1272, one Gram negative *Salmonella typhimurium* MTCC 1252 and nisin producing culture *Lactococcus lactis* subsp. *lactis* MTCC 440 were kindly obtained from Institute of Microbial type culture collection, Chandigarh, India. All test strains except *Lactococcus lactis* MTCC 440 were grown on nutrient agar at 37^0C. The experiment was done using the culture *Lactococcus lactis* MTCC 440 for studying the microscopic examination, cell morphology, gram staining, and biochemical tests which included

catalase, oxidase activity, and carbohydrate fermentation patterns. This is a preliminary test procedure carried out to study their biochemical characteristics as literature concerning to the strain *L. lactis* MTCC 440 was not illustrated in detail by researchers. The colony morphology, growth, and physicochemical characteristics of nisin-producing strain were observed at 30^0C on M17 agar. All these strains were maintained in storage at 4^0C until needed.

Nisin purification

The nisin producing *Lactococcus lactis* MTCC 440 was grown in M17 broth (Hi Media Laboratory Pvt Ltd. India) (pH 7.0) seeded with 5 % inoculum of overnight culture and maintained at 37^{o}C for 24 h. After incubation, cells were removed from the growth medium by centrifugation (6000 rpm for 15 min, 4^{o}C). The cell-free supernatant was precipitated with 60% ammonium sulphate (Ranbaxy, New Delhi) saturation. The precipitate was dialyzed with distilled water for 12 h at 4^{o}C. The dialyzed sample was used as Nisin the extract of *L. lactis* to study its antibacterial property. One part of the extract was dried in an oven at warm temperature to powder for MIC determination.

Nisin bioassay

The assay of purified nisin was determined using the well diffusion method. Aliquots of ten and twenty microliters of nisin were added separately in 5 mm diameter wells (sterilized cork borer no. # 2 was used for punching the agar). In the control well, nisin extract was replaced by 20 microliters of sterile distilled water in Mueller Hinton agar plate that had been previously seeded with test strains (Rajaram *et al*. 2010). All test plates were incubated at 37^{o}C for 24 hrs and the diameter of the zones of growth inhibition were measured. The clear zone larger than 7mm was considered as the test strain showing susceptibility to nisin (Moreira *et al*., 2005).

Determination of inhibitory spectrum by Minimum Inhibitory Concentration Test (MIC)

The nisin stock solution was prepared by mixing 1mg of powdered nisin in 1 mL of distilled water to give a final concentration of 1 µgm/1 µL. A pure culture of nisin susceptible test strains (*Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus cereus*) was grown in nutrient broth. The culture was standardized using standard microbiological dilution plating technique to have a final concentration of 10^6cells/mL. Different concentrations of nisin (from 10 to 100µL) were added to corresponding tubes and adjusted to

100µL final volume with distilled water. Overnight cultures of the test strains in nutrient broth were added to all tubes in aliquots of 1mL and incubated at 37^{o}C for 24 hrs. Optical density readings were performed at a wavelength of 650nm (Najjar *et al*., 2009).

Determination of protein

The total protein concentration of nisin in the dialyzed sample was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard and dissolved in distilled water to a final concentration of 200µgm/mL. In a series of test tubes, 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard (BSA) was pipetted out. Sample extract nisin was taken in a separate tube containing 0.1 mL and all tubes were adjusted to 1mL with distilled water. Blank was prepared in another tube with 1mL distilled water alone. All tubes were added with 5 mL alkaline copper solution and left them to stand for 10 min. After incubating, 0.5mL of folin-Ciocalteau reagent was added. Optical density readings were recorded at 660nm. Amount of protein in the extract was calculated by plotting the readings in standard graphs.

Molecular weight determination in SDS PAGE

The molecular weight of nisin was determined by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with coomasie brilliant blue R – 250. Range molecular weight markers (28–64 KDa) with five polypeptides were used as a marker (Rajaram *et al*., 2010).

RESULTS AND DISCUSSION

The growth and culture characteristics of *Lactococcus lactis*

The nisin-producing strain *Lactococcus lactis* MTCC 440 was subjected to physiological and biochemical characterization. The present investigation highlights the isolation, characterization and activity of nisin produced by *L. lactis* MTCC 440 on M17 agar medium and the results are summarized in Table 1. The results are in agreement with the findings of Schleifer *et al*. (1985).

Determination of Biomass and nisin production

Good growth of *Lactococcus lactis* was achieved in M17 broth medium. It was shown that the bacterial biomass reached 0.7 $O.D_{650}$ after 12 hrs and increased to its maximum level in 18 hrs (0.9). Cell count was observed and remained constant after 18 hrs (1 x 10^9 CFU/mL). The findings of Tafreshi

et al. (2010) showed that the cell density at 600 nm reached its maximum 1.9 in 32 hrs. They observed that the cell density increased after 16 h from 0.2 to 1.5 in MRS broth and increased to maximum level 1.8 x 10^9 CFU/ mL. Our study showed that the media M 17 supplemented with 0.5% lactose favoured good cellular growth and the total protein concentration obtained was 3.4 gm/100 mL. The growth conditions of *L. lactis* ATCC 11454 conducted by Jozala *et al* (2005) showed that the addition of 12.5% sucrose into M17 broth stimulated maximum nisin production as 285.8 mgm/L. Chandrapati and Sullivan (1998) observed 50% increment in nisin expression using sucrose as the carbon source in M17 broth with the *Lactobacillus lactis* subsp. *lactis* strain. The initial pH value is a crucial factor for maximizing the nisin expression. The pH of M17 broth was prepared at 7 in our present study. The optimum initial pH values of *Lactococcus lactis* subsp. *lactis* ATCC 11454 strain for maximum nisin production were found at 6.5 to 7.50 which was also confirmed by Jozala *et al*. (2005).

Table 1. Physiological and morphological characteristics of *Lactococcus lactis.*

Physiological, biochemical characteristics	Results
Colony morphology	Milky white, mucoid colonies with entire margin
Gram staining	Gram positive, rod
Growth in M17 broth	uniform turbidity
Fermentation type	Homo fermentative
Ribose, mannitol, maltose, lactose	fermentation positive
Sucrose	fermentation negative
Catalase, oxidase	Negative

Determination of bactericidal effect of purified nisin

Nisin has bactericidal effect against *Staphylococcus aureus, Micrococcus luteus,* and *Bacillus cereus.* The maximal activity was observed against *Staphylococcus aureus* and *Bacillus cereus.* Nisin showed killing effect on *Staphylococcus aureus* at 20 mm/20µL, and 11 mm/10µL, on *Micrococcus luteus* at 11 mm/20µL, and 7 mm/10µL and *Bacillus cereus* at 12mm/20µL and 10mm/10µL diameter (Fig. 1). The test strains of *Salmonella typhimurium* and *Listeria monocytogens* were not inhibited by nisin. The present study is not in accordance with the study conducted by Tippayatum and Vanee (2007). They found nisin possessed antibacterial activity against several Gram positive bacteria including *Listeria monocytogens* DMST 17303. A similar study conducted by Breidt *et al*. (1993) reasoned that the bacterial strain showing resistance to nisin could be due to blockage or

alteration of a nisin receptor on the outside of the cells, preventing nisin from binding. It showed discrepancy in the results of the diameter of the zone of inhibition between 10 and 20µL in our present study. The agar diffusion test in the present study, as expected, there was not exact increase in the diameter of the clearance zone by increasing the concentration of nisin. The major limitation of plate diffusion assays is that nisin diffusion agar media is proportional to the concentration of nisin only over a limited linear range and nisin levels should be restricted to this range. Beyond this range, the zone diameters cannot be accurately related to the concentration of nisin (Flores *et al.,* 2003).

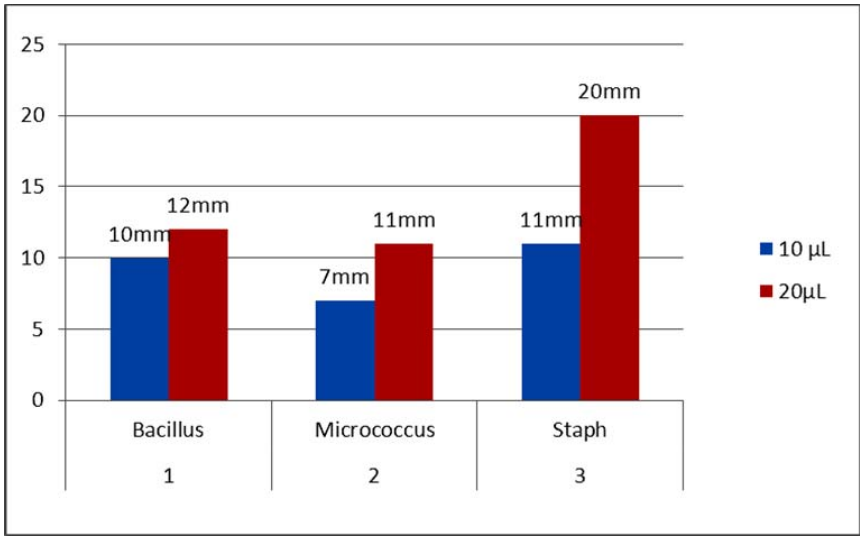

Fig. 1. Antagonistic effect of nisin on susceptible test strains by agar diffusion test

Determination of Minimum Inhibitory Concentration of nisin

The MIC of nisin against susceptible strains showed that MICs of nisin against *Staphylococcus aureus, Micrococcus luteus and Bacillus cereus* were at a concentration of 50, 80, and 70µg/mL, respectively (Table 2). This is in contrast to the results obtained by Tippayatum and Vanee (2007). They found that nisin was inhibitory against *Staphylococcus aureus*, and *Bacillus cereus* at a minimum concentration of 5 mg/mL and 23 mg/mL, respectively. The natural variation in the sensitivity of Gram positive bacteria towards nisin is considerable. Even between closely related species,

minimal inhibitory concentrations (MIC) range from 5µg/L to 5 mg/L (Bennik *et al*., 1997).

Table 2. Determination of Minimum Inhibitory Concentration of nisin.

Test strains	Results (µgm/mL)
Bacillus cereus	70
Micrococcus luteus	80
Staphylococcus aureus	50

Molecular weight determination

The molecular weight of nisin was determined by SDS PAGE gel electrophoresis. Two protein bands were observed when stained with coomassie blue and it clearly indicated the purity of the nisin. The molecular weight of the nisin was calculated to be 3000 and 7000 Da. Similar results were recorded by Gross and Morell (1967).

CONCLUSION

The bactericidal property of nisin produced by *Lactococcus lactis* MTCC 440 was studied by agar well diffusion and minimum inhibitory concentration methods. The study showed that nisin inhibited most of the gram positive bacterial strains used. This indicated that nisin, the product of this organism, can be used as an effective bactericidal agent and the strain *L. lactis* could be used as probiotics with further evaluation on acid and bile tolerance.

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