

## Production and Characterization of Bacteriocin Produced by *Lactobacillus Viridescence*(NICM 2167)

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### ABSTRACT

The present study focused on the production optimization of bacteriocin by *Lactobacillus viridescence* NICM 2167 followed by its purification and characterization. The bacteriocins are antimicrobial peptides produced by many Gram positive and Gram negative bacteria. The bacteriocin produced by LAB (lactic acid bacteria) received attention in recent years due to their potential application as natural preservatives in food. Bacteriocin produced by *Lactobacillus viridescence* showed broad range of antimicrobial activity against food borne pathogens. Production parameters were optimized showing highest production of bacteriocin in MRS broth with pH= 7.0 incubated at 37°C for 48 h. Bacteriocin was purified in two steps involving ammonium sulphate precipitation followed by gel filtration using Sephadex G-100. Purified bacteriocin with single band on SDS-PAGE showed molecular weight of 8.3 kDa. This purified bacteriocin was stable over wide range of pH (4-10) as well as temperatures (4°C-121°C) suggesting it as a potent candidate for preservation of various foods.

**Key words:** *Lactobacillus viridescence* NICM 2167, bacteriocin, antimicrobial activity, gel filtration

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## INTRODUCTION

Lactic acid bacteria (LAB) are widely used in the food industry as starter culture for fermentation. *Lactobacilli* are being utilized in against infectious diseases since decades and have ability to protect against many pathogens (Bernet-Camard et al. 1994). The *Lactobacilli* is widely utilized as a probiotic organism. Many lactic acid bacteria such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* etc are known to produce antibacterial substances including bacteriocins, which can inhibit the growth of several pathogenic bacteria.

Bacteriocins are byproducts of bacterial metabolism. Bacteriocins are agents which are encoded in the genetic material carried by plasmid with the purpose of killing or inhibiting closely related species or even different strains of the same species. Bacteriocins arise from the need for survival in over populated environment or culture. Thus the cells containing encoding for bacteriocins have the capacity of destroying the surrounding cells without the bacteriocin plasmid and the event is compensated by the rate of which the bacteriocin containing cells is able to transpose the plasmids with the required function into other cells. Eventually only cells containing a plasmid encoding bacteriocin will leave in the culture (Avonts and Vuyst 2001). Bacteriocins are antimicrobial peptides and proteins produced by lactic acid bacteria. Although bacteriocins may be found in Gram-positive and Gram-negative bacteria (Riley and Wertz 2002; Nandane et al. 2007), those produced by LAB (Lactic acid bacteria) have received particular attention in recent years due to their potential application in food industry as natural preservatives (Ennhar et al. 1999).

Bacteriocins can be used as food additives like, Nisin produced by *Lactobacillus lactis* is the best-studied and the only antibiotic approved in more than 60 countries including USA to be used in food industry (Twomey et al. 2002) and a marketed preparation with pediocin PA-1 producer is available (Rodriguez et al. 2002). Several studies have indicated that LAB starter cultures or co-culture are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria. The latter trait has mainly been documented for fermented sausages, fermented vegetables and olives and dairy

products (Ennhar et al. 1999). *Lactobacillus* bacteriocins are grouped as class-I characterized bacteriocins (Lantibiotics), class-II bacteriocins (Heatstable, non-lantibiotics), class-III (heat-labile proteins with large molecular mass) and class-IV (hydrophobic and heat-stable proteins, associated with lipids or carbohydrates). The antibiotics in general have wider spectrum of activity than the non-antibiotics.

So in the present study attempts have been made to produce, purify and characterize the bacteriocin produced by *Lactobacillus viridescence* (NCIM 2167).

## MATERIAL AND METHODS

### Material

#### Chemicals

De Man Rogosa Sharpe media, Nutrient broth, Nutrient agar, ammonium sulphate, sodium carbonate, trisodium citrate, copper sulphate, polyphenol reagent, bovine serum albumin, ethanol, HCl, NaOH, Phosphate buffer were purchased from Hi media, India. All other chemicals used were of analytical grade.

### Culture

*Lactobacillus viridescence* (NCIM 2167) was procured from National Collection of Industrial Microorganisms (NCIM), Pune and was used for the production of bacteriocin.

### Indicator organisms

To study the effect of the bacteriocin produced by the strain under study various food-borne pathogens and spoilage causing microorganisms like *Bacillus cereus* (NCIM 2156), *Bacillus megaterium* (NCIM 2087), *Escherichia coli* (NCIM 2066), *Salmonella typhimurium* (NCIM 2501), *Pseudomonas aeruginosa* (NCIM 2036), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (NCIM 2079) were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India.

### Bacteriocin assay

The antibacterial activity of the bacteriocin isolated from *Lactobacillus viridescence* was determined using the well diffusion method as described by Franz et al. 1996. Wells (50 µL) that had been cut in agar plates previously seeded with

the indicator bacteria. The plates were incubated at 37°C for 24 h and after incubation the diameter of zone of inhibition was measured.

### Optimization of production parameters

#### Effect of incubation time on bacteriocin production

MRS broth was prepared, inoculated with fresh culture of *Lactobacillus viridescence* and incubated at different incubation time like 24, 48 and 72 h. The growth was measured at 600 nm and cell free culture medium was used to quantitate protein concentration.

#### Effect of pH and temperature on bacteriocin production

The MRS broth having different pH 4, 5, 6, 7, 8, 9 and 10 were prepared and separately inoculated with fresh culture of *Lactobacillus viridescence* and incubated for 48 h. Similarly for production temperature optimization MRS broth was prepared, inoculated with fresh culture of *Lactobacillus viridescence* and incubated at different temperatures like 10°C, 20°C, 30°C, 37°C and 45°C for 48 h. The growth was measured at 600 nm and cell free culture medium was used to quantitate protein concentration.

#### Production of bacteriocin

Overnight grown culture of *Lactobacillus viridescence* (NCIM 2167) was propagated in de Man Rogosa Sharpe broth at 10% of inoculum volume and incubated for 48 h at 120 rpm at 37°C. After incubation the whole broth was centrifuged at 11180g for 15 min and the cell free supernatant was used as a crude bacteriocin (Ogunbanwo et al. 2003). Protein concentration of the bacteriocin in the sample was determined by the method of Lowry et al. 1951.

#### Purification of bacteriocin

The cell free culture supernatant (crude bacteriocin) was saturated with 70% ammonium sulphate and stored at 4°C to precipitate out the proteins. The pellet was collected after centrifugation at 11180 g at 4°C for 30 min. The pellet was dissolved in 0.1 M phosphate buffer (pH 7.0) and dialyzed against the same buffer at 4°C overnight [9]. Concentrated bacteriocin sample was further purified by gel filtration using Sephadex G-100. Five grams of Sephadex G-100 was activated according to the standard procedure.

Further swollen, deaerated gel was packed in column and column is washed with 0.1 M phosphate buffer (pH 7.0). Concentrated dialyzed bacteriocin sample was loaded on the packed column, then it was eluted with 0.1 M phosphate buffer (pH 7.0) and samples fractions of 3 mL were collected at the rate of 0.25 mL/min. Protein content of fractions were analyzed by measuring its absorbance at 280 nm and for bacteriocin plate assay were used. Molecular weight of purified bacteriocin was analyzed by SDS-PAGE analysis with 12% of resolving gel and 4% of stacking gel in a midi-electrophoresis equipment along with standard protein markers (Jhample et al. 2015).

#### Activity assay

The bactericidal activity of culture supernatant, partially purified, and purified bacteriocin produced by *Lactobacillus viridescence* (NCIM 2167) was calculated by serial dilution of respective bacteriocin samples. Above mentioned samples were diluted in the range of 10<sup>-1</sup> to 10<sup>-10</sup> and the smallest detectable zone of inhibition corresponding to the diluted bacteriocin was used to calculate AU/mL (Sharma et al. 2011).

#### Characterization of bacteriocin

##### Effect of pH

Purified bacteriocin was mixed with equal volume of buffers with pH values ranging from 4 to 10, with an increment of 1 and incubated for 30 min at Room temperature. Bacteriocin samples treated with different pH values then assayed against indicator bacteria by well diffusion method.

##### Effect of temperature

Purified bacteriocin was incubated at different temperatures like 4, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C for 30 min and at 121°C for 15 min. Bacteriocin samples treated with different temperatures then assayed against indicator bacteria by well diffusion method.

## RESULTS AND DISCUSSION

*Lactobacillus viridescence* (NCIM 2167) was selected on the basis of its ability to produce bacteriocin which was active against many potential food pathogens. Bacteriocin production from *Lactobacillus viridescence* (NCIM 2167) is

novel source which has not been investigated so far.

Antimicrobial activity of the bacteriocin produced by *Lactobacillus viridescence* was analyzed by agar well diffusion technique on potential food pathogens. Bacteriocin present in culture supernatant showed the antimicrobial activity against various Gram positive and Gram negative microorganisms. The antimicrobial activity was observed against microorganisms like *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* whereas; *Alkaligenes faecalis* and *Bacillus subtilis* were not susceptible to it (Table 1).

**Table 1-** Antimicrobial activity of crude bacteriocin

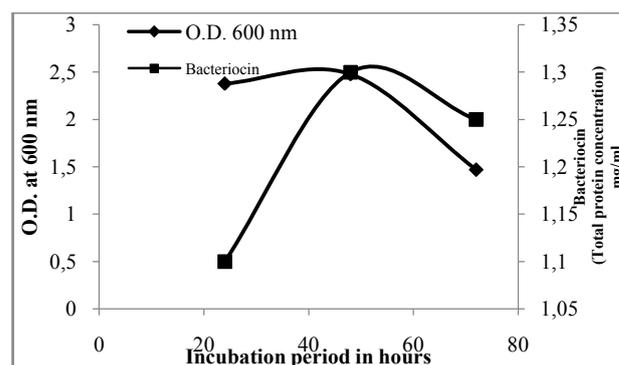
Test organisms	Zone of inhibition
<i>Bacillus cereus</i>	+
<i>Bacillus megaterium</i>	+
<i>Bacillus subtilis</i>	-
<i>Escherichia coli</i>	+
<i>Salmonella typhimurium</i>	+
<i>Staphylococcus aureus</i>	+
<i>Pseudomonas aeruginosa</i>	+
<i>Alkaligenes faecalis</i>	-

('+' sign shows presence of zone of inhibition; '-' sign indicates absence of zone of inhibition)

As bacteriocin is peptide in nature various reports reported that protease has ability to degrade bacteriocins (Sharma et al. 2011). But all the indicator organisms tested viz. *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Alkaligenes faecalis* can produce potent protease (Drapeau et al. 1972; Brunder et al. 1997; Anwar and Saleemuddin 1998; Thangam and Rajkumar 2002; Bhagwat et al. 2015). Thus it clearly shows that only few proteases could break down the bacteriocin as per their specificity towards respective bacteriocin. Amongst *Escherichia coli* showed the better results, hence it is used for further studies. Bacteriocin from culture supernatant showed the total activity of  $6.2 \times 10^8$  AU/mL (Table 2). It showed a good antagonistic activity against a number of potent food pathogens, which shows its efficacy and potential application as a natural food preservative. Moreover bacteriocins are supposed to act only on closely related species, which limits their application as a good

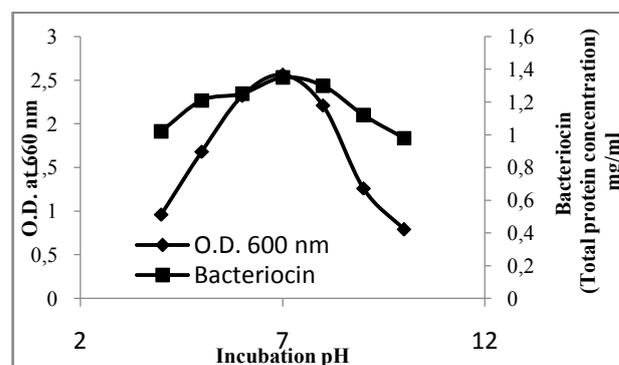
preservative (Cleveland et al. 2001). In contrast, our study revealed that bacteriocin produced from *Lactobacillus viridescence* was found to be active on various microorganisms suggesting its broad spectrum of activity. Similar results were observed in case of bacteriocin produced from *Bacillus Subtilis* R75 which was found to be active on number of species, thus it proves that bacteriocins from different microbes have specific inhibition spectra (Sharma et al. 2011).

The protein concentration was found to be more at 48 h of incubation than 24 and 72 h of incubation, respectively same results were obtained for growth of *Lactobacillus viridescence*. Hence, the optimum period of incubation for production is 48 hours (Fig. 1). Similar reports were observed in case of bacteriocin production from *Lactobacillus plantarum* where optimum period was found to be at 48 h, in contrast production of bacteriocin from *Bacillus subtilis* R75 required 72 h of incubation (Sharma et al. 2011; Amortegui et al. 2014).



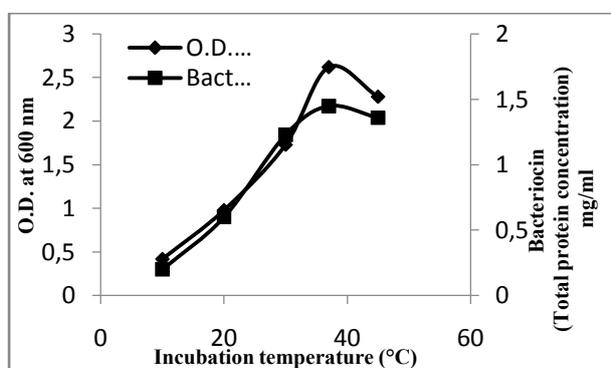
**Figure 1** -Effect of incubation period on production of bacteriocin

In pH optimization studies, the concentration of protein was found to be highest at pH 7, whereas considerable decrease was observed at both acidic as well as alkaline pH (Fig. 2).



**Figure 2** - Effect of pH on production of bacteriocin

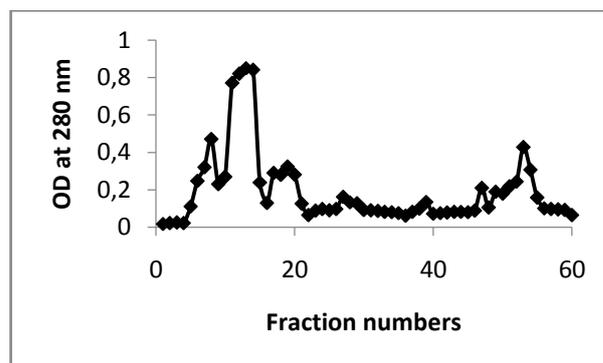
Most of the reports of bacteriocin production from *Lactobacillus* showed its production at acidic to slightly neutral range of pH, on the contrary our study stated highest production at neutral range of pH (Deraz et al. 2005; Amortegui et al. 2014). While in case of temperature optimization studies, better results were found at 37°C having highest protein content as well as cell biomass suggesting it as an optimum temperature for the production of bacteriocin. The protein concentration as well as growth at temperature of 10°C was very low as compared to others. (Fig. 3) Similar results were observed in case various other microorganisms where bacteriocin production was taken at 37°C temperature (Sharma et al. 2011; Amortegui et al. 2014).



**Figure 3** - Effect of temperature on production of bacteriocin

Bacteriocin with wide spectrum of antimicrobial activity was further subjected to purification process. In two step purification process, firstly partial purification was achieved by subjecting cell free supernatant to ammonium sulfate precipitation at 70%. Further purification was carried out by size exclusion chromatography using Sephadex G-100. Enhanced activity of partially purified bacteriocin exhibited total activity of  $6 \times 10^7$  AU/mL, whereas after complete purification by gel chromatography it showed total activity of

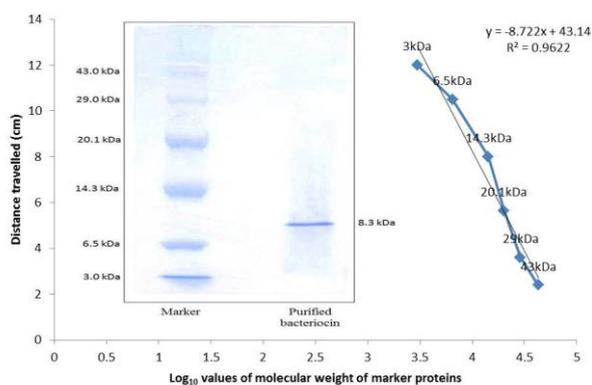
$3 \times 10^7$  AU/mL. In final purification step, active fractions (52-54) were pooled together and which showed 4.69 fold purification of bacteriocin with recovery of 1.032% (Fig. 4 and Table 2). The purified bacteriocin appeared as a single band in SDS-PAGE with molecular weight of approximately 8.3 kDa (Fig. 5). This purified bacteriocin showed antimicrobial activity against all the three tested organisms viz, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* confirming its activity (Fig 6). Low molecular weight of purified protein measured in present study is a characteristic feature of bacteriocin. Similarly bacteriocin produced by *Lactobacillus plantarum* ST13BR had molecular weight of 9.5 kDa (Sankaret al. 2012). A bacteriocin produced by *Bacillus Subtilis* R75 had a molecular mass of 12kDa based on gel filtration analysis (Sharma et al. 2011). The bacteriocins of bacteria belonging to class-I and II have molecular weight <5 kDa and <10 kDa respectively hence bacteriocin purified in this study can be called as class-II bacteriocin group (Bodaszewska-Lubas et al. 2012).



**Figure 4** - Elution profile of bacteriocin purification from *Lactobacillus viridescence* (NCIM 2167)

**Table 2**- Summary of the purification profile for bacteriocin from *Lactobacillus viridescence*

Purification steps	Total activity (AU/ml)	Total protein (mg)	Specific activity ( $10^6$ )	Purification fold	Recovery (%)
Culture filtrate	$6.2 \times 10^8$	271.2	2.28	1	100.0
Ammonium sulfate	$6 \times 10^7$	25.3	2.37	1.039	9.32
Sephadex G-100	$3 \times 10^7$	2.8	10.71	4.69	1.032

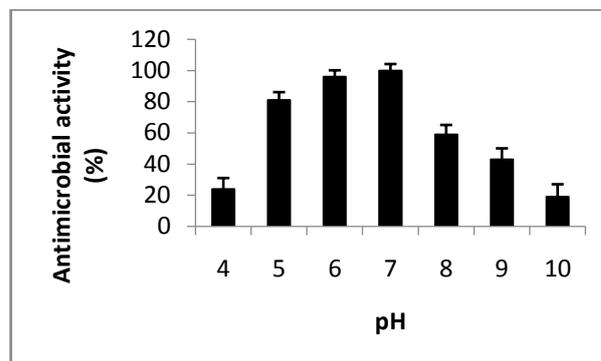


**Figure 5-** SDS-PAGE analysis of purified bacteriocin

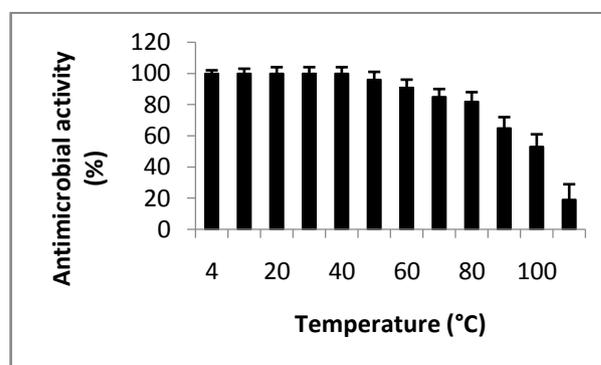


**Figure 6 -** Antimicrobial activity of purified bacteriocin against 1) *Escherichia coli* 2) *Bacillus cereus* 3) *Staphylococcus aureus*

Bacteriocins have potent applications in preservation of various types of food products and hence purified bacteriocin was further characterized for its efficiency to work under different environmental conditions. In present study purified bacteriocin showed its activity at pH range of 4 to 10, but significantly it was active at acidic to neutral range of pH ranging from 5 to 7 (Fig. 7). Similarly significant activity at pH 6 and 7 was shown in case of bacteriocin produced from *Bacillus subtilis* R75 (Sharma et al. 2011), whereas bacteriocin from *Lactobacillus plantarum* LE5 and LE7 was found to be active at wide range of pH values ranging from 2 to 12 (Amortegui et al. 2014). In temperature stability studies it was found that purified bacteriocin was stable at wide range of temperatures (Fig. 8). Similar results were shown in case of bacteriocins produced by *Bacillus Subtilis* R75 as well as *Lactobacillus plantarum* LE5 and LE7 (Sharma et al. 2011; Amortegui et al. 2014). Purified bacteriocin in this study even had shown activity after treatment at 121°C which opens an avenue for its use in such food products which needs to be operated at high temperatures. It can also show the activity against food pathogens which are heat resistant and hence could be exploited as a potent source of bacteriocin.



**Figure 7-** Effect of pH on the activity of purified bacteriocin. Mean error bars in the graph represent the mean  $\pm$  standard error



**Figure 8 -** Effect of temperature on the activity of purified bacteriocin. Mean error bars in the graph represent the mean  $\pm$  standard error

## CONCLUSION

In this study we have described complete purification and characterization of newly isolated class II type of bacteriocin from *Lactobacillus viridescence* (NCIM 2167). In this study optimized production conditions yielded a bacteriocin which was purified at its finest level. Purified bacteriocin was having wide range of physicochemical stabilities as well as it was active against a range of food pathogens. So all these characteristics attributes showed bacteriocin as a new and promising source with multiple future prospective.

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