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Antimicrobial Activity Studies of Bactoriocin Produced by *Lactobacilli* Isolates from Carrot Kanji

[1]Harshada M. Sowani and [2]Prakash Thorat
[1]Walchand Centre for Biotechnology,
Walchand College of Arts and Science, Solapur, MS, India
[2]Department of Microbiology and Research Center,
Shri Shivaji Mahavidyalaya, Barshi-413 411 Dist-Solapur, MS, India

Abstract: Problem statement: In the present study, *Staphylococcus aureus* a causative agent of food poisoning is selected as a test organism to study the antimicrobial effect of bacteriocin. *S. aureus* produces number of exotoxins and enterotoxins which enters the body via contaminated food causing illness. **Approach:** In this case the use of antibiotics is one of the ways of treatment, but in addition to this if we advise such patients to consume the carrot kanji then it will cause better effect because carrot kanji is the naturally fermented food beverage consisting of microflora mainly the *Lactobacilli.* **Results:** The *Lactobacilli* have ability to produce antimicrobial compounds called bacteriocin. Isolation of bacteriocin was carried out from the naturally fermented carrot kanji. The bacteriocin produced by *Lactobacilli* was dialysed and used for the further studies. The well diffusion method is used to study the antimicrobial activity, effect of temperature, pH, enzymes on bacteriocin. From the diameter of zone of inhibition the activity of bacteriocin was determined. The sensitivity of bacteriocin at different pH range showed that at neutral pH the diameter of inhibition zone was greater than that at alkaline as well as acidic pH. Upto 100°C the bacteriocin activity was 80% but as temperature range increased upto 121°C it reduced sharply to 28%. **Conclusion/Recommendations:** In addition to this the effect of *alpha amylase*, *trypsin*, *catalase* enzyme on bacteriocin activity was also studied which shows positive results with alpha amylase, reduced activity with trypsin and catalase remained unaffected.

Key words: Bacteriocin, *Lactobacilli*, Carrot Kanji, *Staphylococcus aureus*, antimicrobial activity, antimicrobial effect, alpha amylase, further studies, well diffusion method

INTRODUCTION

Lactobacilli are Gram positive, non spore forming, non motile, rod shaped, catalase lacking organisms occur in naturally fermented food and drink (Sahota *et al*., 2008; Karovicova and Kohaajdova, 2005). In northern India, carrots are processed in a special traditional way to prepare a ready to serve fermented drink which is deep purple in color known as Kanji (Ivanova *et al*., 2000; Joshi *et al*., 2006) Kanji production typically relies on spontaneous natural fermentation. It is nutritionally rich as it shows the presence of a viable microbiota that stimulate the growth of beneficial organisms within the gastrointestinal tract, have antimicrobial property (Berry *et al*., 1989; Sahota *et al*., 2008; Erten *et al*., 2008) The shelf life of kanji is about 7 days at 30°C during winters. For isolation of *lactobacilli* from carrot kanji MRS medium is used (De *et al*., 1960; Garrity, 2005). MRS medium was developed by De Man, et al. to to support luxuriant growth of all *lactobacilli* from oral, fecal, dairy and other sources *lactobacilli*. MRS medium contains peptones and dextrose which supply nitrogen and carbon. Tween 80, acetate, magnesium and manganese provide growth factors for culturing a variety of *lactobacilli*. These ingredients may inhibit the growth of some organisms other than *lactobacilli*. *Lactobacilli*, produce special antimicrobial compounds such as bacteriocins which are highly specific antibacterial proteins (Boris *et al*., 2001) prevents food spoilage and provides additional protection against Bacillus, *Staphylococcus aureus* and *Clostridial* spores in canned foods.

Most of the gram positive bacteriocins are a membrane active compound that increases the permeability of the cytoplasmic membrane. They often show a much broader spectrum of bactericidal activity than colicins. They fall within two broad classes, viz

Corresponding Author: Harshada M. Sowani, Walchand Centre for Biotechnology, Walchand College of Arts and Science, Solapur, MS, India

the lantibiotics and non lantibiotic (Jamuna and Jeevaratnam, 2004; Aly *et al*., 2006). Bacteriocin production in *lactobacilli* is growth associated, it usually occurs throughout the growth phase, maximum at logarithmic phase and ceases at the end of this phase (Narenderb *et al*., 2010; Joshi, *et al*., 2006) After production bacteriocin is characterized by determining its sensitivity towards different pH and temperature range (Nowroozi1 *et al*., 2004; Yildrim and Johnson, 1998; Boris *et al*., 2001) its protein content also measured (Lowry *et al*., 1951).

Present investigation, focused on the isolation of *Lactobacilli* from fermented carrot kanji, bacteriocin production and its antimicrobial activity on *Staphylococcus aureus*. *Staphylococcus aureus* an important pathogen causing food poisoning due to a combination of toxin-mediated virulence, invasiveness and antibiotic resistance (Marteau *et al*., 2001; Loir *et al*., 2003) The bacteriocin produced by *lactobacilli* isolated from kanji was used against *Staphylococcus aureus*. Bacteriocins are highly specific antibacterial proteins (Narenderb *et al*., 2010; Joshi *et al*., 2006) produced by strains of bacteria active mainly against some other strains of bacteria.

MATERIALS AND METHODS

Preparation of carrot kanji and *Lactobacilli* Isolation: Freshly harvested red colored carrots procured from local market were washed with clean water and peeled (Berry *et al*., 1989; Sahota *et al*., 2008). They were cut into small pieces (3-4×1 cm) of equal size. Kanji was prepared using carrots (150 gm), common salt (NaCl) (30 gm), mustard powder (6 gm), chili powder (1.2 gm) and water 1 liter. The ingredients were mixed well and filled in sterile conical flask of 1000 mL capacity and the flask was cotton plugged. The spontaneous natural fermentation was carried out at 35°C for 4 days (Berry *et al*., 1989; Sahota *et al*., 2008) For isolation of *lactobacilli* De Man Rogosa Sharpe (MRS) medium (De *et al*., 1960) was used. The MRS medium contains peptone (1 g), yeast extract (0.5 g), meat extract (1 g), glucose (2 g), dipotassium hydrogen phosphate (0.20 g), triammonium citrate (0.20), tween 80 (0.1 mL), magnesium sulphate (0.20 g), manganese sulphate (pinch), agar (2.5 g), distilled water (100 mL), pH (6) provides growth factors for culturing a variety of *lactobacilli*. The *Lactobacilli* are strict anaerobes, so after streaking, the MRS plates were incubated in the anaerobic jar at 37°C for 48 h. The well isolated colonies on MRS agar were studied as per the tests given in the Bergey's manual of systematic bacteriology (Garrity, 2005).

Bacteriocin production and its partial purification: The bacteriocin producing strain was grown in 250 mL of MRS broth for at 37°C till it reaches to the late logarithmic phase by determining the O.D. at 660 nm (Boris *et al*., 2001) The cells were separated out by centrifuging broth for 15 min at 4°C. To the supernatant which is used as bacteriocin, ammonium sulphate was added and about 40% saturation was achieved (Nowroozi *et al*., 2004; Yildrim and Johnson, 1998; Boris *et al*., 2001). After stirring on magnetic stirrer, it was kept undisturbed at 4°C overnight. Precipitate formed was collected by centrifugation and dissolved in 20 mM sodium phosphate buffer with pH 7, dialyzed and used for the further studies as a partially purified Bactoriocin (Nowroozi1 *et al*., 2004; Joshi *et al*., 2006; Sivakumar and Saif, 2010).

Determination of Protein: Protein concentration of bacteriocin in supernatant was determined by the method of (Lowry *et al*., 1951), using bovine serum albumin as the standard (Lowry *et al*., 1951).

Antimicrobial effect of bacteriocin against *Staphylococcus aureus*: It was tested on nutrient agar, by well diffusion method (Nowroozi1 *et al*., 2004; Yildrim and Johnson, 1998) under aerobic condition. Prepoured nutrient agar plates were overlaid with 5 mL nutrient soft agar containing 0.2 mL of indicator cultures. Wells of size 5 mm diameter were cut off in the agar plate using a sterile borer and 50 μL of the bacteriocin was placed in each well and kept in refrigerator for 1 h and thereafter incubate overnight at 37°C. The plates were examined for zone of inhibition around the wells.

Effect of enzymes on bacteriocin activity: The above dialysed bacteriocin was incubated for 2 h in presence of 1 and 0.1 mg mL^{-1} of trypsin, alpha amylase and catalase respectively according to (Ivanova *et al*., 2000) Antimicrobial activity was recorded by using well diffusion method as described earlier.

Characterization of bacteriocin:

Sensitivity to pH and heat: To test the sensitivity to pH 5 mL aliquot of bacteriocin was taken in a test tube and the pH values of the contents were adjusted to pH range (2.0-8.0) individually, using NaOH or HCl and the activity was assayed as described earlier against staph. aureus by well diffusion method. To test heat sensitivity 5 mL aliquot of bacteriocin in different test tube was overlaid with paraffin oil to prevent evaporation and then heated at 60-120°C (in steps of 20°C) for 10 min. and these aliquot of respective temperature was tested against *S. aureus* by well

diffusion method (Nowroozi1 *et al*., 2004; Karovicova and Kohaajdova, 2005).

RESULTS

From the fermented carrot kanji, *lactobacilli* were isolated. The well isolated predominant eleven isolates of *lactobacilli* (LB1-LB11) on MRS agar having different cultural morphology were picked up randomly and studied their morphological characters. The isolated colonies were colorless, flat and of irregular shaped and cell morphology showed small rods. All isolates showed gram positive nature and able to grow at 30°C and 50°C but unable to grow at 15°C.

Further identification was carried by performing the biochemical tests and sugar fermentation pattern using current taxonomic status according to Bergey's manual of determinative classification system (Garrity, 2005).

All isolates showed acid production with Glucose, Fructose, Galactose, Maltose and Lactose fermentation while these isolates did not showed catalase activity and hydrolysis of starch and nitrate reduction. The results are summarised in Table 1.

As all the isolates showed the same characteristics and therefore we selected two isolates viz. LB1 and LB2 out of eleven for bacteriocin production and its antimicrobial activity studies. The antimicrobial activity of bacteriocin produced by isolates LB1, LB2 was determined against *S. aureus*. The diameter of inhibition zone for LB1 and LB2 was observed to be 25 and 18 mm respectively (Fig. 1).

Heat stability of bacteriocin by well diffusion method (Fig. 2) was studied by measuring diameter of inhibition zone at temperature 60C, 100C, 120°C (Table 2) and it showed 100% activity at 60°C for up to 10 min it remains stable and so if we considered this as 100% activity then at 100°C, 80% activity was observed. However after incubation for 10 min at 120°C under pressure, considerable decrease in the antimicrobial activity took place Fig. 2 (Table 2).

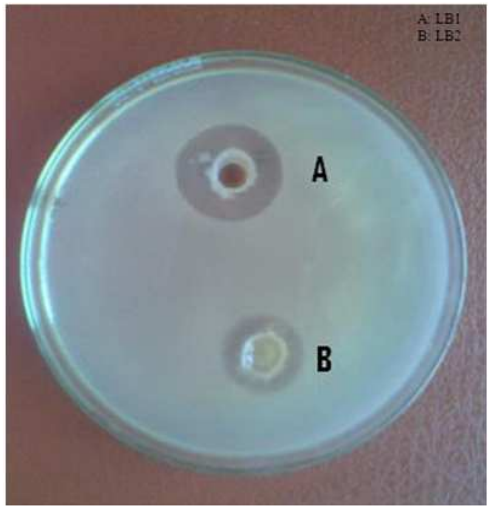

Fig.1: Zone of inhibition of *S. aureus* against bacteriocin produced by (A) LB1 and (B) LB2 by agar well diffusion method

Fig. 2: Zone inhibition of *Staphylococcus aureus* against heat treated bacteriocin samples at (a) 60°C (b) 100°C (c) 120°C

Table 1: Biochemical characterization of 11 *lactobacilli* isolate (LB) isolated from 4 days fermented carrot kanji

Isolate code	LB1	LB2	LB3	LB4	LB5	LB6	LB7	LB8	LB9	LB10	LB11
Nitrate Reducti	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	++	++	++	++	++	++	++	++	++	++	++
Maltose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Starch hyrolysis	+	+	+	+	+	+	+	+	+	+	+
Genus identification	*Lactobacilli* spp										

Notation: (+) Acid production; (+, +) Acid and gas production

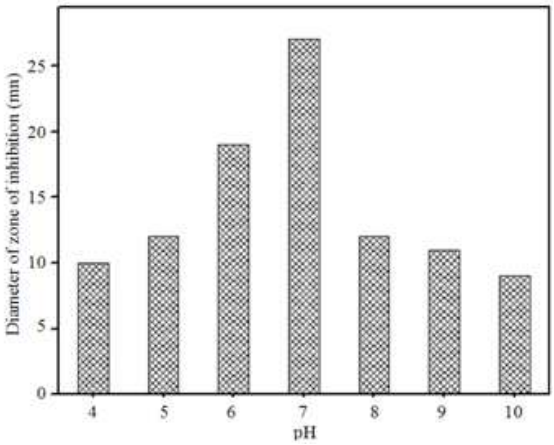

Fig. 3: Effect of pH on antimicrobial activity of bacteriocin

Table 2: Effect of temperature on antimicrobial activity of partially purified bacteriocin from isolated LB1

Temperature (°C)	Diameter of zong of inhibition (mm)
60	25
100	20
121	7
Control	25

As diameter of inhibition zone for LB1 was more, it is more active against *S. aureus* than LB2. Therefore, the bacteriocin produced by LB1 was taken to verify the heat stability.The effect of trypsin, catalase, alpha amylase enzyme on bacteriocin activity was studied. The effect of trypsin, catalase, alpha amylase enzyme on bacteriocin activity was studied.

Maximum activity of partially purified bacteriocin against *S.aureus* was noted at pH 7 and if we consider this as 100% then at acidic pH (pH 6) 80% activity was retained . But at alkaline pH, the antimicrobial activity get highly reduced Fig. 3.

DISCUSSION

As diameter of inhibition zone for LB1 was more, it is more active against *S. aureus* than LB2. Therefore, the bacteriocin produced by LB1 was taken to verify the heat stability.

The effect of trypsin, catalase, alpha amylase enzyme on bacteriocin activity was studied. Antimicrobial activity was reduced after treatment of dialysed bacteriocin sample with trypsin whereas treatment with catalase resulted in no activity change indicating that H_2O_2 was not responsible for inhibition. Treatment with alpha amylase resulted in increased diameter of zone of inhibition suggested that dialysed bacteriocin is glycosylated. This is in contrast to results obtained by Ivanova *et al*. (2000).

Heat stability of bacteriocin by well diffusion method (Ivanova *et al*., 2000) showed that it remains stable at 60°C for up to 10 min and so if we considered this as 100% activity then at 100°C, 80% activity was observed. However after incubation for 10 min at 120°C under pressure, considerable decrease in the antimicrobial activity took place. And pH 7 showed maximum antibacterial activity.

CONCLUSION

MRS media seemed to be more suitable medium for *lactobacilli* isolation and bacteriocin production. The heat stability of bacteriocin indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. The activity of bacteriocin at pH 6 was noted 80% so it can be used in acidic foods like pickle, sausages and yogurt. It might be secondary metabolites. It would remain effective during food processing. Hence, the study revealed that bacteriocin from Lactibacillus species isolated from Lactibacillus species isolated from natural lactic fermentation of carrots possesses a wide spectrum of inhibitory activity against *S. aureus* so it has a potential application as a probiotic.

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