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Production, Purification, Stability and Efficacy of Bacteriocin from Isolates of Natural Lactic Acid Fermentation of Vegetables

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Summary

The antimicrobial activity of partially purified bacteriocin produced during natural lactic acid fermentation of carrot, radish and cucumber was assessed and characterized. Out of ten strains, the isolated strain CA 44 of *Lactobacillus* genus from carrot fermentation produced bacteriocin with maximum antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, though it was more effective against *E. coli* than others. Bacteriocin was stable at up to 100 °C but its activity declined compared to that at 68 °C and was completely lost at 121 °C. The maximum antimicrobial activity was retained within the pH range of 4–5, but it was adversely affected by the addition of papain. Bacteriocin was also effective against *B. cereus* in different fruit products (pulp, juice and wine) indicating its potential application as a biopreservative in fruit products.

Key words: antimicrobial, bacteriocin, lactic acid fermentation, *Lactobacillus*, *Staphylococcus*, *Bacillus cereus*, *E. coli*, pathogenic microorganism, stability, biopreservative

Introduction

Preservation of vegetables by lactic acid fermentation is an ancient practice involving lactic acid bacteria (LAB), which predominantly produce lactic acid besides certain compounds such as bacteriocin, which has antimicrobial activity against other groups of microorganisms. The antimicrobial activity of bacteriocins produced by LAB has been detected in foods such as dairy products, meats, barley, sourdough, red wine, fermented vegetables, *etc*. (*1–5*). Therefore, the strains of lactic acid bacteria have also potential to act as a biopreservative or natural food preservative (*6–8*). The bacteriocins produced inhibited food spoilage and pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Listeria monocytogenes* and *Clostridium perfringens* which are recalcitrant to traditional food preservation method (*9*). The use of bacteriocins or the microorganisms that produce them is attractive to the food industry in the face of increasing consumer demand for natural products and the growing concern about foodborne diseases. It has also necessitated the need to exploit the biologically derived antimicrobial substances produced by LAB. It is not clear if any bacteriocin is produced in the vegetables fermented by LAB in natural or inoculated fermentation. The bacteriocin produced by the strains isolated from naturally fermented vegetables has neither been characterized nor checked for its efficacy in various food products. Therefore, keeping in view the above objectives the present investigations were carried out and the results obtained are discussed here.

Materials and Methods

Fermented vegetables

Vegetables (carrot, radish and cucumber) procured from the markets were washed, peeled and grated/sliced.

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The grated carrot and radish were fermented with dry salt 2 % (by mass) at 27 °C, whereas sliced cucumbers were fermented in 3 % (by mass per volume) brine at 32 °C. Predominant microflora were isolated from these samples.

Pathogenic bacterial cultures

Standard bacterial cultures, *viz*. *Escherichia coli* (0165), *Staphylococcus aureus* (B-43-5) and *Bacillus cereus* procured from Central Research Institute (CRI), Kasauli, were used in bacteriocin screening procedures and all the cultures were maintained as per the recommended practices.

Isolation and identification of bacteriocin producing bacteria

The bacteriocin producers from naturally fermented carrot, radish and cucumber were isolated by pour plate method technique as per the conventional method (*10*) using MRS agar. After incubation for 24–48 h at 32 °C, typical colonies were isolated and purified. The isolates were differentiated on the basis of their morphological, cultural and physiological characteristics such as oxidase test, utilization of citrate as a sole carbon source and catalase test (*10,11*), and accordingly were tentatively identified up to the genus level (*12*).

Screening of isolates for antimicrobial activity

Antimicrobial activity of the bacterial isolates against all the pathogenic microorganisms was determined by well diffusion method (*13–16*) under aerobic conditions. Agar plates were inoculated with 100 μL of each target microorganism after growing them in a broth and diluting appropriately. Wells (3 mm) were cut into the plates and 100 μL of cell-free culture supernatant fluid of the isolated strain was placed into each well. The inhibitory activity against *E. coli* was tested on EMB agar whereas *Staphylococcus aureus* and *Bacillus cereus* were tested on nutrient agar. Plates were kept at cool temperature for 2 h and then incubated at 37 °C for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. The bacterial isolate showing the widest zone of inhibition against the target microorganism was selected for further studies.

Partial purification of bacteriocin

Isolated strain having maximum antimicrobial zone was grown in MRS broth at 37 °C for 24 h. After incubation, the broth was centrifuged at 5000 × *g* for 10 min and the cells were separated out. Supernatant was used as a crude bacteriocin. Different concentrations of ammonium sulphate were added to the supernatant. After stirring on a magnetic stirrer, it was kept undisturbed at 4 °C overnight. Precipitates formed were collected by centrifugation at 10 000 × *g* for 10 min and redissolved in 20 mmol sodium phosphate buffer with pH=6.0. Inhibition zone of different fractions was recorded in comparison with the crude bacteriocin.

Characterization of bacteriocin

Heat stability

A volume of 5 mL of bacteriocin in different test tubes was overlaid with paraffin oil to prevent evaporation and then heated at 68 and 100 °C for 10 and 20 min, respectively, and at 121 °C for 15 min under pressure. The heat-treated bacteriocin samples were then assayed for antimicrobial activity as described earlier.

Effect of pH

A 5-mL aliquot of partially purified bacteriocin was taken in test tubes and the pH values of the contents were adjusted to 2–9 individually, using either diluted NaOH or HCl (1 M NaOH or 1 M HCl solution). After allowing the samples to stand at room temperature for 2 h the activity was assayed as described earlier.

Effect of proteolytic enzyme (papain)

A 5-mL aliquot of bacteriocin preparation was taken in test tubes and treated with papain (100 TU) 1 mg/mL at pH=7. The test tubes with and without the enzyme (control) were incubated for 2 h at 37 °C and heated for 3 min at 100 °C to denature the enzyme. Both the control and the samples were assayed for antimicrobial activity by using well diffusion method.

Determination of preservative effect of bacteriocin

The food products, *viz*. juice (apple), pulp (apricot) and prepasteurized wine (plum) were sterilized and inoculated with *Bacillus cereus* at 10^8 CFU/mL. Initial count of inoculated samples was recorded and bacteriocin supernatant at a concentration of 0.05 to 0.5 % was added. After 24 and 72 h, the plate count was recorded and compared with the control (without bacteriocin).

Results and Discussion

Based on morphological and biochemical tests, all the isolates were identified as belonging to lactic acid bacteria (LAB) group except RA33, which was identified as yeast. The isolate CA44 (giving maximum antimicrobial activity) was Gram-positive, rod shaped, negative for catalase and peroxidase test, having circular and white colonies on the MRS media. The strain was also positive for galactose, arabinose, mannitol, sorbitol, sucrose, glucose, trehalose, lactose, raffinose and negative for maltose, citrate and arginine test. Isolate CA44 from carrot produced the maximum inhibition zone against all the tested microorganisms and was maximum against *E. coli*. The best conditions for bacteriocin production by *Lactobacillus plantarum* in batch fermentation were the salt concentration ranging from 2.3 to 2.5 % and temperature ranging from 22–27 °C (*17*). *Lactobacillus plantarum* strain isolated from fermented carrots which produced bacteriocin with antibacterial activity against *Staphylococcus aureus* and spheroplasts of Gram-negative bacteria (*18*) and *Lactococcus lactis* ssp. *cremoris* was also isolated from radish fermentation (*1*).

An increase in antimicrobial activity after partial purification of crude bacteriocin by ammonium sulphate precipitation took place (Fig. 1). The fraction with the highest bacteriocin activity was precipitated with 20–30 %

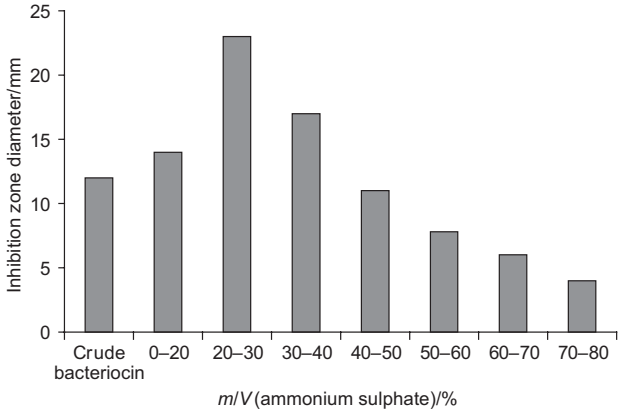

Fig. 1. Increase in antimicrobial activity of bacteriocin from *Lactobacillus* sp. isolate (CA44) using ammonium sulphate fractionation

(by mass per volume) ammonium sulphate. The antimicrobial activity (in terms of inhibition zone diameter) increased from 12 to 23 mm. There was 1.91-fold increase in the partially purified bacteriocin activity than that of crude bacteriocin. Earlier, the inhibitory activity of bacteriocin isolated from malted barley was precipitated from cell free supernatant using 40 % ammonium sulphate saturation, and resuspended in 2 mmol sodium phosphate buffer, pH=6.0 and purified using chromatography (*19*).

Partially purified bacteriocin was found to be stable at 68 °C for up to 20 min. At 100 °C for 10 min it could retain 55 % of antimicrobial activity, while at the same temperature for 20 min, only 28 % of activity could be retained (Table 1). However, after incubation for 15 min at 121 °C, the complete loss of activity took place. Compared to the earlier reports on bacteriocin, residual activity was lower in our study than reported earlier (*20*). Furthermore, since tolerance of bacteriocin to heat is known to depend on the stage of purification, pH, presence of culture medium, other protective components, *etc.* that might have influenced the antimicrobial activity in our findings too. The heat stability of bacteriocin discussed here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. Furthermore, when comparatively low temperature is employed for processing compared to high temperature being used at present, the retention of nutrients would be higher. However, more studies on these aspects are needed.

The partially purified bacteriocin showed maximum activity against the target microorganisms at pH=5.0 (Fig. 2), but after pH=5.0 the activity of the bacteriocin gradually but continuously decreased. At pH=9.0, the antimicrobial activity was drastically reduced to more than 2.5 times that of the control. Thus, the bacteriocin was found active over a wide pH range with the highest activity at low pH range of 4–5. Earlier, the bacteriocin produced by a newly isolated *Bacillus* species strain 8A was found active over a pH range of 5–8 but was inactivated when incubated outside these limits (*9*). Another bacteriocin produced by *Lactococcus lactis* D53 and 23 was active over a wide pH range with the highest activity shown at low pH range of 3–5 (*13*), as was the case with the bacteriocin from *Pediococcus* sp. (*21*). Bacteriocin activity was completely lost when treated with proteolytic enzyme (papain), which is in agreement with the earlier report (*22*). The bacteriocin pediocin ACH from *Pedicoccus acidilacti* was sensitive to proteolytic enzymes and was completely inactivated by several proteolytic enzymes (*22,23*). The stability of bacteriocin to different conditions reflects that such compounds can withstand the conditions normally encountered in food processing, so would remain effective during processing.

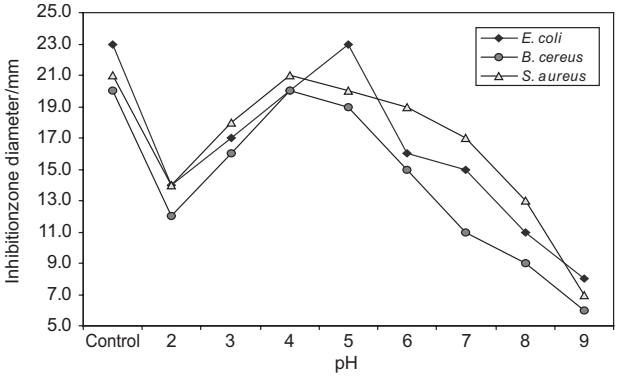

Fig. 2. Effect of pH on antimicrobial activity of partially purified bacteriocin from *Lactobacillus* sp. isolate (CA44)

The partially purified bacteriocin from isolate CA44 was also tested for preservative effect against *B. cereus* (Table 2), and clearly the preservative effect in juice, wine and pulp increased with the increase in the concentration of bacteriocin. Maximum reduction of *Bacillus cereus* population of 92 % was observed in wine followed by juice (87 %) and pulp (63 %) at a concentration of 0.5 %.

Table 1. Effect of temperature on antimicrobial activity of partially purified bacteriocin from isolated *Lactobacillus* sp. (CA44)

Temperature/°C	*t*/min	Inhibition zone diameter/mm		
		E. coli	*B. cereus*	*S. aureus*
68	10	23 (100)	19 (100)	20 (95)
	20	22 (95)	19 (100)	20 (95)
100	10	15 (65.21)	13 (68.42)	11 (55)
	20	10 (43.47)	9 (47.36)	6 (28.57)
121	15	0	0	0
Control (without heat treatment)	–	23	19	21

Values in parentheses represent retention of antimicrobial activity (in %)

Table 2. Preservative effect of partially purified bacteriocin from *Lactobacillus* sp. isolate (CA44) in juice, wine, and pulp against *Bacillus cereus*

ψ(bacteriocin)/%	Preservative effect*/%		
	Juice	Wine	Pulp
Control	0	0	0
0.05	12	16	10
0.1	34	37	17
0.2	50	55	29
0.3	69	72	57
0.4	83	86	60
0.5	87	92	63

$$\text{*Reduction of population/\%} = \frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

However, in control (without bacteriocin), no reduction was observed in the count of *B. cereus*. The results (Fig. 3) further revealed that microbial count drastically decreased in wine and the same pattern was followed in juice too. In pulp, only a concentration of bacteriocin above 0.2 % drastically decreased the microbial count. Highest antimicrobial activity of bacteriocin against the target microorganism in wine could partly be attributed to inhibitory effect of ethanol. Briefly, the results indicate that bacteriocin possessed several desirable characteristics of a biopreservative.

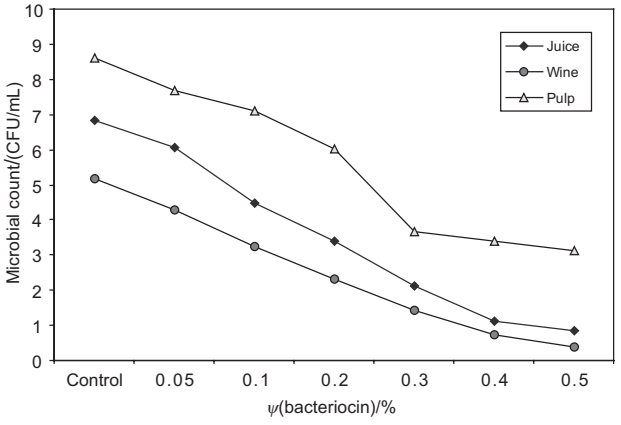

Fig. 3. Reduction in population of *Bacillus cereus* in juice, wine and pulp with the addition of bacteriocin

Conclusion

The study revealed that bacteriocin from *Lactobacillus* sp. isolated from natural lactic acid fermentation of vegetables possesses a wide spectrum of inhibitory activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus.* Therefore, it has a potential for application as a biopreservative in different food products as such or in combination with other preservation methods. Since lactic acid fermentation is employed mostly for development of products, especially for flavour and taste of the fermented products, the production of bacteriocin in such products assumes more significance as biopreservative apart from imparting probiotic effect to the product.

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