

Development of antimicrobial packaging materials for food preservation using bacteriocin from *Lactobacillus casei*

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Abstract

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacteria. Among lactic acid bacteria (LAB), bacteriocins are produced by *Streptococcus*, *Pediococcus*, *Lactobacillus*, etc. In recent years, bacteriocin-producing LAB have attracted significant attention because of their generally recognized as safe status and potential use as safe additives for food preservation. Incorporation of bacteriocins into packaging films to control food spoilage and pathogenic organisms has been an area of active research for last decade. Antimicrobial packaging film prevents microbial growth on food surface by direct contact of the package with the surface of food. The objectives of this study were to isolate bacteriocin-producing LAB from Yakult®, develop antimicrobial packaging system and evaluate their antimicrobial effects on selected spoilage and pathogenic microorganisms. For this reason, the antimicrobial packaging film was made by using the bacteriocin by *Lactobacillus casei* and coating it or adsorbing it onto the surface of different packaging materials. The antimicrobial activity of the coated films was tested by agar diffusion assay against the test organisms *Escherichia coli* and *Staphylococcus aureus*. The results obtained proved that bacteriocins can be used to inhibit both the test organisms. Thus antimicrobial packaging systems can be developed using bacteriocins thereby reducing the risk of pathogen development, as well as extending the shelf life of foods.

Introduction

Microbial spoilage of food is one of the major concerns of food industries as it leads to economic losses. Consumption of such spoiled food can cause food-borne infections and intoxications due to the presence of microbial pathogens such as *Staphylococcus aureus*, *entero-pathogenic Escherichia coli*, *Shigella dysenteriae* and their toxins in it. Thus, it is

extremely important to monitor various factors from the production of the food and its final distribution to the consumers to prevent food spoilage. Packaging has a significant role in the food supply chain. Food packages have an active role in processing, preservation and in retaining quality of foods. This has led to develop packages with antimicrobial properties. Antimicrobial agents such as bacteriocins, if incorporated into the packaging materials, will migrate into the food through diffusion and reduce the growth rate of spoilage and pathogenic microorganisms. Antimicrobial packaging film prevents microbial growth on food surface by direct contact and thereby prolong the shelf-life and maintain food quality and safety.^{1,2}

Bacteriocins are proteins with antimicrobial activity, produced by different groups of bacteria. Many lactic acid bacteria (LAB) produce bacteriocins with broad spectra of inhibition. Several LAB bacteriocins find applications in food preservation, as they are desirable members of the intestinal microflora. Also, these bacteria have *Generally Recognized as Safe* (GRAS) status. Bacteriocins are of great interest as they can be added to foods in the form of concentrated preparations as food preservatives or shelf-life extenders. Immobilized bacteriocins can also be used to develop bioactive food packages.³⁻⁵ The present study was carried out to develop antimicrobial packaging system using bacteriocins. Various different packaging materials were coated with bacteriocins produced by *Lactobacillus casei* var *shirota* obtained from a probiotic drink Yakult®. The effectiveness of the developed antimicrobial films against common food spoilage and pathogenic organisms was also studied.

Materials and Methods

Cultivation of *Lactobacillus casei* var *shirota* from Yakult® and extraction of bacteriocin

Lactobacillus casei var *shirota* was obtained from Yakult®, a fermented milk drink (Danone, Milan, Italy). The product was chosen as it contains a known species of LAB, which is safe for consumption. This also eliminates the need to perform the safety studies for the bacteriocins obtained from the isolate obtained from a food source. For cultivation of the cells, 10 mL of Yakult® was inoculated in 100 mL de Man, Rogosa and Sharpe (MRS) broth and was incubated at 30°C for 48 hours. The cultures were checked for purity on MRS agar plates by performing gram staining and catalase test. The gram-positive, non-sporulating rods, which were catalase negative, were used for large scale cultivation in MRS broth.

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After 24 hours of incubation at 30°C, cells were separated by centrifugation at 4000 rpm for 15 min at room temperature. The cell free supernatant (CFS) culture broth was collected. The pH of the supernatant was adjusted to around 6.5 using 1N NaOH to eliminate the effect of organic acids.^{6,7}

Antimicrobial activity of bacteriocin against *Escherichia coli* and *Staphylococcus aureus*

The antimicrobial activity of CFS containing bacteriocin was determined using the disk diffusion technique as described by Yang and colleagues. *E. coli* and *S. aureus* were used as the test cultures as these are some of the potential pathogens found in food. The optical densities of the culture suspensions were adjusted to 0.1 at 540 nm and were spread on the surface of sterile Nutrient agar plate. Sterile Whatman filter paper discs with a diameter of 6mm were soaked in the neutralized CFS and placed on the surface of the seeded agar plate. Uninoculated MRS broth was used as control. The plates were incubated at 30°C for 24 hours. Diameter of the inhibition zones around the discs were measured and recorded.⁸⁻¹⁰

Thermal stability of bacteriocins

The activity of proteins is affected at extremes of temperature. Since bacteriocins are proteinic in nature, its presence in the extract can be detected by studying its antimicrobial activity in the residual extract obtained after exposing it to different temperature conditions. To test the thermal stability, 10 mL of culture supernatant was heated for 30 minutes at 4°C, RT, 37°C, 55°C, 100°C. Agar well diffusion assay method was used for the detection of the residual bacteriocin activity of the heat treated CFS. 24-hour old cultures of *E. coli* and *S. aureus* were bulk seeded into sterile Nutrient agar. A sterile cork borer of 6mm diameter was used to make wells on the medium. 4 wells were made in each of the plates. 0.1 mL of the CFS was introduced into the different wells. Prediffusion was carried out for 1 hour at 4°C. The plates were then incubated at 30°C for 24 hours and were observed for inhibition zones around the wells.^{11,12}

Development of antimicrobial packaging films using bacteriocins and evaluating its effectiveness

The materials selected to develop antimicrobial coating were glassine paper used to package butter, laminates used to construct tetrapacks, plastic coated hard paper used to construct paper cups for holding ice-creams and beverages. The package materials were cut into small squares of dimensions 2×2 cm and were oven sterilized. Sterile pieces were then soaked for 1 hour in the CFS solution containing bacteriocin to develop surface antimicrobial coating. The coatings were air dried by placing them in sterile petri plates and left overnight at room temperature. The dried films were placed on Nutrient agar plate on which the above-mentioned test organisms were surface spread. For controls, air-dried pieces of packaging materials coated with *St. MRS* broth for 1 hour were used. The plates were incubated at 30°C for 24 hours and were observed for antimicrobial activity.¹³

Results and Discussions

Cultivation of *Lactobacillus casei* var *shirota* from Yakult® and extraction of bacteriocin

Lactobacillus casei var *shirota* from Yakult® was cultivated in MRS broth. The culture was further isolated on MRS agar. It was found to be pure. Small, mucoid colonies were observed, which were catalase negative. On Gram staining of the colony, gram-positive rods were noted. The CFS was obtained by centrifugation process to recover bacteriocins pro-

duced by *L. casei*. It was found to be acidic in nature. Neutralization of CFS was thus carried out using NaOH. This was further used for antibacterial studies.

Campos and colleagues in 2006 extracted the bacteriocins produced by *Lactobacilli* and *Enterococci* and characterized them.¹¹ Deraz and colleagues in 2006 carried out SDS PAGE for purification and identification of acidocin, a bacteriocin produced by *L. acidophilus*.⁸ Bio preservative efficacy bacteriocins produced by *L. fermentum* and their application in food products was studied by Udhayashree and Senbagam in 2012. It was found to improve the shelf life of products such as apple juice and fish.¹⁴ Several reports suggest that the antibacterial activity of the bacteriocin extract was found to be maximum after 24 hours of cultivation of *Lactobacilli* in MRS broth, i.e. during the early stationary phase and remained stable only for a short period, followed by a decrease.^{15,16}

Antimicrobial activity of bacteriocin against *Escherichia coli* and *Staphylococcus aureus*

The CFS culture was found to inhibit both *E. coli* and *S. aureus*. The measured diameters of zones of inhibition are tabulated below (Table 1). The antibacterial activity of the centrifuged CFS is thought to be due to the presence of bacteriocins since the potency of the culture metabolite is retained after the pH adjustment.

Table 1. Antimicrobial activity of bacteriocin against *Escherichia coli* and *Staphylococcus aureus*.

Test organisms	Diameter of inhibition zones, mm
<i>Escherichia coli</i>	16
<i>Staphylococcus aureus</i>	23

Table 2. Effect of temperature on the bacteriocin stability.

Temperature of exposure, °C	Diameter of inhibition zones, mm	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
4	0	0
28	12	22
37	18	20
55	8	10
100	0	2

Table 3. Effectiveness of the bacteriocin coating on packages.

Package type	Diameter of inhibition zones, mm	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Glassine paper	14	12
Laminates	8	10
Plastic coated hard paper	12	8

Thus, the recorded inhibition levels are not due to interference by lactic acid produced by the culture in the supernatant fluid.

Bacteriocins are known to harbor the narrow antibacterial spectra of activity^{9,17} although some have been reported to show broad activity spectrum. The antimicrobial assays using the cell-free supernatant of the LAB cultures isolated from *ugba* and *okpiye*, two locally fermented Nigerian food condiments showed that *S. aureus* was the most sensitive organism tested, while *Listeria monocytogenes* was the most resistant. It was also active against *E. coli*.¹⁸ The work carried out by Sharma and colleagues in 2014 showed that the crude bacteriocin produced by *L. casei* exhibits antibacterial activity against several food borne pathogens like *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*.¹⁹ Several factors influence the antibacterial activity of the bacteriocins. According to Sezer and Guven, the culture medium, incubation conditions, target microorganisms as well as the sensitivity of methods used in determining the antimicrobial activity are important factors.²⁰

Thermal stability of bacteriocins

The bacteriocin produced by the *L. casei* var *shirota* was found to be sensitive to heat treatment. The antibacterial activity of the CFS was completely lost at extreme low and high temperature (Table 2). A considerable loss of activ-

ity was recorded after the treatment at 55°C. The loss of antibacterial activity suggest that the bactericidal agent synthesized by the LAB are bacteriocins with a proteinaceous active moiety which remains stable only at a narrow range of temperature and gets inactivated at extreme temperatures.

Several reports on the ability of bacteriocins to withstand the high temperature conditions have been published.¹² The work carried out by Campos and colleagues have shown that the bacteriocin activity remain stable after extended refrigerated storage and freezing–thawing cycles.¹¹ This fact suggests that bacteriocin produced by the LAB strains may find application as biopreservatives in minimally processed vegetables. Similar kind of work carried out in past have shown that the bacteriocin obtained from other *L. casei* was found to be heat stable (5 min at 100°C).¹⁹ No significant activity loss was also recorded by Nwuche when the CFS was incubated in a water bath at 80°C for 30 min.¹⁸ At temperatures as high as 121°C, bacteriocins have been shown to retain activity and stability.²¹ This property of high temperature tolerance may be important in the food industry where some food preparation procedures involve a heating step.

Effectiveness of the package materials coated with bacteriocin

The antibacterial activity of the package materials developed by coating of bacteriocin was studied against *E. coli* and *S. aureus*. The coated packages were found to be inhibitory for both the tested organisms. The bacteriocin adsorbed on the packages diffuses through the medium thereby inhibiting the growth of organisms. These results showed that packaging materials coated with bacteriocin can give a good protection against bacterial growth (Table 3).

The efficacy of bacteriocin coatings on the inhibition of pathogens has also been demonstrated in other studies. Dawson and colleagues evaluated the effect of lauric acid and nisin impregnated soy based films on the growth of *Listeria monocytogenes* on turkey Bologna.²² Hoffman and colleagues studied the antimicrobial effects of corn zein films impregnated with nisin, lauric acid and EDTA. Their results showed that *L. monocytogenes* cell numbers decreased by greater than 4 logs after 48 h of exposure to films containing Lauric acid or nisin alone.²³ The study carried out by Cleveland and colleagues in 2001 have proved that bacteriocins are safe, natural antimicrobials and can be used for food preservation.¹² Mauriello and colleagues developed active polythene films antilisterial film by using the bacteriocin produced by *L. curvatus*. Food products like pork steak and ground beef contaminated by *L. monocytogenes* were packaged in these films, which were found to be effective

against the contaminant thereby preventing spoilage.¹³

Conclusions

The study revealed that the bacteriocins produced by *L. casei* possessed antibacterial activity against *E. coli* and *S. aureus*, the potential bacterial contaminants associated commonly with food. Therefore, coating of the biopolymers can be done to develop antibacterial packages in food industries. Since the culture is obtained from a probiotic drink Yakult®, the bacteriocins it produces is safe for consumption. It is found to be inhibiting, however it is important to optimize the parameters for the maximum production of bacteriocin by LAB and determine optimum conditions for its maximum antibacterial activity. Further, the sensitivity of a wide range of food spoilage organism should be tested for the bacteriocins produced by *L. casei*. Further, high *in vitro* bacteriocin activity does not always corresponded to high activity in *in vivo* environment. A model system that effectively replicates a food microenvironment can be developed for such a study. Also, the stability of coated packaging films over a period of time should be determined.

In conclusion, the bacteriocins produced by LAB, which are safe can be used to develop inert, active, antibacterial packaging films to control the post-processing contamination that lead to foodborne illness.

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