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Production and Partial Purification of Lactocin produced from *Lactobacillus lactis* and its Bactericidal activity against Food spoiling bacteria

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Abstract

The antimicrobial activity of partially purified bacteriocin produced from raw milk samples against common food spoiling pathogens including Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis and Bacillus cereus were assessed and characterized. To test the bactericidal effect of lactocin, various concentrations of pH were used. Bactericidal activity was estimated using agar well diffusion technique by concentration of 25µL, 50µL, 75µL and 100µL. The bacteriocin of Lactobacillus lactis at pH 4 shows high bactericidal activity against Klebsiella pneumoniae. While at pH 7, it shows high bactericidal activity against Bacillus subtilis and Klebsiella pneumoniae, followed by high bactericidal activity against Bacillus subtilis at the pH 9. This activity indicates its potential application as a biopreservative in various food products.

Key words: Bactericidal, Bacteriocin, K*lebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*, Biopreservative.

Introduction

As the globalization of industries increases a rapid expansion in the formulation of the chemical preservatives, the safety and effectiveness of the new chemical is yet to understand thoroughly. Antibiotics are effective against microorganisms, but the reality is no antibiotic inhibits all microorganisms[1]. Also many plants like *Punica granatum*[2], *Moringa oleifera*[3] shows effective antimicrobial properties against few pathogens. There are various physical and chemical methods to preserve the food. But the use of chemicals is harmful to our digestive system. For this reason the food technologies deviating their research towards the screening of eco-friendly safe and broad spectrum preservatives[4]. In the recent years the food industry faced the need of increasing the possibilities for better conservation and of the food products. Today the conservation is commonly performed by sterilization or by adding sugar, salt, organic acids or by smoking. However, some of these compounds change the taste quality and the appliance of others is not healthy[5]. Food preservation processes are dependent of the biological activity of micro organism for production of a range of metabolites that can suppress the growth and survival of an undesirable micro flora in foods[6]. Many bacteria of different taxonomic branches are residing in various habitats produce antimicrobial substances that are active against other bacteria. Even a fungal strain, *Aspergillus* isolated from textile waste water has shown the ability to decolourise textile dye reactive blue MR[7] and another species *Aspergillus sulphureus* has shown the ability to decolourise the dye reactive black HFGR[8] . Fermentation of various food stuffs by LAB is one of the best methods of biopreservation. The LAB had the ability to produce antimicrobial compounds called bacteriocins. In recent years, bacteriocin had grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of food enhanced shelf life or safety. Recent scientific evidence supports the role of probiotic LAB in mediating many positive health effects[9]. Food experts wanted to increase the production of many convient and minimise processed refrigerated food products to meet the demand of health conscious consumers. At present, there are several concerns about the safety of foods. In general, Foods are refrigerated and vacuum packaged and extended shelf-life. However, they may contain pathogenic bacteria that could multiply under storage condition. To control growth of the pathogenic bacteria during storage, several techniques, such as reducing water activity, maintaining low pH low storage temperature and incorporation of suitable preservatives, had been recommended, bacteriocins of food grade lactic acid bacteria are produced as normal by-products of microbial metabolism made attractive as natural preservatives. Bacteriocins are particularly attractive preservatives[10]. In addition bacteriocins are protein in nature should be readily digested n the human gastro intestinal tract. They can function as natural food preservatives through the inhibition of spoilage or pathogenic bacteria and ultimately contribute to food safety. Recent approval by the U.S food and Drug Administration (FDA) of the bacteriocin nisin for use in processed cheese spreads has stimulated interest in the potential application of other antimicrobial compounds produced by food-grade microorganisms[11]. Bacteriocins are proteins or peptides with bactericidal activity produced by various gram positive and gram negative bacteria with various mechanism of action. Bacteriocin exhibit bactericidal activity against species closely related to producer strain. The best-characterised are subtilin of

B.subtilis, megacin of *B.megaterium*, bacteriocin of *B.cereus*, *B.thuringinesis* is of major interest in bacteriocin research. 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 food borne diseases. It has also necessitated the need to exploit the biologically derived antimicrobial substances produced by LAB. Recent findings indicate that even mesocarp extract of *Cocos nucifera* can be used as a potential antimicrobial agent[12]. Therefore, keeping in view the above objectives the present investigations were carried out and the results obtained are discussed here.

Material and Methods

Isolation and identification of Bacteriocin producing bacteria: Bacteriocin producing raw milk samples were collected from aavin dairy, Erode, Tamilnadu. Predominant microflora was produced from these samples by serial dilution of the samples followed by spread plate technique[13] as per the conventional method using nutrient agar. After incubation at 37^0 C for 24 hours, numerous colonies were developed. Furthur using spread plate technique, large, thick, greyish white, moist, smooth, opaque colonies and large, opaque, irregular, mucoid colonies were selected assuming that it may be L*actobacillus sp*. To get pure cultures of these isolates, the plates were carefully sub-cultured on nutrient agar plates and incubated at 37^0C for 24 hours. The isolates were further identified using growth on selective medium[14] where the selected colonies were streaked on nutrient agar plates using quadrant streaking method and incubated for 24 hours at 37^0C. The colonies developed were subjected to gram staining[15] and were examined under oil immersion objective (10X and 45X) for its motility.

Furthur the isolates were subjected to biochemical characterization[15] such as indole production test, methyl red test, voges proskauer test and citrate utilization test and accordingly were tentatively identified as *Lactobacillus lactis.* This culture was inoculated into nutrient broth and nutrient agar slants and then incubated at 37^0C for 24 hours and stored in refrigerater as an inoculum.

Characterization of Production medium based on pH[16]: The isolates of *Lactobacillus lactis* have been grown in separate 100ml MRS broth with 1% of inoculum to optimize the pH at which bacteriocin production was high. For the optimization, both the isolates were inoculated into the production medium at different pH such as pH 4, 7 and 9. Then the media were incubated at for 24 hours. Then the culture was centrifuged for the separation of crude bacteriocins for further assay and purification process of bacteriocins.

Extraction and Partial purification of bacteriocin: After incubation, the bacterial culture was filtered using Whatman no: 1 filter paper. The filtrate was then centrifuged at 8000 × *g* for 20 min and the cells were separated out. Supernatant was used as a crude bacteriocin. The charges on a protein in solution can be neutralized by the addition of salts and this has been used in purification of proteins[17]. For this, the supernatant of the bacterial cultures were treated with solid ammonium sulphate at pH 4 to 10% saturation and held for 3 hours at 4^0 C with stirring. The mixtures were centrifuged at 6000 rpm for 15 minutes. The precipitates were recovered and suspended in 5ml of 50mM sodium phosphate buffer at pH 7. Then the protein sample precipitated was subjected to salting out procedure.

Process of Purification by osmosis[17]: The protein solution to be desalted was taken inside the dialysis bag, pretreated with 10 mM EDTA and 2% sodium bicarbonate.The dialysis bag was then suspended in a large vessel containing about 100 fold excess water preferably dilute buffer (50 mM of phosphate buffer) with the help of a glass rod. The salt molecules pass freely and get diluted by the large volumes of the fluid in the external medium. Repeated changes of the dialysis fluid help in reducing the salt concentration inside the bag to negligible levels. To concentrate the protein, the sample in dialysis bag is suspended in sucrose solution where the water inside will move out and get absorbed by sucrose. Sucrose being impermeable remains in the partially purified protein. The protein concentration from the partially purified sample by dialysis was further purified using column chromatography.

Ion exchange Chromatography[18]: The chromatography column was washed using distilled water one or two times. Then the column packed with Silica. The column was washed with solution A (50 ml of 25mm Tris HCL +50mm NaCl). The dialyzed samples were poured into the column. The proteins were then eluted using solution B (ions of 25 mm Tris HCL +75 mm NaCl). The elutants were collected in the same test tubes. The process of elution was carried out using solution C. The eluted samples were then tested for activity.

Screening of isolates for Bactericidal activity: The purified proteinaceous compounds from *Lactobacillus lactis* were subjected for its bactericidal activity against common pathogens involved in food spoilage. The organism selected includes *Bacillus subtilis, Staphylococcus aureus*, *Klebsiella pneumonia* and *Bacillus cereus. The bactericidal activity against these organisms* was determined by agar diffusion method under aerobic conditions. Mueller Hinton agar plates were inoculated with 100 mL of each target microorganism after growing them in a broth and diluting appropriately. Then the wells were cut in the medium using well cutter. The protein samples were added to the wells at 25µl, 50µl, 75µl and 100µl concentration. The plates were then incubated at 37^0 C for 24 hours. After 24 hours, the activity was measured by the presence of inhibitory zone.

Results and Discussion

Based on the results obtained from serial dilution of the milk sample followed by spread plate technique and biochemical characterization tests, confirms that the culture obtained from the milk sample was *Lactobacillus lactis* and this was clearly shown in table 1. This result was supported by the report that

Lactobacillus species is predominantly present in milk[19]. Furthur nutrient agar is suitable for the production of bacteriocin at 37^0 C for 24 hours[20]. Few bacterial species were identified by determination of morphological cultures, physiological and biochemicals characteristics in goat's milk strains[21]. The morphology of the bacteria was also determined by gram-staining reaction and examined by a phase contrast microscope in *Lactobacillus salivarius*[22].

For the production of bacteriocin (lactocin), the isolates were inoculated into the production medium at different pH such as pH 4, 7 and 9. MRS broth is suitable for the growth of *Lactobacillus lactis* strains[22]. Bacteriocin production is dependent on the pH level of the medium used[23]. Also the effect of incubation temperature, incubation period and initial pH of the medium were suitable for the production of bacteriocin[24]. MRS agar medium was suitable for bacteriocin assays of *Lactobacillus sp.* because it stimulates high molecular weight and lipid containing bacteriocin production[25]. Activity of bacteriocin differs greatly with respect to pH. Maximum inhibitory activity was noticed at pH 4 to pH 10[16]. Temperature, pH as well as nutrient availability seem to play a crucial role in bacteriocin production[26]. Modification of nutrient composition of cultivation media should be considered for maximal production of bacteriocin that has potential use as a food biopreservative[27]. From these results and reports, it is evident that the production of bacteriocin highly depends on parameters like temperature, incubation period, pH etc. The bacteriocin thus obtained is further subjected to ammonium salt precipitation. After partial purification of crude bacteriocin by ammonium sulphate precipitation, shows increased antimicrobial activity[17]. The partially purified bacteriocin is further subjected to purification by dialysis and ion - exchange chromatography. By this, 80% of the samples get purified.

This purified bacteriocin (lactocin) was tested for its bactericidal effect against various food borne pathogens in various concentrations of 25µl, 50µl, 75µl and 100µl at altered pH levels using agar well diffusion technique. Antimicrobial activity of the bacterial isolates against the pathogenic microorganisms was determined effectively by agar well diffusion method[28]. Under aerobic conditions, the bacterial isolates shown wide zone of inhibition against the target microorganisms. The results obtained shows that the bacteriocin of *Lactobacillus lactis* at pH 4 posse's high bactericidal activity against *Klebsiella pneumonia* when compared with other test organisms and the concentration employed was 100µl. The inhibitory zone (14mm) represented the antibiotic potency of bacteriocin, which was clearly presented in table 2. This result was authenticated by the report that, bacteriocin controlled the food spoiling microorganism of both gram positive and gram negative bacteria[29]. Bacteriocin activity differs greatly with respect to sensitivity to pH[30]. When the pH was altered to 7, the activity was high against *Bacillus subtilis* (12mm) and *Klebsiella pneumonia(10mm)* in the same concentration of 100µl as shown in table 3. Bacteriocins show antibacterial activity at a pH range of 4.0 to 7.0 in *Lactobacillus acidophilus*[31]. At the pH of 9, the bactericidal activity was high against *Bacillus subtilis (12mm)* and the concentration employed was 100µl, which was clearly indicated in table 4. From this study, it was evident that the bactericidal effect of bacteriocin is highly dependent on the pH. Increase in the level of pH above 9 shows reduction in the inhibitory activity. pH levels between 4 to 9 is the opt pH concentration for good inhibitory activity of bacteriocin from *Lactobacillus sp* against a wide spectrum of various pathogenic organisms. This was further strengthened from the report that bacteriocin from *Lacto bacillus sp* possess a wide spectrum of inhibitory activity against *Staphylococus aereus* and *Bacillus cereus*[32]. Also the bacteriocin isolated from *L.acidophilus* strains, lost its inhibitory activity when the pH was raised above 7 gradually[33].

Table - 1
Biochemical characterization of *Lactobacillus lactis*

S. No	TESTS	OBERVATION	RESULTS
1.	Gram staining	Gram negative rods	+
2.	Motility	Motile	+
3.	Indole test	Cherry red ring was formed	+
4.	Methyl red test	Orange colour was formed	+
5.	Voges proskauer	Remain yellow colour – No colour changes	-
6.	Citrate utilization	Remain green colour – No colour changes	-

Table -2
Bactericidal activity of lactocin against common food spoiling organism at pH 4

Organisms	Zone of inhibition in mm			
	25 µl	50 µl	75 µl	100 µl
Bacillus subtilis	-	2	2	4
Staphylococcus aereus	2	2	4	4
Klebsiella pneumoniae	-	6	8	14
Bacillus cereus	-	4	6	6

Table – 3
Bactericidal activity of lactocin against common food spoiling organism at pH 7

Organisms	Zone of inhibition in mm			
	25µl	**50µl**	**75 µl**	**100 µl**
Bacillus subtilis	4	6	10	12
Staphylococcus aereus	-	-	8	8
Klebsiella pneumoniae	2	4	8	10
Bacillus cereus	-	-	6	8

Table – 4
Bactericidal activity of lactocin against common food spoiling organism at pH 9

Organisms	Zone of inhibition in mm			
	25 µl	**50 µl**	**75µl**	**100 µl**
Bacillus subtilis	8	6	8	12
Staphylococcus aereus	-	-	4	4
Klebsiella pneumoniae	-	-	-	-
Bacillus cereus	-	-	-	-

Conclusion

Research on microbes aiming at exploiting their unknown beneficial characteristics was increasing day by day. Microorganisms associated with gold jewelries[34], Synthesis of methyl cinnamate from *B.licheniformis*[35], Rhizobacteria associated with sunflower[36], Ecophysiological and cytopathological impact of Bacillus *thuringiensis*[37] were the authentication for this. The present investigation suggests that the lactocin can be used to kill or control the growth of above food spoiling bacteria after the investigation of effective dose and self life of food materials. It was concluded that bacteriocin or bacteriocins-like inhibitory substances are produced by wide variety of gram positive bacteria. *Lactobacillus lactis* was one among the best producer for bacteriocin (lactocin). Observation made during centrifugation, ammonium salt precipitation, dialysis and chromatography indicated that native *Lactobacillus lactis* is associated with a large bacteriocin complex. The ability of *Lactobacillus lactis* to inhibit other bacteria was more besides than other strains. Inhibitory substances produced by *Lactobacillus lactis* meet the criteria that would allow us to define it has bacteriocin (lactocin). A broad spectrum of activity (being Gram positive bacteria) against wide variety of bacteria including gram negative, on essential protein moiety, a bacterial mode of action. Therefore, it has a potential for application as a biopreservative in different food products as such or in combination with other preservation methods.

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