Partial characterization and optimization of parameters for Bacteriocin production by Probiotic Lactic acid bacteria

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ABSTRACT

Present paper has some background on the scientific research about lactic acid bacteria as probiotics and their bacteriocins for healthy nutrition of fermented food. In the present investigation, we have isolated nine isolates of lactic acid bacteria (L₁ – L₉) from curd and screened for bacteriocin production. The (L₂) strain possessing strong antimicrobial activity against enteropathogenic bacteria such as Staphylococcus aureus and Pseudomonas aeruginosa was selected for bacteriocin production. Our findings clearly confirm that the antagonistic activity of (L₂) strain is due to the bacteriocin production. The bacteriocin was sequentially purified and its molecular weight was determined to be 35 k Da by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The influence of culture conditions on the production of bacteriocin by (L₂) strain was evaluated. The bacteriocin activity was stable from pH 5-10 and the temperatures ranging between 30 °C to 60 °C and by utilizing glucose and yeast extract as carbon and nitrogen sources. Maximum bacteriocin activity of 12800 AU/mL was obtained at pH 6 and 13000 AU/mL at 30 °C and increased bacteriocin production was noted, when MRS broth was supplemented with glucose and yeast extract. This bacteriocin may have a potential use as food biopreservatives and may help in improving the gut environment by combating several pathogenic microorganisms. These substances in appropriate concentrations may be used in veterinary medicine and as animal growth promotor instead usual antibiotics, as well as an additional hurdle factor for increasing the shelf life of minimal processed foods.

Keywords: Lactobacillus, probiotic properties, fermentative production, antimicrobial properties, partial characterization.

INTRODUCTION

Lactic acid bacteria have been of great importance as starter cultures and LAB with probiotic potential have been reported to have many beneficial effects to the human hosts on inhabiting the gut mucosa [1]. A potential probiotic bacterium must qualify certain selection criteria as described by Gibson et al. [2] like acid and bile stability, antimicrobial production and antagonistic activity. Antimicrobial proteinaceous compounds produced by bacteria that are active against other bacteria, despite varying greatly in chemical nature, mode of action and specificity have traditionally been defined as bacteriocins [3]. Bacteriocins form a heterogeneous group with respect to the bacteriocin producing bacterial species, molecular size, antimicrobial spectrum, stability, physical and chemical properties, mode of action [4, 5] and have been detected in all genera of Lactic Acid Bacteria. Research on bacteriocins from LAB has expanded during the last decades. The use of these substances in extending shelf life of vegetables and milk or its products has provided successful results [6]. In India, a wide variety of traditional fermented foods made from ingredients like milk, cereals, pulses and vegetables have been developed for the benefit of human health from ancient times. The primary microorganisms responsible in bringing about the desirable attributes in the final products are those belonging to Lactic Acid Bacteria (LAB).
The gastrointestinal micro biota presents a significant barrier that must be overcome for a pathogen to initiate an infection. The concept of preventing or ameliorating intestinal infections through dietary interventions designed to manipulate commensal bacteria, or as a means of introducing transiently colonizing probiotic strains, has received much attention in recent years. Such strategies could potentially decrease antibiotic use and associated problems of antimicrobial resistance. Probiotic organisms have been proposed to play roles in improving digestive function, in the reduction of chronic inflammation, and in hastening recovery from intestinal disease [7-9]. Bacteriocins are a heterogeneous family of small, heat-stable peptides with potent antimicrobial activity that are produced by many bacterial species, including many probiotic strains [10]. Bacteriocins produced by Gram-positive bacteria have a bactericidal or bacteriostatic effect on other species and genera, but activity is usually limited to other Gram-positive bacteria [11]. Bacteriocins have been used by the food industry to reduce the use of chemical preservatives in foods with limited shelf life, or those foodstuffs that present a high risk for pathogen contamination [12].

In recent years, much attention is being given to a large variety of bacteriocinogenic Lactic acid bacteria from different sources, because their bacteriocins are considered to be safe in the form of food bio preservatives, since they can be degraded by gastrointestinal protease [13, 14]. Although, Lactobacilli show a high impact on effective protection to human health, there is obvious evidence that Lactobacilli from different origins possess antimicrobial properties at different levels [15]. Thus, the present study was undertaken to study the inhibitory activity of partially purified bacteriocins of the LAB isolates from curd on human pathogens and also to evaluate the production of these bacteriocins under different physical and cultural conditions like pH, temperature, carbon, nitrogen sources and in different salt concentration.

**MATERIALS AND METHODS**

**Sample collection**
Cultures of probiotic *Lactobacillus spp.* were isolated from curd and maintained in the Department of virology, S. V. University, Tirupati, Andhra Pradesh, India, was used throughout the study. The culture was maintained on MRS agar slants.

**Isolation of Lactic acid bacteria**
For the isolation of LAB, the samples were inoculated into De Man Rogosa Sharpe’s (MRS) agar [16] and incubated at 37 °C for 48 h. After incubation, the colonies were randomly selected from the MRS agar plates. The colonies were propagated on the same media until the pure culture were obtained. Purification of the culture was confirmed by Gram’s staining. Pure cultures were again cultured on MRS agar slants and stored at 4 °C until used.

**Identification of Lactic acid bacteria**
Identification of the selected isolates with the desired antimicrobial activity was carried out using morphological, physiological, microscopic and biochemical methods as described by Michael et al. [17]. Biochemical characteristics observed included gas production, different sugar fermentation, Indole Methyl red, Vogues prauskeur and Citrate utilization (IMViC), catalase, gelatin hydrolysis, starch hydrolysis, urease and oxidase tests.

**Screening of isolates for Antimicrobial activity**
The isolated *Lactobacillus spp.* were screened for antimicrobial activity against enteropathogens, using agar well diffusion method on MRS agar plates as described by Ivanova et al [18]. The enteropathogens collected from the SVIMS hospital, Tirupati were used in the present study. Indicator bacterial entero pathogens *E. coli, Enterococcus faecalis, Pseudomonas fluorescense, Pseudomonas auregenosa, Staph aureus, Salmonella typhimium, Proteus mirabilis, Bacillus megaterium1684 and Xanthomonas campestris* were cultured in nutrient broth for 24 h at 37 °C and seeded onto the surface of MRS agar plates. Wells were created on the seeded agar plates and 50 µL of *Lactobacillus* culture supernatant obtained after centrifugation at 10,000 rpm for 5 min was added to each well. The culture supernatant was adjusted to pH= 6.5-7.0 with 1 N NaOH to nullify the lactic acid activity. Following incubation, the plates were examined for the presence of clear zone of inhibition (mm) of the target bacteria (Enteropathogens) i.e extending from the culture of *Lactobacillus spp.* was measured and based on the presence or absence of zone the results are presented as +ve or −ve.

**Bacteriocin bioassay:**
The *Lactobacillus spp.* having probiotic properties and antimicrobial activity isolated from curd sample was selected for bacteriocin production. Bacteriocin producing bacteria was cultured in MRS + Tween 80+ buffer medium containing sodium citrate, sodium acetate and dipotassium hydrogen phosphate 0.2 % each (pH= 6.8) at 37 °C for 24 hours in a rotary shaker at 100 rpm. The amount of bacteriocin production was calculated as arbitrary units.
The arbitrary unit of the bacteriocin was defined as the reciprocal of the highest serial two fold dilution showing a clear zone of growth inhibition and is expressed in arbitrary units per mL [19].

**Partial purification of bacteriocin:**

For the partial purification of bacteriocin, adsorption desorption method was used [20]. The culture was heat killed and cooled to room temperature. The pH of culture aliquot was adjusted to 6 – 6.3 and shaken at 100 rpm at room temperature for 2 hrs to adsorb bacteriocin to the producer cells. The adsorbed bacteriocin molecules on to their surfaces was harvested by centrifugation at 6000 rpm for 15 min and washed with sterile 5 mM Na-P buffer (pH= 6.5). The pellet obtained was resuspended in 100 mM NaCl solution (pH=1.5–2.0) for desorption. Cell-free supernatant was collected by centrifugation at 8000 rpm for 15mins and dialyzed in 1000 Da cut-off dialysis bag (sigma) for 24 hrs against double-distilled water with frequent changes of water. The dialysate was then lyophilized. Adjustment of the cell- free supernatant to pH= 6.0 with 1 M NaOH prevented the inhibitory effect of lactic acid.

**Determination of molecular size of bacteriocin by SDS-PAGE:**

The molecular size of the purified bacteriocin was determined using SDS – PAGE as described by Sambrook et al., [21]. Sterile glass plates were assembled, 20 mL of 18 % separating gel were dispensed, 2 mL of butanol was then overlaid onto the gels, allowed to polymerize, after which the overlay was poured off and then the gel surface rinsed with deionized water. To the above gel, 8 mL of 5 % stacking gel was overlaid and fixed in an electrophoresis apparatus. Now the partially purified bacteriocin or dialysate was subjected for SDS-PAGE through 18 % separating gel and 5 % stacking gel for the determination of molecular weight. 20 µL of partially purified bacteriocin were loaded in separate wells. Following electrophoresis the gel was removed from the setup containing marker proteins and stained with coomassie brilliant blue and destained to visualize the bands. The apparent molecular weights of the sample were determined by comparison with the mobility of the standard markers.

**Optimization of media components for the production of bacteriocin**

The effect of the medium components on the bacteriocin activity was studied by growing the strain in different medium components.

**(a) Effect of pH and temperature on bacteriocin production**

To determine the effect of pH on bacteriocin production, four batches of 100 mL MRS broth was prepared and adjusted to pH=5,6,7,8 respectively with 1 N HCl or 1 N NaOH, and then autoclaved. Each medium was inoculated (1% v/v) with an overnight culture of bacteriocin producing organism (L2) and incubated at 37 °C for 24 h. After incubation the absorbance values were determined at 580 nm and the bacteriocin activity was determined [22].

To determine the effect of temperature on bacteriocin production, 100 mL each of sterilized MRS broth in four different sets of flasks was inoculated (1% v/v) with an overnight culture of bacteriocin producing organism Lactobacillus spp. L2 and incubated at various temperatures of 30 °C, 40 °C, 50 °C and 60 °C for 24 h. After incubation the absorbance values were read at 580 nm to determine the bacteriocin activity [23].

**(b) Effect of carbon sources on bacteriocin production**

The effect of carbon source on the production of bacteriocin was carried out using 2% (w/v) glucose, lactose, sucrose, fructose and maltose as carbon sources. The sterilized medium was inoculated with an (1 % v/v) overnight culture of bacteriocin producing organism Lactobacillus spp. L2 and incubated at 37 °C for 24 h. The absorbance values were read at 580 nm to determine the bacteriocin activity.

**(c) Effect of nitrogen sources on bacteriocin production**

Different nitrogen sources such as beef extract, yeast extract, peptone, tryptone and ammonium chloride which were supplemented at 2 % (W/V) to study their effect on bacteriocin activity by bacteriocin producing organism Lactobacillus spp. L2. The medium was sterilized and inoculated (1 % v/v) with an overnight culture of bacteriocin producing organism Lactobacillus spp. L2 and incubated at 37 °C for 24 h. Absorbance values were determined at 580 nm and the bacteriocin activity were determined.

**(d) Effect of NaCl on bacteriocin production**

The effect of NaCl on bacteriocin production was determined by preparing MRS broth, with different salt concentrations like 1 %, 3 % and 5 % respectively. The flasks were inoculated (1 % v/v) with an overnight culture of bacteriocin producing organism Lactobacillus spp. L2 and incubated at 37 °C for 24 h. Absorbance values were determined at 580 nm [24].
**RESULTS**

A total of nine *Lactobacillus* spp. were isolated and identified from curd. The physiological and biochemical characteristics of the *Lactobacillus* isolates were studied and screened for the probiotic properties like thermo, acid and osmo tolerance, antimicrobial activity and their degree of inhibition against the entero pathogens. Among these, three of the strains represented as L_2_, L_4_ and L_5_ were found to be bacteriocin producing *Lactobacillus* spp., when tested against the indicator strains, and the isolate L_2_ was found to be more potential and the results are shown in Table 1.

Table 1. *Lactobacillus* spp. showing antimicrobial activities against enteropathogens

<table>
<thead>
<tr>
<th>Enteropathogens</th>
<th>Lactobacillus spp. Producing Bacteriocin Lactobacillus Strains</th>
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<tbody>
<tr>
<td></td>
<td>L_2_</td>
</tr>
<tr>
<td>E. coli</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus megaterium 1684</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>+</td>
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<tr>
<td>Pseudomonas florescence</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>+</td>
</tr>
<tr>
<td>Listeria innocua</td>
<td>-</td>
</tr>
<tr>
<td>Xanthomonas campestris</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus spp</td>
<td>+</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>+</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: No inhibition = _; Inhibition = +

![Fig. 1. Zone of inhibition against enteropathogens by L_2_, L_4_ and L_5_ isolates by well diffusion method.](image-url)
Screening of antimicrobial property

The antagonistic activity of the selected three *Lactobacillus* isolates L2, L4, L5 to inhibit the growth of enteropathogens was investigated. All the three isolates inhibited the growth of *E.coli*. The data of inhibition are reported in Table 3. The L2 isolate has strongest inhibitory activity against the gastro intestinal enteropathogens like *E.coli, Enterococcus faecalis, Pseudomonas fluorescence, Pseudomonas auregenosa, Staph aureus, Salmonella typhimurium* and *Proteus mirabilis* (Fig.1) and less antagonistic activity was observed against *Bacillus megaterium*1684 and *Xanthomonas campestris*. The *Lactobacillus* spp. L2 having probiotic properties and strong inhibitory activity was selected for the optimization of bacteriocin production using various sources. Furthermore the tested isolates (strains) were able to inhibit the growth of human enteropathogens.

![Image of protein bands and pH values](image.jpg)

**Fig. 2. LANE 1: Partially purified bacteriocin showing protein band  Marker well**

**Fig. 3. Bacteriocin production at different pH.**

**Bacteriocin by SDS-PAGE**

In order to determine the molecular size of the bacteriocin isolated from L2 isolate, the partially purified bacteriocin was subjected to SDS-PAGE analysis. As shown in (Fig. 2) the estimated molecular mass of partially purified...
bacteriocin was found to be (35 K Da) as evidenced in SDS-PAGE. Possession of bacteriocin by L2 isolate is an indication that the bacteria can be used as probiotic and as biopreservative.

**Optimization of media component for the production of bacteriocin**

(a) **Effect of pH on bacteriocin production**

The effect of pH on bacteriocin production is shown in (Fig. 3). Results showed that the MRS broth adjusted to pH=6 fostered the best production of bacteriocin by the L2 isolate (12800 AU/mL) followed by the pH values 7, 5 and 8, where the low levels of bacteriocin was recorded. From these results, it can be concluded that optimal production of bacteriocin occurs at a pH=6

**Effect of temperature on bacteriocin production**

The bacteriocin activity was tested at different temperatures 30 °C, 40 °C, 50 °C and 60 °C and the activity was found to vary from 400 to 13000 AU/mL, the maximum arbitrary unit was measured as 13000 AU/mL at 30 °C, (Fig. 4), followed by 40 °C, 50 °C and 60 °C incubation temperature. Significant reduction in the bacteriocin production was noticed as the temperature is increased. Growth temperature seems to play an important role in bacteriocin activity.

![Fig. 4. Bacteriocin production at different temperatures](image)

The values represented in the graph were the mean of two consecutive experiments.

(b) **Effect of carbon sources on bacteriocin production**

In the presence of glucose as the carbon source the bacteriocin production, was maximum of 12000 AU/mL, followed by lactose, sucrose, fructose and maltose. Less activity was observed with fructose and maltose (Fig. 5).

![Fig. 5. Bacteriocin production in different carbon sources](image)
(c) Effect of Nitrogen sources on bacteriocin production

In this study, different nitrogen sources were supplemented in MRS broth for the bacteriocin production. The increased bacteriocin activity levels of 1600 AU/mL were recorded (Fig. 6) when the producer strain was grown in the presence of yeast extract compared to other nitrogen sources in the medium consisting of yeast extract as the nitrogen source, 1600 AU/mL. However, in the presence of peptone 1400 AU/mL of bacteriocin activity was recorded following other nitrogen sources tryptone, beef extract and ammonium chloride.

![Fig. 6. Bacteriocin production in different nitrogen sources](image)

The values represented in the graph were the mean of two consecutive experiments.

(d) Effect of NaCl on bacteriocin production

Regarding various salinity (NaCl %) tested from 1 % to 5 % NaCl, the bacteriocin activity was varied from 800 to 12800 AU/mL and the Ls isolate showed maximum bacteriocin activity in 1 % NaCl concentration of about 12800 AU/mL followed by 3 % (1600 AU/mL) and 5 % (800 AU/mL), where 1 % NaCl was found to be suitable for the bacteriocin production (Fig. 7). Bacteriocins have been reported to be inhibitory against several other bacteria [25,26].

![Fig. 7. Bacteriocin production in different salinity](image)

The values represented in the graph were the mean of two consecutive experiments.
Bacteriocin production by optimized submerged fermentation

The designed optimized medium showed significant variation in bacteriocin production by submerged fermentation. The production levels were found to be dependent on the culture condition and medium component. It could be observed that there is an increased expectation on production of bacteriocin from 13000 AU/mL to 15800 AU/mL with the optimum conditions (Table 2).

Table 2. Selected factors assigned for bacteriocin production

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>6</td>
</tr>
<tr>
<td>Temperature</td>
<td>30°C</td>
</tr>
<tr>
<td>Glucose</td>
<td>2g/100ml</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2g/100ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>1g/100ml</td>
</tr>
</tbody>
</table>

DISCUSSION

Lactic acid bacteria have been shown to inhibit the growth of many enteric pathogens *in vitro* and have been used in both human and animals to treat the gastrointestinal disorders [27]. Bacteriocins produced by Lactic acid bacteria have received considerable attention during recent years for their possible application as bio preservatives in food, with the hope that the use of chemical preservatives in foods will be reduced. The *Lactobacillus* L2 has been shown to strongly inhibit some of the usual potentially harmful microorganisms. Maximal bacteriocin activity could be obtained by supplementing a culture medium with growth limiting factors such as sugars, vitamins and nitrogen sources, by regulating pH or by choosing the best adapted culture medium [28]. In order to exploit the potential utilitarian benefit of the antimicrobial properties of the isolated *Lactobacillus spp*. L2 the present study was undertaken to determine the optimal cultural conditions for greater bacteriocin yield in constituted growth medium. Various physico chemical factors are seemed to affect the bacteriocin activity. By utilizing glucose as carbon source, yeast extract as nitrogen source and 1% NaCl, highest bacteriocin production was noticed. The above results are in agreement with [29] also reported that highest bacteriocin activity was obtained when glucose was used as a carbon source. Every microorganism has a minimal, maximal and an optimal pH for growth and metabolism. Microbial cells are significantly affected by the pH of their immediate environment, because they apparently have no mechanism for adjusting their internal pH. Thus, studying the effect of pH on the antimicrobial activity produced by our isolates was an important criterion of this study. The effect of pH of the medium and temperature on the production of bacteriocin was also investigated. The medium at 30°C incubation temperature and pH=6 fostered the best production of bacteriocin by the *Lactobacillus spp*. L2 isolated from curd sample. The results obtained in our study regarding the pH tolerant bacteriocins are comparable with the previous results [30]. The optimized medium increased the bacteriocin production of 15800 AU/mL, when compared with the initial production 13000 AU/mL. From the results proved that it can be used in fermented acidic foods like pickle or yoghurt. Nisin is the first used as a food biopreservative in 1931 and first received approval by food and Drug Administration (FDA) to be used in pasteurized processed cheese in 1988 [31]. Like nisin the bacteriocin produced by *Lactobacillus spp*. L2 in the present study also has the potential to develop probiotics and biopreservative characters.

Previous studies reported that many of the antimicrobial compounds produced by lactic acid bacteria are bacteriocins with a proteinaceous nature; while other non-protein agents are also produced [32].The general mechanism of bacteriocin action which has been suggested is disruption of the electrochemical gradient across the cytoplasm membrane by pore formation. The molecular weight determination of the bacteriocin was done by SDS-PAGE. The molecular size of the bacteriocin has been reported as (35 K Da) obtained by SDS-PAGE analysis which was quite high in comparison with bacteriocin like pediocin PA 1.0 (16.5 K Da) [33]. The 5.5 K bp plasmid of *L. acidophilus* is currently being sequenced to determine the exact location of the structural gene of bacteriocin [34, 35].

CONCLUSION

Results of this investigation showed that the bacteriocin suspension of *Lactobacillus spp.* (L2) grown in MRS broth had the best inhibitory effect against enteropathogens. The present study demonstrated the production of bacteriocin by *Lactobacillus spp.* (L2) under optimized culture conditions and the bacteriocin was partially purified and characterized. To date, Nisin is the only bacteriocin that has found practical application in some industrially processed foods. It is anticipated that advances in bacteriocin research and combination treatment for food preservation and enteropathogens will benefit both the producer and the consumer of the food industry.
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REFERENCES
[7]. Gotteland M., Brunser O, Cruchet S. Aliment Pharmacol Ther. 2006, 15, 1077-86.