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## ORIGINAL ARTICLE

# Fermented Fruit Juice as a Source of Bacteriocidal Compounds

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**ABSTRACT**
*The activity of Gram positive and Gram-negative bacteria to inhibit the growth of other bacterium is due to the presence of bacteriocin in them. Bacteriocins are the ribosomally synthesised proteins produced by both gram positive and gram negative bacteria. These compounds have found its application in industrial and health sectors. The present study was mainly conducted to isolate bacteriocidal agents from lactic acid bacteria present in fruit juice. Fruit juice namely, orange juice was subjected to overnight fermentation and serially diluted in MRS Agar medium to obtain the lactic acid bacteria. The isolated LAB in pure culture was identified after biochemical (Gram staining, sugar fermentation, citrate test, catalase test) and molecular characterisation as Lactobacillus casei. The crude bacteriocin was produced using standard methods from the LAB isolate. The crude Bacteriocin obtained showed great antimicrobial activity against food borne pathogens such as L.monocytogenes, S.typhi, and other common pathogen namely K.pneumoniae. This bacteriocin also showed variation in activity when subjected to different external factors such as pH, temperature and enzyme activity.Hence, we can validate the use of a waste product i.e. fermented fruit juice for commercial purposes such as food preservation and for medicinal purposes such as development of antibacterial drug.*
***Keywords****: Bacteriocidal, Bacteriocin, Lactic Acid Bacteria, Fruit Juices, Antagonism, Antibiotic*

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## INTRODUCTION

Certain bacteria possess the ability to kill other bacteria due to the presence of certain proteins in them known as bacteriocins. Bacteriocins are antimicrobial peptides of ribosome that are considered to be an important tool to defend microbes [1].Though the bacteriocins produced by different bacteria work differently their basic mode of action involve perforating the target membranes thereby disturbing the energy potential. The Bacteriocins produced by different bacteria act in different ways. Bacterocins can be isolated from the microflora of milk, yoghurt, fruit juices, water, Soil, stool, and even samples from chemicals [2]. Milk and other dairy products are rich in the bactriocin producing bacteria such as Lactic Acid bacteria. LABs even used as starter culture in flavoring products because of their antimicrobial nature thereby improving the quality of products. Nisin, a bacteriocidal compound produced from a LAB is widely used in dairy products preservation and has already replace the nitrate formerly used in preservation of cheese from *Clostridium* contamination. Cheese, yoghurt, meat products are all the natural sources of lactic acid bacteria. Fermentation in these products yields lactic acid. Hence, natural food preservatives such as bacteriocins alleviate the food spoilage thereby increasing the shelf life period. If incorporated to food stuffs, may help eliminate the microbes in vegetables that have been inhabited in the vegetables through the cut surfaces. Hence, the use of bacteriocins could be used as an alternative to chlorine or ozone treatment of food stuffs. A starter culture prepared with these antimicrobial peptides can successfully be used for bio preservation [3]. Fruit juices also contain acids and upon fermentation they produce lactic acid bacteria.

Bacteriocins produced by lactic acid bacteria also help in maintaining good human health. Milk is rich in this bacteria and this contributes towards maintaining a good environment in the stomach. Bacteriocins

produced by non-pathogenic lactobacilli too contribute in keeping up the vaginal micro biome [4]. Fruit juices also contain lactic acid bacteria when they undergo fermentation. The present work was aimed at isolation and identification of lactic acid bacteria from fermented fruit juice sample and production of bacteriocin from the same.

## MATERIAL AND METHODS

### Isolation of Lactic Acid Bacteria from fruit juice

Orange juice was collected from a nearby juice store at Kollam. It was then allowed to ferment overnight. The fermented juice was then serially diluted from $10^{-1}$ to $10^{-8}$ in 0.85 % saline solution. Further 100 µl of dilutions were transferred into MRS Agar plates and kept for overnight incubation in the bacteriological incubator maintained at 37ºC.Among the incubated petri plates, $10^{-8}$ diluted plates with less isolated microbial colony was chosen. Pure culture was isolated after quadrant streaking and the obtained pure culture was sub cultured in both liquid and solid medium. The identification of the pure culture was done by biochemical tests and molecular methods.

### Biochemical Characterisation of the isolated Bacterium

The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics [5, 6]. The used tests were: Gram reaction; production of catalase, acid production from carbohydrates (1 % w/v) in MRS broth with phenol red as indicator; production of acid and gas from 1 % glucose, nitrate reduction in nitrate broth; indole production in tryptone broth and citrate utilisation tests.

### Molecular characterization of the isolate

The bacterial DNA was isolated as per the methods of Troyer *et al.,* [7]. Agarose gel electrophoresis was performed in order to check the quality of the DNA. The extracted DNA was subjected to 16S rRNA amplification and further for sequencing at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

### Production of Crude bacteriocin

The isolated and identified bacterial culture was inoculated in 200mL MRS broth containing 15% glycerol and kept in an orbital shaking incubator for 3 days and later stored at 4ºC upto a week Later this broth was subjected to centrifugation for 10 minutes with 5000 rpm. The cells got separated and clear supernatant was obtained above. The cell free supernatant was then collected and stored as crude bacteriocins.

### Screening of the Bacteriocidal activity of the crude Bacteriocin

To screen the bacteriocin producing microbes, agar well diffusion method was adopted. Three bacteria were chosen namely, *Klebsiella pneumonia, Lysteria monocytogenes and Salmonella typhi* as test organisms. These test organisms were cultured in nutrient broth and incubated over night for its growth. To identify the bacteriocin activity, 3 petriplates with Mueller –Hinton agar were prepared and autoclaved. The test organisms were swabbed on to the prepared petri plates. Wells were made in each plate with the help of a well-borer for adding 50µL and 100µL of bacteriocin and a positive control (antibiotic). The plates were then incubated overnight at 37 ˚C. After incubation, the zone of inhibition was measured.

### Effect of pH on bacteriocin

To study the effect of pH , bacteriocin pH were adjusted as acidic, basic and neutral with the aid of pH meter .The Meuller – Hinton agar plates were prepared and the test organisms were then swabbed on each petriplates. Three wells were punctured using a well borer on each petriplate. From the three test tubes 100 µl of treated bacteriocins were transferred into the three wells using a pipette. The plates were then incubated overnight. .The zones of inhibition on the plates were measured and recorded .

### Effect of Temperature on bacteriocin .

To study the effect of temperature on bacteriocin activity, bacteriocin was subjected to three different temperatures namely 60 ˚C, 100 ˚C , 121 ˚C. After the plates preparation, three wells were made with a well borer and 100 µl of each of heat treated bacteriocins were added to the wells. The plates were incubated for one day. The zones were measured and recorded.

## RESULTS AND DISCUSSION

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are similar to yeast and paramecium killing factors, and are structurally, functionally and ecologically diverse. Applications of bacteriocins are being tested to assess their application as narrow-spectrum antibiotics [8]. In the present study, the isolation of a lactic acid bacteria was done from a fruit juice.

 

**Biochemical Characterization**

The isolated culture was found to be Gram positive and rod shaped in morphology (Fig.1)

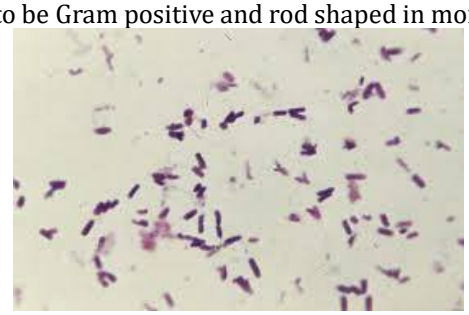

Fig.1 Gram Positive Lactobacilli

The results of biochemical characterization are as follows:

| Biochemical Test | Result |
|---|---|
| Catalase Test | Negative |
| Citrate Test | Negative |
| Fermentation of : | |
| Glucose | Acid Production |
| Sucrose | Positive |
| Rhamnose | Negative |
| Lactose | Positive |

Thus according to Bergey's manual the isolated species was identified as *Lactobacillus casei.*

**Molecular characterization of the isolate**

The isolated bacterial DNA was subjected to Agarose Gel Electrophoresis to confirm its quality. The DNA was then amplified via PCR and later given for sequencing. The sequence was analysed in NCBI-BLAST and was found to be similar to that of *Lactobacilus casei* with 100% query cover and 99.40% identity. The obtained sequence was submitted to Genbank and the obtained accession number is OK465109.

>LAB Isolate

GTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTT
TTTTAGGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGGAAACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTC
TGGTCTGTAACTGACGCTGAGGATCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGG
AGTACGAGCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCTGGTCTTGACATCTTTTTGATCACTGAGAGATCAGGTTTCCCCTTCGGGGGC
AAAATGACAGGTGGTGCATGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCTA

**Screening the antimicrobial Activity of the Produced Bacteriocin**

For both the obtained crude bacteriocins, maximum zones of inhibition were seen on the addition of 100 µL bacteriocin.(Table 1)

**Effect of pH on bacteriocin activity**

The activity of bacteriocin was tested at different pH. Bacterocin showed high zone of inhibition in an acidic medium than that in a basic or neutral medium.. Its activity decreased as the pH was increased to an alkaline pH.(Table 2)

 


Fig. 2a,2b,2c- Showing the zones of inhibition after the addition of Bacteriocin

**Effect of Temperature on bacteriocin activity**

Bacteriocin activity was tested at different temperatures (60 ˚C, 100 ˚C, 121 ˚ C ).As the temperature increased, the activity of bacteriocin decreased (Table 3). Zone of inhibition was found higher at bacteriocin treated at 60 ˚C. Zone of inhibition was less for higher temperature (at 121 ˚ C).

 

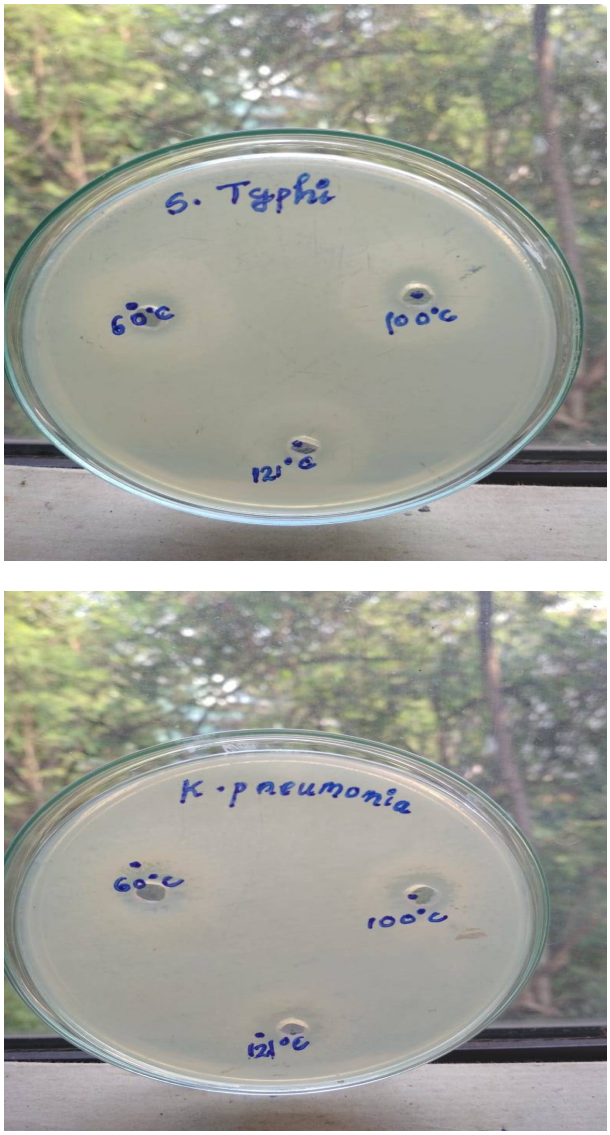


Fig.3a,b Effect of temperature on the activity of Bacteriocin

The crude bacteriocin had considerable antibacterial effect against the three selected bacterial cultures. The efficiency of crude bacteriocin was observed by treating the bacteriocins at different temperatures (60°C, 100°C and 121°C) and different pH (acidic, basic and neutral). The results reveal that bacteriocins activity decreased when the temperature was raised while the activity increased under acidic pH conditions. From the available studies researchers have revealed that bacteriocins are stable over a broad range of temperature. In study of *Stoffels et al.,,* [9] when analyzing the effects of bacteriocins isolated from Carnobacterium species it was reported that bacteriocins are stable between 30°C-80°C. Another study reported that even at 60°C retained more than 60% of their activity for 30 minutes and declined subsequently [10]. Various studies have reported that bacteriocins of the two strains of LAB considered to extremely heating stable. In another study, the effect of heat was determined from bacteriocin of these two strains. It was reported that these bacteriocins from LAB can withstand heat treatment after 15 minutes at 121°C without altering the antibacterial activity. Studies have focused and brought to the limelight the effects of potential of hydrogen on the optimal activity of bacteriocins [11,12]. Previous findings have determined this influence of pH and revealed the 100% active stability of bacteriocins was observed between pH 6-7 and at pH4 more than 70% of activity was retained. Bacteriocins differ greatly with respect to their sensitivity to different pH. Studies on the effect of various pH values ranging from 1-12 show that optimal activity is attained at pH 4 and 5. Most bacteriocin produced by Lactobacilli is considered to be more tolerant of acid than alkaline values. Hence, the present study affirms the use of waste product such as fermented fruit juice as a source of bacteriocidal compounds for use in food and medical industries.

 

| Test Organism | Bacteriocin from Fruit Juice(50uL) | Bactricoin from Fruit Juice 100uL |
|---|---|---|
| *Salmonella Typhi* | 8mm | 15mm |
| *Klebsiella pneumonia* | 11mm | 14mm |
| *Listeria monocytogenes* | 11mm | 17mm |

Table.1 .Zone of inhibition produced by the Bacteriocin on the test organisms

| | **Zone of inhibition** | | |
|---|---|---|---|
| **Test Organisms** | **Bacteriocin in Acidic pH** | **Bacteriocin in Basic pH** | **Bacteriocin in Neutral pH** |
| *Salmonella Typhi* | 21 mm | 10 mm | 19 mm |
| *Klebsiella Pneumonia* | 22 mm | 12 mm | 16 mm |
| *Listeria Monocytogens* | 23 mm | 13 mm | 20 mm |

Table 2. Effect of pH on the antimicrobial activity of Bacteriocin

| | Zone of inhibition | | |
|---|---|---|---|
| **Test Organism** | **Bacteriocin in 60˚C** | **Bacteriocin in 100˚C** | **Bacteriocin in 121˚C** |
| *Salmonella Typhi* | 25 mm | 23 mm | 18 mm |
| *Klebsiella Pneumoniae* | 12 mm | 11 mm | 10 mm |
| *Listeria Monocytogens* | 20 mm | 15 mm | 13 mm |

Table 3. Effect of temperature on the antimicrobial activity of Bacteriocin

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