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USE OF ANTIMICROBIAL BACTERIOCINS PRODUCED FROM INDIGENOUS LACTOBACILLUS SPP. AS BIOPRESERVATIVES INPINEAPPLE JUICES

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ABSTRACT

Within the beverage industry, fruit juices are rapidly growing segment among non-alcoholic beverage because of their nutritional benefits. But they are prone to spoilage due to microbial activity which makes it inconsumable. Besides the physical method which includes pasteurization, chemical preservatives are widely used in fruit juices and beverages to improve their shelf-life where potassium sorbate and sodium benzoate are most common among various preservatives. The demand of natural, safe and environmental friendly preservatives has been increasing. That is why, bacteriocins, lactoperoxidase, herb leaves and oils, spices, and some organic acids are gaining significance now a days and considered as Generally Recognized As Safe (GRAS) additives. Bacteriocins being bioactive antimicrobial peptides which is extracellularly released by various bacteria specifically probiotic and readily degraded by proteolytic enzymes in the human body. This suggests the possibilities of its use as biopreservative in fruit and vegetable juices apart from dairy usage. In the present study various types of food and dairy products were used to obtain indigenous Lactobacillus spp. and then protenacious fermented product obtained after their fermentation in liquid MRS (deManRogosa and Sharpe medium) medium was used in the preservation of pineapple juice after their proper investigations.

KEYWORDS: Biopreservatives, Bacteriocins, Probiotic, Proteolytic enzymes, Fruit juices.

INTRODUCTION

Approx. a century ago, Elie Metchnikoff was the first to push the significance of intestinal lactobacilli in microflora in keeping up wellbeing and life span. The term 'probiotic' has been promoted by R. Fuller, and is characterized as a live microbial feed supplement which influences the host by improving its intestinal microbial environment. To increase the shelf life of food various preservatives are used. These preservatives may have some harmful effects so if possible, foods without preservatives may be used. There are many harmful effect of food preservative.[1]

In recent past, bacteriocins have fascinated substantial attention regarding their safe use as food preservatives, since their easy digestibility in human digestive system.[2] As natural food preservatives, bacteriocins fulfills demand of consumers for high quality safe foods without involvement of chemical preservatives. But, use of bacteriocins can be limited as food additives for various reasons, like efficient elimination of pathogen or its high price.[3]

There are distinct mode of actions of Bacteriocins which are mainly associated with either bactericidal effect with or without cell lysis or bacteriostatic or cell growth.[4] The bacteriocins mostly produced by LAB exert antibacterial effect against Gram-positive bacteria, by targeting the cell envelope-associated mechanisms.[5]

METHODS AND MATERIALS

Isolation and & Screening of *Lactobacillus* spp. with Antimicrobial Potential

(1) Several type of samples including as raw milk, fermented milk, curd, buttermilk, cottage cheese, khoa, dhokla batters, Dosa batter, rotting food & vegetable waste etc. were used for the screen out the microbial species of genus *Lactobacilli* which yielded varied number of colony forming units (CFUs) of *Lactobacillus* species.
(2) The isolation of *Lactobacillus* spp. was performed on MRS media plates as it allows the selective and vibrant growth of *Lactobacillus* spp.[6-7]
(3) The antimicrobial potential of 14 different *Lactobacillus* isolates against *E. coli* (MTCC-1687) and *S. aureus* (MTCC- 737) was performed.[8-9] The procedure included the culture of pure *Lactobacillus* isolates in liquid MRS medium for 48 hours at 37oC followed by use of cell free fermentation extract for antimicrobial activity by well diffusion method.

Biochemical Characterization of Antimicrobial *Lactobacillus* spp.

The biochemical tests included in present study were catalase test, indole test, methyl red reduction test, Vogas-Proskuare test, citrate utilization test, starch hydrolysis test and carbohydrate fermentation tests including lactose, sucrose, maltose and mannitol as source ofcarbon in the basal medium.

Detection of Isolates secreting Proteinaceus Antimicrobial Component

Out of the 14 indigenous lactobacillus isolates only 8 isolates were reported to show antimicrobial potential against the test microbial species. To determine the antimicrobial protein component that must be secreted by indigenous lactobacillus isolates in culture broth, the cell free extract of sample broth of all the selected 8 isolates were treated with protease enzyme where the loss of antimicrobial activity of the extracts was the indication for the presence of proteinaceus antimicrobial component in the cell free extracts.

Extraction of Antimicrobial Proteins

The extraction and precipitation of protein from the cell free fermentation extracts produced at optimized pH and temperature condition in 100 ml volume of MRS broth was performed by salting out method. The salting out procedure generally involves the saturation of fermentation liquor upto 70% with solid ammonium sulphate.[10]

SDS PAGE analysis of antimicrobial proteins

20µl of prepared protein samples was mixed with 20µl Laemmli's 2X sample loading buffer the composition. This preparation was then heated 95^{o}C for 5 minutes in boiling water bath. After bringing them down to room temperature, 15 to 20µl of this prepared samples ware loaded into the wells of SDS-PAGE gel for electrophoretic separation and investigation.Composition of Laemmli loading buffer is;

S.N.	Components
1.	125 mMTris, pH 6.8
2.	4% SDS
3.	20% Glycerol
4.	10% β-Mercaptoethanol
5	0.0025% Bromphenol Blue

Biopreservation Activity of Bacteriocin

The freshly prepared pineapple juice was filtered through nylon sieve followed by passing through sterile set up of whatman filter paper and was dispensed in sterile test tubes followed by adding 100 µl of 1X, 10X and 100X diluted main stocks of extracted proteinaceus components . The experimental sets were observed for the preservative action of the proteinaceus components of indigenous *Lactobacillus* spp. origin compared to 1% benzoic acid. The tubes were kept at room temperature for 4 weeks and was investigated for microbiological parameters after the period of incubation.

Microbiological Evaluation of Juice

(1) The pineapple juice was subjected to standard plate count method for microbiological evaluation. 0.1 ml of juice sample was used to spread on to the nutrient agar plates and the plates were incubated at 37ºC for 24 hrs.

(2) The bacterial colonies formed after incubation was counted on the instrument called digital colony counter. The microbial colonies were checked in both experimental and control set of juices. In present investigation also, in terms of microbiological evaluation the pineapple juices were subjected to total bacterial count, *Coliform* count, *Pseudomonas* count and total fungal count at 10^{-7} dilution.

(3) At the start of experiment the microbial load of fresh pineapple juice coded as FJ was monitored, where the total bacteria count at 10^{-7} dilution was observed to be only 2 on nutrient agar plates but there were no coliform, pseudomonas and fungal counts observed on McConky's agar plate, Cetrimide agar plate and potato dextrose agar plate respectively.

RESULTS AND DISCUSSIONS

Isolation, Screening, & Biochemical Characterization

Out of the 14 indigenously isolated *Lactobacillus* spp. only 8 isolates shows inhibitory effect towards test microbial species used in present work. Only two LAB isolates with codes LB-2 and LB-7 out of all 8 LAB isolates showed inhibition towards both the test microbial strains namely *E. coli* (MTCC-1687) and *S. aureus* (MTCC- 737). The bacterial isolates LB-5, LB-11, LB-12 and LB-14 were reported with inhibitory activity against test *E. coli* (MTCC-1687) only whereas the other isolates with codes LB-6 and LB-13 were observed to be positive for inhibitory activity against the tests *S. aureus* (MTCC- 737).

Biochemical Characterization of Antimicrobial *Lactobacillus* spp.

All the bacterial isolates considered under present investigation were reported to be Gram+ve bacillus type of bacteria and also they were endospore –ve when stained with Malachite Green stain. The indigenous *Lactobacillus* isolates showed no reaction in catalase test, IMViC test and starch hydrolysis test as their biochemical character but showed variable response in carbohydrate fermentation tests.

Detection of Isolates secreting Proteinaceus Antimicrobial Component

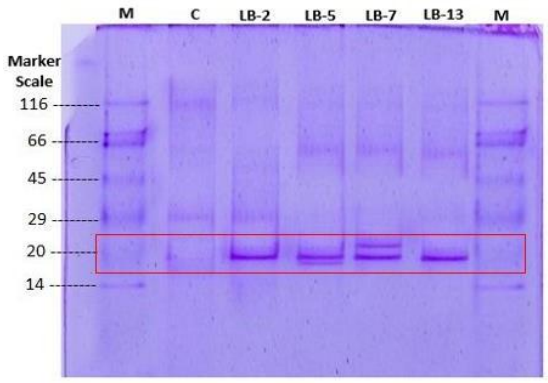
Only 4 indigenous *Lactobacillus* isolates coded as LB-2, LB-5, LB-7 and LB-13 were reported to lose their antimicrobial potential upon treatment with protease enzyme while rest other isolates were discontinued for further experimentation since their antimicrobial activity was retained after protease treatment which might be due to the presence of unknown organicacid.

Extraction of Antimicrobial Protein

The final optimized pH and temperature conditions are pH-7.5 and temperature 42°C at which the amount of antimicrobial protein secreted by different selected *Lactobacillus* isolates.

SDS PAGE analysis of antimicrobial proteins

This section of the current thesis work is dealing with molecular characterization of antimicrobial protein components secreted by the 4 selected isolates coded as LB-2, LB-5, LB-7 and LB-13 in cell free extract during fermentation process at optimize pH and temperature conditions. Upon careful observation in comparative way, the protein samples loaded from LB-2 and LB-13 have 1 single sharp protein band just beyond the level of 20 KD molecular weight marker, while the sample LB-5 and LB-7 were separated into 2 bands at the range of 20 KD molecular weight marker where one band shared the position exactly same compared to the positions of bands from samples LB-2 and LB-13 respectively. But sample LB-5 yielded 1 band beyond the common band while LB-7 yielded 1 band above the common band at the range just above the 20 KD molecular weight marker.



Clear and sharp banding pattern of proteins in sample loaded enclosed in red border box, at the level of 20 KD marker range is indicative of presence of bacteriocin like biological substances

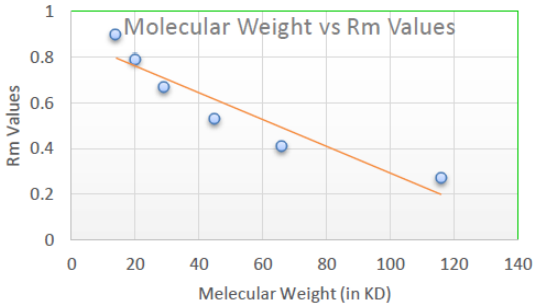
In term of molecular weight assessment the proteinaceus substances with antimicrobial properties obtained from fermentation activity of indigenously isolated *Lactobacillus* species fall in the range of 20 KD in present investigation. It has been quoted in many research articles earlier that the molecular weight of antimicrobial proteins like bacteriocins ranges of2 KD to 37 KD.[11]

The molecular weight of the common band was estimated to be the 17.31 KD in all the samples while the lane *L-4* and *L-5* has an extra band other than the common bands with molecular weights measuring 15.8 KD and 23.36 KD respectively. These molecular weights of the proteins were estimated by comparing the Rm values obtained on software "*Gel Analyzer Ver. 19.1*" with the help of MS-Excel 2013.

Microbiological Evaluation of Juice

The order of the effective preservation activity based on the least score in juice samples can be arranged as FJ>JB-3>JB-1>JB-2>JC+ve>JB-4>JC–ve. This describes that among all the juices samples JB-3 with bacteriocin B-3 produced due the fermentation activity of isolate LB-7 at optimized conditions is supposed to be efficient biopreservative action on juice that could be comparable with the microbial scoring of fresh juice out of the bacteriocins obtained from other lactobacillus isolates considered in present study namely LB-2, LB-5 and LB-13. Even the efficacy of B-1, B-2 and B-3 are observed to be better than the efficacy of 1% benzoic acid while the 4th bacteriocin B-4 from LB-13 isolates lags behind the efficacy of 1%benzoic acid in pineapple juice after 4 weeks of storage.

S.N.	Band from top to bottom	Molecular Weight of Bands	R_m Values of Bands
1.	Band – 1	116 KD	0.27165353
2.	Band – 2	66 KD	0.4055118
3.	Band – 3	45 KD	0.53149605
4.	Band – 4	29 KD	0.6692913
5.	Band – 5	20 KD	0.78740156
6.	Band – 6	14 K	



CONCLUSION

With reference to the objective of our present study we can conclude that protenacious antimicrobial substances produced from the fermentation of some of indigenous *Lactobacillus spp.* can be used as Biopreservative in pineapple juices.

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