DETERMINATION OF BIOPRESERVATIVE EFFECTS OF BACTERIOCIN ON SUGARCANE JUICE ISOLATED FROM LACTIC ACID BACTERIA

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Abstract:- Biopreservation in food and juices, has become a topic of major concern in recent years.The aim of the study was to isolate, identify and characterize bacteriocin producing Lactic acid bacteria (LAB) from different foods products like curd, cheese, tomato & chicken and to exploit their potential as biopreservatives in sugarcane juice. The Lactobacillus strains screened for their bacteriocin producing ability by performing antimicrobial properties against gram positive and gram negative pathogens such as S.aureus, E.coli & P.aeruginosa. Out of ninteen isolates, only isolate no 2, 3, 6, 16 and 18 were selected on the basis of microscopic and biochemical characterization. PCR sequencencing of bacterial isolates shows high similarity with reference isolates of L.plantarum. Sugarcane juice was found to be shown bioprservative efficiency upto 48 hrs using 5% CFS of bacteriocin as a bioprservative. Bacteriocinogenic lactic acid bacteria and their isolated bacteriocins are considered safe additives useful to control the frequent development of pathogens and spoiling microorganisms in foods and juices. These substances in appropriate concentrations may be hurdle factor for increasing the shelf life of minimal processed foods.

Keywords: Bacteriocin, Lactic acid bacteria, Biopreservation.

I. INTRODUCTION:-

Modern technologies in food processing and implementation of food safety standards have reduced the loss due to food spoilage and the related food-borne illness. However, the chemical based preservatives and food additives have their own side effects which emphasize the importance of using natural preservatives.

One of the concerns in food industry is the contamination by pathogens, which are frequent cause of food borne diseases. The microbiological spoilage of foods is due to thebiochemical activity of microorganisms as they grow in food, causing changes in the food's appearance, odor, texture, or taste. Since food borne pathogens do not typically give an organoleptic indication of their presence, the organoleptic changes caused by spoilage microorganism serve as a warning to the consumer that the food could be unsafe for consumption, thus protecting millions of people from food borne illness (1) Lactic Acid Bacteria constitute a group of genus that has the following common features: cocci, rods and a basic composition of DNA below 50 mol% G+ C. They are typically Gram positive, mesophilic, can grow within 5°C to 45 °C under aerobic, anaerobic or microaerobic conditions and are asporogenous. In addition, they are oxidase and catalase negative, cannot reduce nitrate to nitrite and are incapable of producing indole or hydrogen sulphide. This group consists of numerous genera: *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Streptococcus, Enterococcus, Aerococcus, Bifidobacterium and Pediococcus* (2,3,4). Lactic acid producing bacteria (LABs) produce antimicrobial substance Bacteriocins which are ribosomaly synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect (5).

Lactic acid bacteria are used extensively in food processing such as diary, beverage and meat products (6). The most important role of the lactic acid bacteria (LAB) is to inhibit growth of spoilage and pathogenic bacteria in food (7). They reduce the pH in food, low enough to inhibit the growth of most of other microorganisms including common human pathogens, thus increasing the self life of fermented food (8). The bacteriocin application is also included in improvement of safety and quality of the fermented food (9,10). As considering the potentiality as broad spectrum activity and limitation as narrow spectrumactivity of bacteriocin, they could be utilized as an alternative to antibiotics. The aim of the study is to isolate, purify and characterize the bacteriocin and used as biopreservatives in sugarcane juice.

II. MATERIAL AND METHOD:-

Sample collection

Curd, cheese, tomato, and chicken samples were purchased from local market of Lucknow, India for isolation of *Lactobacillusspp.* and stored aseptically at 4^0C.

Isolation and identification of lactobacillus isolates

One gram of each food sample was taken aseptically and homogenized in 10 ml of sterile buffered peptone water (BPW) and incubated at 37 ^{0}C for 24-48 hours. After incubation sample was processed following serial dilution and desired dilutions was spread on MRS media to isolate bacteria. The plates were incubated at 37 ^{0}C 24-48 hours anaerobically.

Screening of *Lactobacillus* isolates on Selective Media

For screening, *Lactobacillus* isolates was inoculated on MRS Vancomycin media and MRS Clindamycin & ciprofloxacin media. Incubated anaerobically in desiccator for 24-48 hrs at 37^0C.

Microscopic and Biochemical Characterization of *Lactobacillus* Isolates

The isolates were identified using standard morphological, microscopic and biochemical characterization. The bacteriocin producing isolates was identified as per Bergey's Manual of Systemic Bacteriology. Gram staining was done to identify the morphology structure and

biochemical test- Catalase, Starch Hydrolysis, Casein Hydrolysis, Methyl Red, Carbohydrate Fermentation Glucose, Lactose, Sucrose, Mannitol |& Sorbitol was performed.

Partial Purification of Bacteriocin

The isolated strain was grown in MRS broth (pH-6.0) and was incubated at 37°C for 48 hours anaerobically. A supernatant that may contain bacteriocin, a cell-free solution was obtained by centrifuging the culture at 10,000 rpm for 10 minutes at 4°C. After centrifugation the supernatant was collected in a fresh sterile tube. CFS was purified with 0.5 micron filter. The CFS was adjusted to pH-6.0 using 1N NaOH.

Estimation of Total Protein

The amount of protein in the CFS was estimated by Lowry's method. Absorbance at 660 nm was measured in spectrophotometer and estimated the amount of protein present in the given sample.

Determination of Antimicrobial Properties of Bacteriocin

The strains of bacteria *S. aureus, P.aeruginosa* & *E. coli* were provided by Rapture Biotech, Lucknow for this particular study. The inhibitory activity of CFS protein was screened by agar well diffusion assay on MRS media. Four wells were cut and in one well put 50ul CFS of curd and in second well 100ul of curd in same way another two well filled with 50ul and 100ul of CFS of cheese were added. The plates incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Biopreservative Efficiency of Bacteriocin In Sugarcane Juice:

Sugarcane juice was obtained aseptically in the laboratory. Then pasteurize the juice at 80^{o} C for 5 min and cooled immediately. Check the different parameters of juice like pH, acidity and microbial evaluation (control). 5% bacteriocin was added in juice and incubate for 72 hrs. On 0 hr, 24 hrs, 48 hrs and 72 hrs juice were check at different parameter of pH , total acidity and microbial evaluation. The result was recorded and compare with control.

III. RESULT:-

Isolation and Identification of Bacterial Strain

In the present study, a total of 4 different food samples like curd, cheese, chicken and tomato were collected to isolate *Lactobacillus* species. On the basis of gram staining, all isolates were gram positive rods. Various biochemical test were performed to identify the LAB strains. Based on these studies, the test isolates showed the absence of catalase, and casein hydrolysis test showed positive. A total of 19 isolates were characterized and identified to species level utilizing carbohydrate fermentation test, biochemical and physiological characteristics as indicated in table 1 and 2.

Table-1 Morphological characterization of the isolated bacterial strain

Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Pigment	White creamy
Opacity	Opaque
Grams reaction	Positive
Cell shape	Cocci in chains, rods
Spore (s)	Negative
Motility	Non motile

Table2-Biochemical characterization of the isolated bacterial strain

Isolates no	MRS	MRS+V	MRS+Clindamycin+ciprofloxacin	Catalase	Casein	Starch	MR	G	L	Su	M	So
							Carbohydrate tests					
1	+	-	+	+	+	+	+	+	+*	+	+	+
2	+	+	+	-	+	+	+	+	-	+	+	+
3	+	+	-	-	+	+	+	+	-	+	+	+
4	+	+	-	+	+	+	+	+	+*	+	+	+
5	+	+	+	+	+	+	+					

6	+	+	+	-	+	+	+	+	+	+	+	+
7	+	+	+	-	+	+	+	+	+	+	+	+
8	+	+	+	-	-	+	+					
9	+	+	+	-	+	+	+	+	+	+	+	
10	+	+	-	-	+	-	+	+	+	+	+	
11	+	+	+	-	+	-	+	+				
12	+	+	+	-	+	-	+	+	+	+	+*	
13	+	+	+	+	-	-	+	+				
14	+	+	+	-	+	-	+	+				
15	+	+	+	-	+	+	+	+				
16	+	+	+	-	+	-	+	+	+	+	+	+
17	+	+	+	+	+	+	+					
18	+	+	+	-	-	-	+	+	+	+	+	-
19	+	+	+	-	-	-	+					

(G-Glucose , **L**- Lactose, **S**-Sucrose,**M**-Mannitol and **So**- Sorbitol)

Quantitative Estimation of Protein using Lowry, et al. Method

The quantitative estimation of protein was studied using Lowry, et al. method. These studies showed that all the LAB isolates showed the presence of protein content and the quantitative estimation was carried out using UV- spectrophotometer with the absorbance at 660nm and the results are shown in table 3. From 18 isolates, three isolates curd 1, curd 2 and cheese 16 of higher protein concentration were selected and checked further at different time point at 24hrs, 48 hrs and 72 hrs for the optimum time for the maximum amount of bacteriocin production which indicated in table 4.

Table3-Protein concentration of the isolated bacterial strain

Isolates of lactobacillus	**Concentration (ug/ml)**
1	71
2	58
3	42
4	52
6	40
7	48
9	36
10	20
12	10
16	40
18	30

Table4-Protein concentration of the selected bacterial strain at different time interval

Isolates of lactobacillus	**Concentration (ug/ml)**
24 hours	
1	116
2	94
16	50

48 hours	
1	108
2	106
16	102
72 hours	
1	90
2	92
16	91

Determination of antimicrobial properties of bacteriocin

Two isolates curd 2 and cheese16 produce high concentration of protein at 48 hrs were selected and antimicrobial activity was performed. The result shown in table 5indicates that the inhibitory metabolites produced by isolated *Lactobacillus* species were extracellular& diffusible and capable to inhibit the growth of pathogenic bacteria. Maximum zone of inhibition observed at 100ul of bacteriocin against *S.aureus* and *Pseudomonas.*

(a) **(b)** **(c)**

Fig1- Inhibition zone of isolates of curd and cheese against a)*E.coli, b)S.aureus* and c)*P.aeruginosa* pathogen

Table5-Zone of Inhibition of *Lactobacillus* isolates

Microorganism	**Zone of Inhibition (mm)**			
	CURD 2		**CHEESE 18**	
	50ulCFS	**100ul CFS**	**50ulCFS**	**100ul CFS**
Escherichia coli	3	6	4	7
Staphylococcus aureus	3	6	2	9
Pseudomonas spp	2	9	2	8

Biopreservative efficiency of bacteriocin in sugarcane juice

The microbial population, pH and titrable acidity were measured in sugarcane juice sample at different time interval at 0 hrs(control) and after addition of bacteriocin at 0 hrs, 24 hrs, 48 hrs and 72 hrs .They reduced the acidity and drastically decreased the microbial count till 48 hrs incubation revealed that bacterioin effective in minimal processed food.

(a) **(b)** **(c)** **(d)** **(e)**

Fig 2-Biopreservative efficiency of bacteriocin in sugarcane juice a) control b) with CFS at 0hs c) 24 hrs d) 48 hrs and e) 72 hrs

Table6-Biopresevative efficiency of bacteriocin at different parameter

Parameters	**Sugarcane juice**	**0hrs Bacteriocin**	**24hrs**	**48hrs**	**72hrs**
Ph	6.5	7	6.57	7	7
Acidity	1.4	0.7	1.05	0.63	1.26
Cfu/ml	$6.64*10^3$	$6*10^4$	$3*10^4$	$2.9*10^5$	$3.4*10^6$

Amplification and sequencing of 16S rRNA gene

16S rRNA was amplified using universal Bacterial primers:

Primer :

27F - AGA GTT TGA TCM TGG CTC AG
1492R - CGG TTA CCT TGT TAC GAC TT

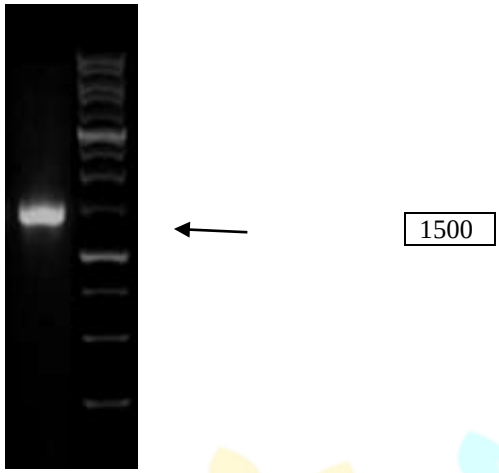

Fig- 3 Amplified DNA

Sequencing

The PCR product was purified and the product is sequenced by Sanger's method of DNA sequencing. The sequencing obtained were assembled and submitted to NCBI data base for analysis.The amplified 16S rRNA was identified as *Lactobacillus* plantarum.

>curd2
AGAGTTTGATCCTGGCTCAGCAAACCGCCGTAACTTTCAGAATAATCTAACGGTAAATCACTTCGACCGCAAACCGTACACCA
ACGCAACTCTGGGGCCACCCCAAACGGTTGCAGTAACTGAATTTCGACCACGTTAGCCACCAACGCGGGGGCCACATCTTGA
TCAATCAGTTGCAGCGCACTGGTCAACTGTTGGTACCACCGCCCCACCGGTTGATTATCTGGAAAAGCGACGTCCATTAAGTT
CATAACGTACGTGGCATAAGCGTTCAAAGCAATGTCTTGACTAATATGTTCAAGATACTGTACTGACTTGACACTATTAATAT
ACGAGAGACCTTGATCACGTAAATCACCAACGTATTCCCCCATCGTGAACGGCAATAACTCCGCCGCCATTTTGAAGCCACGC
CGACGCGCACCGCGGATAAAAACCATCTTCTTGCCATACTCGGCAGTTAAAAACTTGATTAGCATATCGCGTTCCCGGTAGTC
CCGGCGAAAGAGCAAAATGCCGTTAAAATTCGTGACCATTTGTTGGGCCATCCGGATCACCTAGCGATTCTAATAGTCATCTT
GCCGATAGCCATAGCTGGCTAGCAAGTCCTGACGGTCCTTCCAGTTCTCTTGGACTTTTACCCAGAGCTCAAGGTAGACCTTA
TCCCCAAGTAAGTGTTCAATATCACGCCGAGCTAACGAGCCAATCTTCTTGAGCATGCTACCACCCTTACCGATGATGATGCC
CTTTTGCGAAGACCGTTCAATAATAATCGTGGCCTGAACGTGAATTTTTTCTTCATCTTGACGTTTGATCGAATCAATCACGAC
CGCCGTTGAATGGGGAACTTCTTGACGAGTCAATTCAAGAACCTTTTCACGAATCAATTCAGAAATGATAAACCGCTCTGGAT
GATCCGTAATTTGGTCGGACGGATAATACTGCGGTCCTTCCGGCAATTGGCCCTTCAAGGTCGTCAGTAGTTCATCAACGTTA
TTTCCTTCAAGCGCGGAAATTGGATAAACTTCCTTCCACGGCAAAGCGTCTTTATACTGATCCATAATCTCCAAGAGATGATC
TGGATGGACTTGATCAATTTTATTAATCACTAAATAAATCGGTTGTTTAACCGTTTTAAGACGATCGATAATAAAGTTATCCCC
GGCACCACGCCGTTCGTCCGCATTAATCATGAATAGTACGGCATCCACTTCACCCAAAGTTGACAAGGCGGACTTGACCATA
AAGTCGTAACAAGGTAACCG

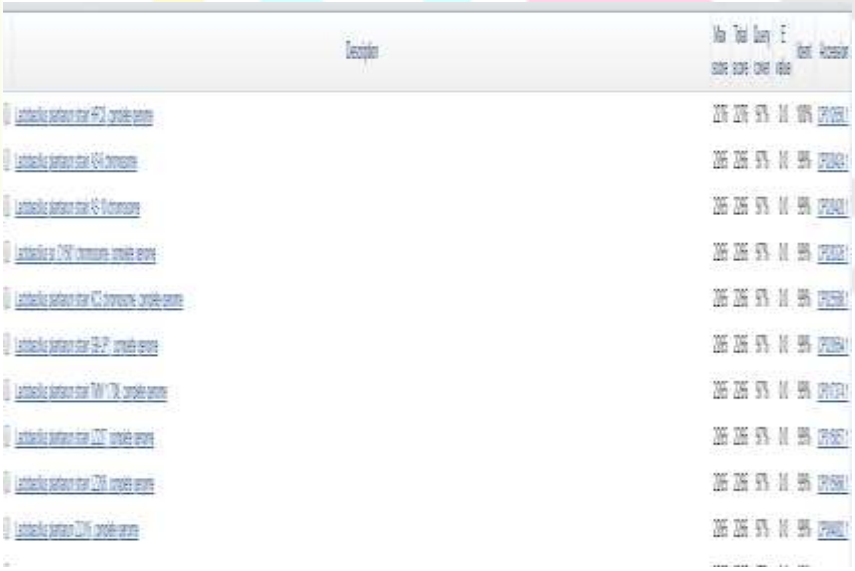

Fig- 4The Top 10 matches for the sequence derived from query

Phylogenetic Analysis

The evolutionary history was inferred using the UPGMA method.

Fig- 5 The phylogenetic position of the query among neighbouring species.

IV. DISCUSSION:-

The present investigation was carried out to access the isolation and characterization of bacteriocin producing LAB strains from different foods and was screened for bacteriocin production, used as bio preservation in sugarcane juice. Isolated LAB from various food samples like yogurt, meat and probiotic drink (11). In present study, ninteen bacterial strains were successfully isolated from different food samples like curd, cheese, chicken and tomato were collected from local areas in Lucknow. Characterization of selected isolates by using morphological characters, cultural characteristics was done as described in Bergy's Manual of Systematic Bacteriology. Microscopic analyses showed that allwere gram positive, rod shaped bacteria. Based on the morphological, biochemical and molecular analysis isolates were identified as *L. plantarum*.

All the isolates were screened for bacteriocin production by inhibiting the growth of pathogenic microbes and found active against *E.coli, S.aureus and P.aeruginosa*. (12) stated that the bacteriocin produced from *L.plantarum* have been found to be inhibitory towards closely related LAB, particularly the mesophilic and thermophilic lactobacilli.

In this present study Sugarcane juice was fond to be shown bioprservative efficiency upto 48 hrs using 5 % CFS of bacteriocin as a bioprservative. Results compared with (13) used a lime juice and lemon juice for bio preservative efficacy of bacteriocins of all four isolates was observed in sweet lime and lemon juices. The minimum inhibitory concentration (MIC) of bacteriocins for all the four isolates was found to be 100 ul in sweet lime. Bioprservative effect on fish and apple juice, the partially purified bacteriocin from isolates of *Lactobacillus* tested for preservative effect maximum reduction was observed in fish compared to apple juice at the concentration of 5 % bacteriocin (in control without bacteriocin no reduction of population was observed). The results further revealed that microbial count drastically decreased in apple juice and in fish (14).

V. CONCLUSION:-

The present study revealed that isolation, identification, and characterization of *Lactobacillus* strains and production of bacteriocin using *Lactobacillus* species isolated from different dairy products and chicken and its bio preservatives effect on refrigerated food products. Out of ninteen isolates, only isolate no 2, 3, 6, 16 and 18 were selected on the basis of microscopic and biochemical characterization. *Lactobacillus sp.* isolated from curd and cheese posses a wide spectrum of inhibitory activity against *E.coli, P.aeruginos* and *S.aureus*. In the presence of bacteriocin there was a reduction in the microbial population in sugarcane juice during storage at room temperature. Therefore, bacteriocins from these bacteria can be used for biopreservation of citrus fruit juices. Therefore, the bacteriocin produced from *Lactobacillus spp.* can be treated as a potential bio preservative for replacing chemical food preservatives which have side effects on the foods which are to be preserved as well as on the health of the consumer.

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