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Bacteriocins for control of undesirable organisms in the preservation of processed frozen foods

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ABSTRACT

Production of antagonistic substances is highly important factor in microbial ecology. Among many different substances known to play a role in bacterial interactions, bacteriocins are the most specific and efficient antagonists. In recent years bacteriocins produced by Lactic Acid Bacteria (LAB) and Non Lactic Acid Bacteria (Non-LAB) have attracted great attention due to their application in food processing and preservation to control undesirable organisms. The present study was carried out where fish samples (most common) were collected from local market of Pune and fish processing and marketing units from konkan region of Maharashtra (Ratnagiri). The fish samples collected for the present work included L jacket, L-Sardine, Ribbon fish, Itoyori, Croaker and Lizard fish and were subjected to isolate bacterial pathogens like Salmonella Spp, Escherichia coli, Bacillus cereus, Staphylococcus aureus and Vibrio parahaemolyticus using Wilson-Blair's-agar, MacConkey'sagar (including Hicrome agar), Nitrate agar, Mannitol Salt agar and Vibrio agar media, respectively. In present work efforts have been taken for screening, isolation, purification and confirmation of bacteriocin from lactic acid bacterial (LAB) and other bacterial strains (Non-LAB). During the study 20 lactic acid bacterial strains (LAB) and 74 (NON-LAB) were randomly isolated from 7 food and vegetable samples including Raw milk, Curd, Butter milk, whey, Cabbage, rose flowers, sewage and soil samples (garden, rhizospheric and fertile soils).
The 16 out of 20 LAB and 32 out of 74 (Non-LAB) isolates showed zones of growth inhibition against one or more of the test pathogens (Escherichia coli, Salmonella sp., Staphylococcus aureus, Bacillus cereus, Vibrio parahaemolyticus). The three promising isolates were studied for morphological, cultural, biochemical properties and identified as Pediococcus pentosaceus strain –I, Pediococcus pentosaceus strain –II and Bacillus aryabhattai by 16s-rRNA gene sequencing method and further G+C content and phylogenetic trees were prepared .
Key words: *Bacteriocins, lactic and Non Lactics, Food preservation*

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INTRODUCTION

Bacteriocins are proteinaceous antibiotic like substances produced by bacteria which can inhibit the growth of similar or closely related bacteria [1]. Globally, in general and in India particularly, huge quantities of foods are spoiled every year especially by microorganisms and cause incalculable losses to producers, handlers, stockiest and industrialists. Many times packaged foods are unapproved and hence rejected as they harbor pathogenic and other microorganisms [2].
Amongst non-vegetarian foods, fish foods include various alive and preserved fishes, their products. The preserved fish and their products include loose and packaged salted, dried and frozen. The frozen fishes and their products are mainly export grade and in very little quantity they are used in local markets [3].
Fish is very valuable source of food for mankind. The flesh of fish is composed of high quality proteins, fats, minerals, vitamins, iodine, phosphorus and it is easily digestible due to low percentage of connective tissues. India has a continental sea shelf of about 59.7 million hectares of which only 20% is explored. India is one of ten largest fish producing nations, but has a share of little over 3% of total fish production of the world. The seafood processing industries were established during world war-II [3]. Although thousands of fish species are known to man only few are commonly used e.g. Tuna, Mackerel, Croaker, Ribbon fish, Basa, Salman, Tilapia, Clams, Shrimps, Oysters, and Lobsters. Now a days Indian marine products are exported to over 70 countries in the world where South East Asian countries are largest buyers followed by European Union .e.g. USA, Japan, Australia, France, Italy, Singapore, Spain, Portugal, Belgium, Greece, Taiwan etc) .

During the financial year 2012-13, the export of marine products was 928215 tons (3511.67 million USD) [3]. During the financial year 2017-18, the India's shipment of 1317244 MT of sea food earned 7.08 billion USD and registered growth of 21.35% as compared to previous years [4].
In present work efforts have been taken for screening of bacteriocins from lactic acid bacterial (LAB) and other bacterial isolates (Non-LAB) and study of their broad spectrum antagonistic potential against common bacterial pathogens found in the processed frozen fishes e.g. pathogenic *Escherichia coli, Salmonella sp., Staphylococcus aureus, Bacillus cereus* and *Vibrio parahaemolyticus* [5,6].

MATERIAL AND METHODS

Collection of Processed Frozen fish samples (Table-1) :

Table-1: Details of fish samples used in the present work:

Sr. No.	Local Name of the fish	Number of samples collected	Packet Size (g)	Scientific name of the fish
1	L-sardine	5	100	*Sardinella longiceps*
2	Ribbon fish	5	100	*Lepturacanthus savali*
3	Lizard fish	5	100	*Synodus indicus*
4	Itoyori fish	5	100	*Nemipterus peroni*
5	Croaker	5	100	*Johnius spp*
6	L-Jacket fish	5	100	*Oligoplites saurus*

Locally processed frozen fish samples were collected from Pune fish market and from Fishery from Ratnagiri, India.

Enrichment and isolation and identification of pathogens from processed frozen fish samples:

The pathogens which are commonly reported in the processed frozen fishes and using standard enrichment and isolation media were isolated from processed frozen fishes and identified with previous references [7,8] (Table-2).

Table-2: Test pathogenic bacteria, media and conditions used for their isolation and further cultivation and identification:

Sr. No.	Gram nature of organism	Pathogenic test bacterium	Media used for Cultivation*	Incubation temperature (ºC)
1	Gram positive	*Staphylococcus aureus* (Coagulase positive)	Mineral Salt medium	37 ºC
		Bacillus cereus	Nutrient agar medium	37 ºC
2	Gram negative	*Salmonella paratyphi--B*	Tetrathionate broth, Wilson-Blair media	37 ºC
		Vibrio parahemolyticus	Alkaline Peptone water, Vibrio-medium	37 ºC
		Pathogenic *E. coli*	MacConkey's agar , Hichrome media	37 ºC

Isolation of LAB and NONLAB from different natural sources:

The following enrichment media and isolation media were used to isolate LAB and NONLAB using spread plate technique at 30^0C for 48-72-h under microaerophilic conditions from samples of natural source material: [7,8]

Natural source material used:

Raw milk, Curd, Butter milk, whey, Cabbage, rose flowers, sewage and soil samples (garden, rhizospheric and fertile soils)

Media used:

a) Enrichment media: [7,8]	For LAB:	deMan Rogosa Sharpe (MRS) broth
	For Non LAB:	Nutrient broth
b) Isolation media used: [7,8]	For LAB:	MRS agar
	For Non LAB:	Nutrient agar and MRS agar

Screening of Lactic Acid bacterial (LAB) and Non Lactic Acid bacterial (Non-LAB) isolates for their inhibitory activity against test bacterial pathogens:

Isolates were screened for their antibacterial potential (inhibitory activity) against pathogenic test bacteria- *Escherichia coli*, *Staphylococcus aureus, Salmonella paratyphi-B, Bacillus cereus and Vibrio parahemolyticus* with paper disc agar diffusion method [8]. The main focus of screening was on selection of only those isolates showing broad spectrum of inhibitory activity. Thus from screening studies, **three promising isolates** were selected i.e. from LAB, two isolates **L 19 and L 20** while from NonLab the one isolate **ISO6RNFA.**

Confirmation of bacteriocin nature in the selected promising antagonistic (showing inhibitory activity) bacterial isolates:

Bacteriocin nature confirmation was done by using following tests as guideline**:** Solvent extraction of bacteriocins: the loss of inhibitory activity (Inactivation) of the extract occurs upon trypsin treatment, inactivation (loss of Inhibition activity) at high temperatures and enzymes like amylase and lipase treatment. [9,10].

Identification of the selected three promising bacteriocinogenic isolates :

The selected three promising bacteriocinogenic isolates were identified by 16-S rRNA gene sequencing method, blast studies of gene sequences and phylogenic trees preparation. The sequences and identity was submitted to NCBI and accession numbers to these three isolates were obtained.

RESULTS AND DISCUSSIONS:

Collection of Processed Frozen fish samples:

The fish samples collected for the present work included L jacket, L-Sardine, Ribbon fish, Itoyori, Croaker and Lizard fish and were subjected to isolate following bacterial pathogens (Table-3):

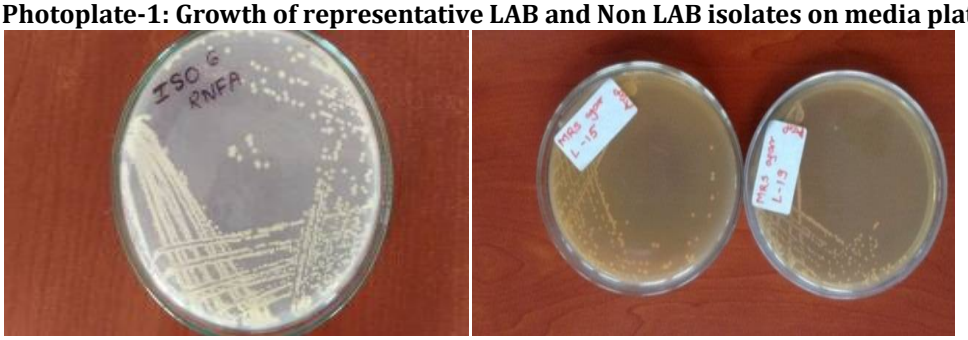
Table-3: Test pathogenic bacteria isolated and identified:

Sr. No.	Gram nature of organism	Pathogenic test bacteria isolated from fish samples
1	Gram positive	*Staphylococcus aureus* (Coagulase positive)
		Bacillus cereus
2	Gram negative	*Salmonella paratyphi--B*
		Vibrio parahemolyticus
		Pathogenic *E. coli*

Isolation of LAB and NonLAB from different natural sources:

Total 20 LAB and 74 NONLAB isolates were obtained by using enrichment media and isolation media using spread plate technique at 30^0C for 48-72-h under microaerophilic conditions and from samples of natural source material (Photoplate-1). [7,8]

Photoplate-1: Growth of representative LAB and Non LAB isolates on media plates:



LAB isolates on MRS agar **NONLAB isolates on Nutrient agar**

Many workers have isolated LAB and other bacteria (NonLAB) from similar sources like those we used in the present studies. The presence of lactic acid bacteria in flowers was first reported very early [11], found in a national park in the United States. The *Lactobacillus floricola sp.* was isolated from mountain flowers [12]. Three lactic acid bacterial strains were isolated from wheat sour dough samples [13] while 08 strains of lactic acid bacteria from Burkina faso fermented milk samples [14]; Previous studies reported isolation of 10 lactic acid bacteria from natural lactic acid fermentation of vegetables, of which one of the isolates i.e. *Lactobacillus spp.* CA44, showed maximum antimicrobial activity (bacteriocin activity) against *Escherishia coli, Staphylococcus aureus*, and *Bacillus cereus.* [9]

Antibiogram spectrum study (Antagonistic / Inhibitory activity) of LAB and Non LAB isolates against Standard pathogenic test bacteria and selection of promising antagonistic bacterial isolates:

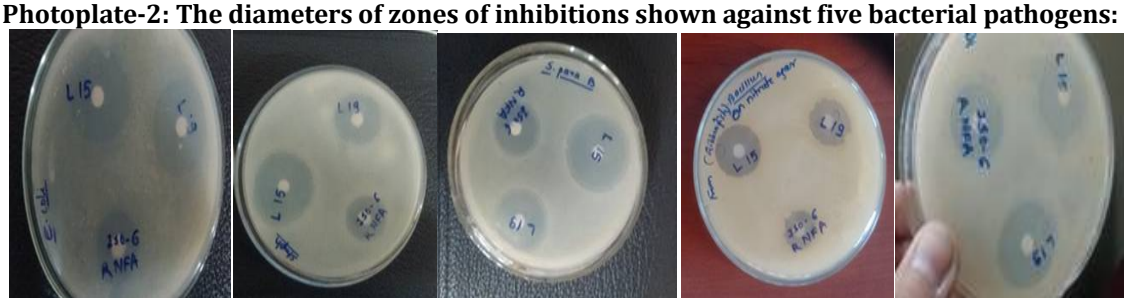
Screening of Lactic Acid bacterial (LAB) and Non Lactic Acid bacterial (Non-LAB) isolates for their inhibitory activity against test bacterial pathogens:

Isolates were screened for their antibacterial potential (inhibitory activity) against pathogenic test bacteria- *Escherichia coli*, *Staphylococcus aureus, Salmonella paratyphi-B, Bacillus cereus and Vibrio parahemolyticus* with paper disc agar diffusion method [8]. The main focus of screening was on selection of only those isolates showing broad spectrum of inhibitory activity. The total of 20 LAB and 74 Non LAB isolates were screened for activity using Paper disc agar diffusion method.

Out of 20 LAB isolates only two isolates viz. L15 and L19 showed inhibitory activity against all above five pathogens tested with average diameters of inhibitory zones in the range of 25-36 mm. Hence L15 and

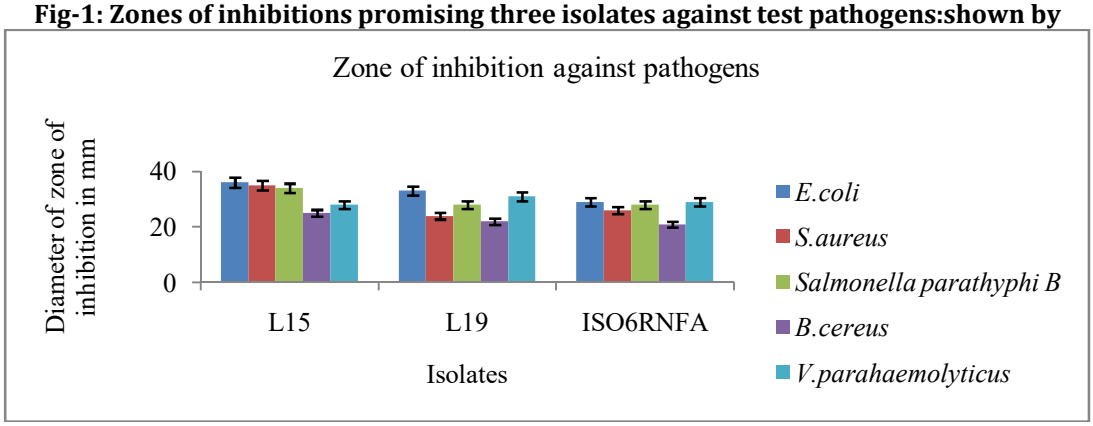
L19 were selected for further study as promising isolates amongst the lot. Out of 74 Non LAB isolates the one isolate viz. ISO6RNFA (NL-51) showed the best spectrum of inhibitory activity with range of 20-30 mm of inhibitory zones against all above test pathogens. Hence this isolate -ISO6RNFA was selected along with L15 and L19 isolates for further study as promising isolates amongst the lot. Thus from screening studies, three promising isolates were selected i.e. from LAB, two isolates L 19 and L 20 while from NonLab the isolate ISO6RNFA (Photoplate-2 and Fig-1).

Photoplate-2: The diameters of zones of inhibitions shown against five bacterial pathogens:



E.coli *S. aureus* *S. paratyphi-B* *B. cereus* *V. parahaemolyticus*

Fig-1: Zones of inhibitions promising three isolates against test pathogens:shown by



All the three isolates have shown good zones of inhibition against all the five bacterial pathogens isolated from frozen processed fishes and they ranged from 20 mm minimum to 36 mm maximum. The all the three isolates were broad spectrum in nature but extent of inhibitions was found decreased from isolates L15, L19 to ISO6RNFA (Fig 1).

Three bacterial isolates i.e. L15, L19 and ISO6RNFA showed broad spectrum of inhibitory (antagonistic) activity with all the five test pathogens used. With *E. coli, S. aureus, Salmonella paratyphi-B, B.cereus and V. parahaemolyticus.*

The 340 lactic acid bacterial isolates from food samples were detected [15]. Out of 359 lactic acid bacterial isolates only 37 were bacteriocinogenic [16], while the previous studies were isolated and reported that the variations with regards to bacteriocinogenic lactic isolates may depend on the types of samples used for isolation, sample sources and isolates. [17]

Confirmation of bacteriocin nature in the selected promising antagonistic (showing inhibitory activity) bacterial isolates:

Bacteriocin nature was confirmed by using following tests as guideline**:** Solvent extraction of bacteriocins: the loss of inhibitory activity (Inactivation) of the extract occurs upon trypsin treatment, inactivation (loss of Inhibition activity) at high temperatures and enzymes like amylase and lipase treatment [9,10] and found that the antagonistic agents of all three selected promising isolates were extracted with chloroform, they were inactivated upon exposure to trypsin, amylase, lipase, and high temperatures and as these properties are of bacteriocin, here bacteriocin nature of antagonistic agents of three isolates was confirmed.

Selected promising isolates: (showing broad spectrum of inhibition of test bacterial pathogens): (genetically identified-16s rRNA gene sequencing):

Table1: NCBI accession numbers of promising isolates:

Sr. No.	Isolate code	Accession number obtained from NCBI	Identified bacteria as
1	L15	MG551544.1	*Pediococcus pentosaceus* strain –I
2	L19	MG551545.1	*Pediococcus pentosaceus* strain –II
3	ISO 6 R-NFA	MG551546.1	*Bacillus aryabhattai*

CONCLUSIONS

1) Three bacterial isolates i.e. L15, L19 and ISO6RNFA showed broad spectrum bacteriocin activity against all the five test pathogens which are commonly reported in processed frozen fishes e.g. pathogenic *E. coli, S. aureus, Salmonella paratyphi-B, B. cereus and V. parahaemolyticus.*

2) The three bacteriocinogenic bacterial isolates were genetically identified as:

L15	MG551544.1	*Pediococcus pentosaceus* strain –I
L19	MG551545.1	*Pediococcus pentosaceus* strain –II
ISO 6 R-NFA	MG551546.1	*Bacillus aryabhattai*

3) The bacteriocins of the three bacterial isolates can be produced commercially and used to control common bacterial pathogenic bacteria found in the processed frozen fishes.

4) The bacteriocins of above three isolates can be tested by preparing consortia.

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