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Research Article

NATIVE BACTERIOCINS AND ITS KILLING ABILITY: A PROMISING APPROACH OF FISH BIOPRESERVATION

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ABSTRACT

Fish was assessed for bacteriocin producing LAB [Lactic Acid Bacteria] like bacteria. LAB was screened from intestine of *Oreochromis niloticus* using Man, Rogosa, and Sharpe (MRS) Lactobacilli agar at 35°C for 48 h. Antagonistic aspects of bacteriocin were studied by agar well diffusion method against *Listeria monocytogenes* (ATCC 657, ATCC= American Tissue Type Cell Culture) *Aeromonas hydrophila* (ATCC 646) and *Pseudomonas aeruginosa* (ATCC 1688). Bacteriocin substantiality was evaluated at 90°C temperature, pH 9 and in presence of different enzymes. Thermostable and thermolabile bacteriocins, proteinaceous in nature, capable to inhibit *Listeria* and *Aeromonas* victoriously were revealed from fish. Most of the cell associated antimicrobials were reported to be effective against *Aeromonas* and *Pseudomonas*. Acidic antimicrobials were functional against *Aeromonas* and *Pseudomonas*, whereas hydrogen peroxide as inhibitory compound was successful only against *Pseudomonas*. Maximum resistances to LAB bacteriocins were communicated by *Pseudomonas* followed by *Aeromonas* and least by *Listeria*. Intrinsic Pediocin like anti-listeria products and undefined thermolabile anti-aeromonas and anti listeria products obtained from research work could be easily used for biopreservation of fish with lest consequences.

Keywords: Bacteriocins, Biopreservation, Fish, LAB.

1. INTRODUCTION

Fish spoilage is an utmost issue during trading and transportation of fish [1]. This is truly due to physical, chemical and microbiological mans [1]. Copious amount of protein and unsaturated fatty acids are liable for degradation of fish muscle condition during storage [1]. Endogenous proteases, by hydrolyzing protein, play an important role in worsening the state of fish muscle [2]. Deterioration is further augmented by microorganism's activities [3]. Thus, some initiatives must be taken to lengthen the shelf life of fish during storage. Several inventive sustaining methods are practiced to get safe products. Traditional preservation techniques not only encompass toxicity of the chemical preservatives but also interfere with sensory and nutritional qualities of fish [4]. Besides, impedance of some microorganisms to most frequently used preservatives has made the situation more difficult for fish industry [5]. Moreover, elevated requirement for secure and minimum additives treated foods favors natural products, which do not harm consumers or the products [1]. Therefore helpful microorganisms and their products are exploited to upgrade quality and microbiological safety of fish food [6]. Although in maximum cases, antimicrobials with antilisterial properties are mainly considered as preservatives ignoring their ability to control other spoilage and pathogenic flora [7, 8]. *Listeria monocytogens* with its ability to grow at storage temperature is definitely a severe threat for preserved fish [9]. Besides *Listeria*, there are many other microbes responsible for fish spoilage. *Aeromonas hydrophila*, ubiquitous in nature [10], is a hazardous contaminant of fish mainly cross contaminates products during storage. *Pseudomonas* group with transitory generation time and increased competence to exploit non protein nitrogenous (NPN) substances spoiled muscles of fish in ice [11].

Bacteriocinogenic properties of lactic acid bacteria (LAB) and their certified use in conventional fermented products make them alluring as biopreservatives [12]. LAB being intrinsic to fish, its antimicrobials would give rise to least consequences in fish [13-16].

Present research thus engrossed on inhibitory compounds of LAB with special priority to bacteriocins which are protein in nature. The study involved inspection of fish for LAB bacteriocins with anti-listeria, anti-aeromonas and anti-pseudomonas potentiality.

2. MATERIAL AND METHODS

2.1. Preparation Inoculum from Fish

Oreochromis niloticus were indiscriminately chosen from the fish market at each sampling time (bimonthly from August 2018 to August 2019). Fish were delivered to the laboratory in aseptic condition. Each fish was then cleaned with deionized water and the surface was sterilized by ethyl alcohol. Each fish was then anatomized, and gut was weighed aseptically for microbial investigation. Tissue was homogenized in autoclaved phosphate- buffered saline [PBS; pH 7.2] to obtain a (wt/vol) suspension of fish [17].

2.2. Isolation and Identification of LAB like Isolates from Fish

The fish homogenates were plated on Man, Rogosa, and Sharpe (MRS) Lactobacilli agar and incubated at 35°C for 48 h under anaerobic condition in an anaerobic gas chamber. A total of 7 distinct colonies were selected at random from the cultured plates and sub cultured several times to obtain pure isolates. Pure Cultures were stored at -20°C in MRS Broth supplemented with 20% glycerol [18].

Tests were performed to determine colony characteristics, morphology of isolates, Gram stain reaction, oxidase test, catalase test. The tests were carried out on each isolate according to the procedures described in literature [19].

2.3. Culture of Indicator Bacteria

Listeria monocytogenes (ATCC 657) *Aeromonas hydrophila* (ATCC 646) and *Pseudomonas aeruginosa* (ATCC 1688) was received from American Tissue Type Culture Collection (ATCC), Hi Media Laboratories Ltd., Mumbai, India. The lyophilized pellet of each individual indicator bacteria obtained in KWIK-STIK and inoculated on non selective media like nutrient agar plate and incubated at 35°C for 24 h. Their growth was additionally confirmed on corresponding selective agar *i.e. Listeria* Selective Agar Base with *Listeria* Selective Supplement for *Listeria* and Glutamate Starch Phenol Red Agar Base for diagnosis of *Aeromonas* and *Pseudomonas*.

2.4. Study of Anti-listeria, Anti-aeromonas and Anti-pseudomonas Inhibition of LAB isolates

Inhibitory operations of LAB like isolates against *Listeria monocytogenes* (ATCC 657), *Aeromonas hydrophila* (ATCC 646) and *Pseudomonas aeruginosa* (ATCC 1688) were assessed by agar well diffusion method [20, 21]. 50µl (10^9 CFU/ml, CFU=colony forming unit) of pathogenic and spoilage flora [22] were grown on nutrient agar plates. Nutrient agar palates were then pierced with wells of 0.5mm diameter. 50µl [23] of LAB like bacteria consist of 10^9 CFU/ml [22] from MRS broth cultures were poured into the well and then incubated at 35°C for 24 h.

2.5. Screening of Inhibitory Compounds from CFS (cell-free supernatant) and its Different forms

2.5.1. Antagonistic Activity of Filtered CFS

MRS Broth cultures (incubated for 48 h) of LAB like bacteria were centrifuged at 8000 g for 10 min at 4°C [24] to obtain crude cell-free supernatant (CFS) by separating cells from the growth medium.

Unprocessed supernatant was then filtered [24] through syringe-driven filter membranes (0.20 mm pore size) to eliminate any remaining bacterial cell. Filtered CFS was then tested for antibacterial activity through agar well diffusion method. 50 µl of cell free supernatants [23] were then applied in wells on nutrient agar plates which were earlier overspread with 50 µl of indicator strain. The nutrient agar plates were incubated for 24 h at 35°C.

2.5.2. Elimination of Organic acid from CFS

Filtered CFS was maintained to pH 9 utilizing 1M NaOH solution [25]. Inhibition of alkaline CFS (ACFS) was then studied against *Listeria monocytogenes* (ATCC 657) *Aeromonas hydrophila* (ATCC 646) and *Pseudomonas aeruginosa* (ATCC 1688) by agar well diffusion method.

2.5.3. Exclusion of H_2O_2 from CFS

Alkaline CFS was managed with catalase (ACCFS) [24] and evaluated again for antibacterial activity.

2.5.4. Examination of Thermal stability of CFS

ACCFS was heated at 90°C for 10min [22], and then inhibition of that CFS (ACHCFS) was checked against the indicator strain by agar well diffusion method.

2.5.5. Effect of Administration of Enzymes on CFS

Reactivity of refined cell free supernatants was examined against proteinase K (Hi Media, India) and trypsin (Hi Media, India) [18]. Inhibitions by enzyme

treated CFS were then determined. The diameters of the inhibition zones were measured [26].

3. RESULTS AND DISCUSSION

Seven specific LAB like isolates were evaluated for inhibitory activities against *Listeria monocytogenes* (ATCC 657) *Aeromonas hydrophila* (ATCC 646) and *Pseudomonas aeruginosa* (ATCC 1688) (Table 1-3). LAB like isolates were picked up from MRS agar plate on the basis of physiological and biochemical characteristics. Round and white to cream coloured colonies with Gram-positive, rod shaped, catalase and oxidase positive were mainly considered. All the isolates developed clean inhibitory zones of different diameters during their bacteriocinogenic activities in agar well diffusion method [Figure 1 a,b,c]. This undoubtedly proves synthesis of antimicrobials as the reason of inhibition rejecting competitive exclusion.

LAB like isolates had ample proficiency against Gram positive *Listeria* as well as Gram negative *Aeromonas* (Table 1, 2). In addition, 57% of the tested isolates (Table 3) were operative against Gram negative *Pseudomonas aeruginosa* (ATCC 1688). This definitely indicates large scale antimicrobial vigor of LAB. Same isolates found to be effective against three indicator bacteria by different antimicrobials (Fig. 2 (L1 to L7 isolates).

Table 1: Antagonism of LAB against *Listeria monocytogenes*

LAB	Effect of 48 hour incubated lactobacillus culture					
	Antimicrobial effect	Crude cfs	Filtered Cfs	Neutralized cfs	Neutralized cfs + catalase	Neutralized cfs + catalase + heat treatment
L1	+	+	+	+	+	-
L2	+	+	+	+	+	+
L3	+	+	+	+	+	+
L4	+	+	+	+	+	+
LF	+	-	-	-	-	-
L6	+	+	+	+	+	+
L7	+	+	+	+	+	+

+= Presence of Inhibition - = No Inhibition

Table 2: Antagonism of LAB against *Aeromonas hydrophila*

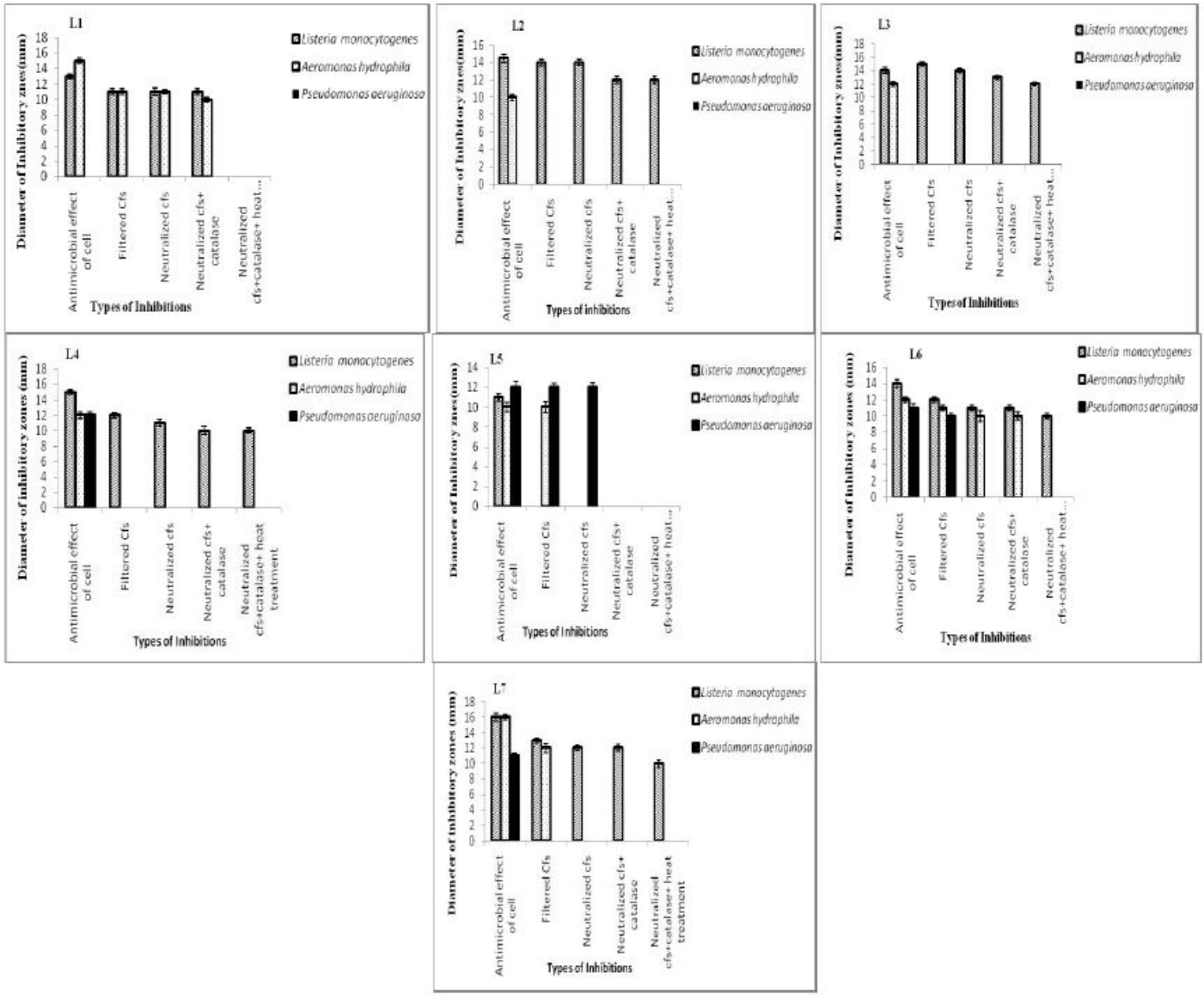
LAB	Effect of 48 hour incubated LAB culture					
	Antimicrobial effect	Crude cfs	Filtered Cfs	Neutralized cfs	Neutralized cfs+ catalase	Neutralized cfs+catalase+ heat treatment
L1	+	+	+	+	+	-
L2	+	-	-	-	-	-
L3	+	-	-	-	-	-
L4	+	-	-	-	-	-
L5	+	+	+	-	-	-
L6	+	+	+	+	+	-
L7	+	+	+	-	-	-

Table 3: Antagonism of LAB against *Pseudomonas aeruginosa*

LAB	Effect of 48 hour incubated LAB culture					
	Antimicrobial effect	Crude cfs	Filtered Cfs	Neutralized cfs	Neutralized cfs+ catalase	Neutralized cfs+catalase+ heat treatment
L1	-	-	-	-	-	-
L2	-	-	-	-	-	-
L3	-	-	-	-	-	-
L4	+	-	-	-	-	-
L5	+	+	+	+	-	-
L6	+	+	+	-	-	-
L7	+	-	-	-	-	-

(a) *Listeria* (b) *Aeromonas* (c) *Pseudomonas*

Fig. 1: Zones of Inhibition of LAB like Bacteria on MRS agar Plate of Indicator strains



(mean ± SE, n =5)

Fig. 2: Diameters of Inhibitory Zones and Types of Antagonism Explicited by Different LABs (L1 To L7)

86% filtered CFS of LAB like bacteria were competent enough to (Table 1) diminish the activity of *Listeria monocytogenes* (ATCC 657). CFS became indecisive against *Listeria* after treatment with NaOH and catalase. This advocates that organic acids and hydrogen peroxide are not liable for inhibition against *Listeria*. Many LAB CFS retained antimicrobial potency against *Listeria monocytogenes* after being heated at 90°C for10 min. Heating reduced the inhibitory activity of the supernatant as evidenced by shrinkage of inhibition zone from cell-culture to various forms of CFS (Fig. 2 [L1 to L7 isolates]). In case of *Aeromonas hydrophila* (ATCC 646), 43% inhibition was mainly outlined by live cells (Table 2). However, 29% inhibition of LAB like isolates against *Aeromonas* was manifested by acid and thermolabile protein like products (Table 2). Antagonism against *Pseudomonas aeruginosa* (ATCC 1688) exerted by cell, acid and hydrogen peroxide (Table 3).

Three isolates with thermostable antilisterial substances conveyed inhibition to *Aeromonas hydrophila* (ATCC 646) entirely via live cells (Fig. 2 [L1 to L7 isolates]). No thermostable inhibitory products were seemed to be working against *Aeromonas*. Anti-aeromonas thermolabile products arose very few. *Aeromonas*, being gram negative, its outer membrane imparted obstruction against bacteriocin like thermostable and thermolabile compounds [27] of LAB.

No protein like thermolabile and thermostable products (Fig. 2[L1 to L7 isolates]) established antagonism against *Pseudomonas aeruginosa* (ATCC 1688). Moreover, successful isolates along with thermolabile and thermostable bacteriocin like anti-aeromonas and anti-listeria products appeared to be ineffectual against *Pseudomonas*. This validates augmented counteraction of *Pseudomonas* as Gram negative bacteria even more than *Aeromonas* to LAB bacteriocin.

Acid generated by LAB execute strong inhibition against indicator bacteria [28]. However, as biopreservative agent of fish acid will influence the taste of the material, so further inspection is required for its use. Hydrogen peroxide is also antibacterial [28]. Peroxide (H_2O_2) not only injures enzymes and membrane lipid but also act as a source of harmful free radicals [29]. Therefore results endorsed administration of the bacteriocin as a fish biopreservative. Further, the research work encourages application of bacteriocins produced by L1 and L6 LAB like isolates as competent biopreservative reagents than bacteriocins solely perform against Gram positive bacteria (Fig. 2[L1 to L7 isolates]).

Treatment of thermolabile and thermostable inhibitory compounds containing CFS with proteinase K and trypsin resulted in loss of activity, confirming its proteinaceous nature. Anti-listeria and anti-aeromonas compounds of the present study could not be niscin [30] or enterocin [31] due to their high pH resilience aptitude. Unspecified thermostable anti-listeria products could be pediocin [32] due to their heat resistance and high pH tolerance competence. Further, some unknown thermolabile anti-listeria and anti-aeromonas products with high pH endurance were also expressed. Live cell linked antagonisms had anti-aeromonas and anti-pseudomonas effect.

4. CONCLUSION

The present research inspires application of bacteriocins of native LAB like strains to be used for shelf life extension of fish. Aftereffects of intrinsic bacteria and their products would be very negligible on microbial system, nutritional attributes and sensory qualities of fish. LAB like strains with probiotics nature might add remedial and precautionary effects on public health. Even so, LAB like isolates should be examined for biogenic amines production and for antibiotic resistances. The paper further reiterates tremendous potential of thermolabile and thermostable proteinaceous products over protective culture to remove pathogenic and spoilage flora of fish. Immune genes of bacteriocins from protective strains can easily enter to pathogenic bacteria through conjugation or transposon because genes and immune gene of bacteriocins abide on same plasmid or chromosome [33]. Bacteriocin of the present work could be a replacement to antibiotics. Protein like antil-listeria and anti-aeromonas compounds further to be investigated for their resistances. Furthermore, proteinaceous antimicrobials are assured food supplement as ingested by gut proteases. Unfortunately no bacteriocin like products was detected against *Pseudomonas*, only acid and peroxide antagonism was recognized. *Oreochromis* intestine needs to be explored further for LAB with protein like inhibitory products against *Pseudomonas*. Consequences of in situ application of anti-listeria and anti-aeromonas bacteriocins need to be scrutinized on chemical and physical conditions of fish food.

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Declaration of Competing Interests

The author mentions that there is no competing financial interest or personal relationship that could have emerged to affect the work of the present paper.

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