Potential Lactic Acid Bacteria from Freshwater Fish Processing Wastes Have Been Isolated and Characterized for use in Fermentative Utilisation of Fish Processing Waste

Dr. Anita Mishra

Vice principal, Department of Biotechnology, Vidyadayini Institute of Science Management and Technology, Bhopal Madhya Pradesh. anita.mishra556@gmail.com

Abstract- Lactic acid bacteria (LAB) that are proteolytic and/or lipolytic were isolated from the visceral wastes of various fresh water fishes. The LAB count was found to be highest in Mrigal visceral waste (5.78 log cfu/g) and lowest in tilapia (4.24 log cfu/g). The selected LAB isolates were characterised morphologically, biochemically, and molecularly. FJ1 (E. faecalis NCIM5367) and LP3 (P. acidilactici NCIM5368) isolates exhibited both proteolytic and lipolytic properties. All six native isolates chosen for characterization exhibited antagonistic properties against a variety of human pathogens. All of the native isolates were susceptible to the antibiotics cephalothin and clindamycin; additionally,All native isolates were susceptible to cephalothin and clindamycin, but resistant to cotrimoxazole and vancomycin. Individually, P. acidilactici FM37, P. acidilactici MW2, and E. faecalis FD3 were erythromycin sensitive. FJ1 (E. faecalis NCIM 5367) and LP3 (P. acidilactici NCIM 5368) strains with both proteolytic and lipolytic properties have the potential to be used in the fermentative recovery of lipids and proteins from fish processing wastes.

Index terms- LAB, lactic acid bacteria, fish processing wastes, visceral waste,*E. faecali.*

INTRODUCTION

Global fish production is currently around 141 million metric tonnes (MMT), and India generates significant foreign exchange by exporting seafood in excess of 2.8 MMT, in addition to 3 MMT of freshwater fishes from the inland fisheries sector. Currently, more than 76 percent of global fish production is used for human consumption, with the remainder used for other purposes [13]. Previously, there was no alternative use for fish waste, so it was dumped[16], causing serious environmental problems. Wastes generated from both marine and freshwater fish processing include scales, skins, visceral mass (viscera, air bladder, gonads, and other organs), head and fins.Fresh water fish processing, unlike the marine fish processing sector, is not well organised, resulting in a different level of waste generation and disposal. Among the various types of waste produced, fish viscera alone accounts for 15-25 percent of total body weight. According to recent estimates, the global generation of fish industry waste exceeds 63 MMT, and it is known to be a good source of recoverable biomolecules [6]. Researchers all over the world are working on methods to recover biomolecules in order to reduce organic loading on the environment and pollution problems [5, 26, 28].Lactic acid fermentation is a well-known technology for recovering biomolecules from various types of solid waste generated by animal processing industries, including fish [2, 14, 26]. Not only are fish viscera rich in different biomolecules, but they are also rich in beneficial lactic acid bacteria (LAB) with probiotic properties [4]. Fermentation with LAB is an efficient method of producing acid in situ, which allows waste to be preserved through ensilation [31] or lipids/proteins to be recovered [2]. Because of their antibacterial, immunodulatory, control of intestinal homeostasis, resistance to gastric acidity, and bile acid resistance properties, LAB have been used as probiotics.

Because of their versatile metabolic characteristics such as acidification activity, proteolytic activity, and the synthesis of metabolites such as bacteriocin, LAB is best known as a starter culture [11]. [10]). Microflora in fish digestive tracts have been

shown to play an important role in the production of antibacterial substances [35]. Fermentation with LAB has a greater impact than other traditional methods such as acid silaging because it is environmentally friendly, cost effective, and provides beneficial effects such as antibacterial activity and antioxidative properties [30]. In comparison to in-situ LAB, the use of starter culture (LAB) makes the fermentation process more controllable.

Because LAB has been shown to be very effective in recovering biomolecules from fish industry waste [3, 12, 21]), isolating LAB from fish industry waste becomes even more important.Against this backdrop, the primary goal of this study was to isolate, characterise, and identify native LAB from fish waste that have both lipolytic and/or proteolytic activity in order to investigate them for simultaneous lipid and protein recovery from fish industry waste.

MATERIAL AND METHOD

Fish viscera were obtained from freshly harvested commercial varieties of freshwater fishes [namely, Indian major carps (Rohu – Labeo rohita; Mrigal – Cirrhinus mrigala; and, Catla – Catla catla), common carp (Cyprinsu carpio), and tilapia (Oreochromis mossambicus)] and transported to the laboratory under iced conditions. Bacillus cereus F4433, Escherichia coli MTCC118, Staphylococcus aureus FRI722, Salmonella paratyphi FB254, Salmonella typhi FB231, Salmonella itriditicus, Listeria monocytogenes Scott A, Yersinia enterocolitica MTCC859, and Micrococcus luteus were the pathogenic bacterial strains used in the study for antibacterial activity.(CFTRI, Mysore, Food Microbiology Department). Microbiological media were obtained from M/s Hi-Media (Mumbai, India), and polymerase chain reaction (PCR) reagents were obtained from M/s Genei (Bangalore, India) (India).

Unless otherwise specified, all other chemicals were of analytical grade. The pH of various samples was measured using a pH metre (Cyberscan 1000, Eutech, Singapore) by directly immersing the combined glass calomel electrode in the sample.

Isolation of proteolytic and lipolytic lactic acid bacteria (LAB)

By plating serially diluted samples on MRS agar, the total LAB counts of the fish visceral waste samples were determined. The counts were expressed as log CFU (colony forming units) per gramme of sample using the average of triplicate plates. LAB were isolated from the viscera of various fresh water fishes, as well as fish visceral waste (FVW) fermented naturally by native LAB (NVW). FVW was created by combining fish visceral waste with either jaggery (10% w/w) or glucose (10% w/w) as a carbon source and 2% (w/w) sodium chloride to inhibit spoilage microbes. LAB that had been enriched in the NVW was isolated further. In physiological saline (1:10 w/v), FVW and NVW were homogenized.

The sample was serially diluted and plated on MRS plates containing 1 percent (w/v) skimmed milk (pour plate method), then incubated under anaerobic conditions at 371°C for 48 hours. The presence of a clear zone around the colonies identified the proteolytic LAB strains. These colonies were selected and tested for lipolytic activity by streaking them on MRS plates containing tributyrin at a concentration of 1% (v/v). Lipolytic cultures were distinguished by the presence of a clear zone around the colonies. Proteolytic and/or lipolytic cultures were further characterised using various biochemical tests. The antagonistic properties of these cultures against Listeria monocytogenes Scott A and Micrococcus luteus were also evaluated.

Identification of LAB with potent lipolytic / proteolytic activity

LAB isolates from FVW were identified using the schemes outlined in Bergey's Manual of Systematic Bacteriology [22]. Because the isolates Pediococcus acidilactici LP3, Pediococcus acidilactici MW2, Pediococcus acidilactici FM37, Enterococcus faecalis FJ1, Enterococcus faecalis FD3 and Pediococcus acidilactici FJ4 demonstrated proteolytic and/or lioplytic activities in addition to antibacterial properties, molecular The 16S ribosomal RNA (rRNA) gene was amplified

for molecular identification using RNA primers (5'TCATCTGTCCCACCTTGCGC3') as forward and R (5'GAGTTTGATCCTGGCTCAGG-3') as reverse, as previously described[20].

The PCR was carried out according to the standard protocol in the Thermocycler Gene AmpPCR system 9700 (Applied Biosystem, Perkin Elmer, Foster City, CA, USA) [29]. To make total cell lysate, the cell pellet was harvested and disrupted in phosphate buffer saline (PBS pH 7.0; IX) containing 1% Triton X-100 (Sigma). The mixture was incubated for 15 minutes in a boiling water bath before being snap-cooled in an ice bath. This lysate was used as a template in the amount of five microlitres. The PCR product was examined using agarose (1 percent) gel electrophoresis after it had been amplified. The PCR product was eluted from the gel, purified with a Qiagen gel extraction kit (Qiagen, Hilden, Germany), and sent for nucleotide sequencing at the University of Michigan.

The PCR product was eluted from the gel, purified with a Qiagen gel extraction kit (Qiagen, Hilden, Germany), and sent to M/s Vimta Laboratories in Hyderabad for nucleotide sequencing (India).Native isolates that had been biochemically characterised were tested for antibacterial spectrum, antibiotic sensitivity, and lipase activity. E. faecalis FJ1 and P. acidilactici LP3 isolates with higher proteolytic and/or lipolytic activity were deposited in the national repository of industrial microorganisms at NCL, Pune (http://www.ncl-india.org) with the accession numbers NCIM 5367 and NCIM 5368, respectively.

Antibacterial spectrum of selected native LAB

Native LAB isolates that demonstrated proteolytic and/or lipolytic activity and were characterised by various biochemical tests were then tested for antibacterial activity against various pathogens. Native LAB isolates were grown in MRS broth for 24 hours at 37°C in a static environment. After incubation, the medium was centrifuged at 6000g for 20 minutes in a Rotina 420R (Hettich, Germany) to collect the supernatant, which was labelled as culture filtrate (CF). CF was neutralised with 0.1 N NaOH and tested for antagonistic activity against various human pathogens using the agar well diffusion method (15).Selected pathogens were spread plated on MRS agar plates that were overlaid with soft Brain Heart Infusion (BHI) agar (0.8 percent) and allowed to grow for 4–6 hours. An aliquot (50l) of neutralised CF was then added to the wells on these plates that contained freshly grown pathogenic strains. The plates were then pre-incubated at 4 C for 2-3 hours to allow the test material to diffuse into the agar before being incubated for an additional 18 hours at 37 C.

Antibiotic sensitivity test for selected native isolates

Antibiotic sensitivity discs (M/s Hi-Media, Mumbai, India) were used to test the antibiotic sensitivity of native LAB isolates. MRS agar plates were overlaid with 8ml of MRS soft agar (0.8%) containing 50l of freshly grown culture and incubated at 4 C for the soft agar media to set. Antibiotic discs were placed on solid media and incubated under anaerobic conditions for 24 hours at 37 degrees Celsius.Antibiotic sensitivity discs (M/s Hi-Media, Mumbai, India) were used to test native LAB isolates for antibiotic sensitivity. MRS agar plates were overlaid with 8ml of MRS soft agar (0.8 percent) containing 50l of freshly grown culture, and the plates were incubated at 4 C for the soft agar media to set. Antibiotic discs were placed on solid media and incubated at 37 C for 24 hours under anaerobic conditions.

Preparation of crude enzyme extract and determination of lipase activity of the selected LAB

In an incubator shaker, native LAB isolates from FVW were grown in MRS broth for 24 hours (150 rpm; Technico, India). Following incubation, the broth was centrifuged at 8000g for 15 minutes (Rotina 420R; Hettich, Germany) to collect the culture filtrate, which was designated as crude enzyme (CE) preparation. CE lipase activity was determined by estimating the free fatty acids (FFA) released by the CE during the hydrolysis of a stabilised emulsion (SE) of vegetable oil, as

described by Borlongan [7].To make the stabilised emulsion, combine 50 mL of sunflower oil, 50 mL of 2% bovine serum albumin, and 3.5 mL of Tween 80. 1ml of crude enzyme was mixed with 1ml of stabilised lipase substrate in 1.5 ml of 0.1M Tris–HCl buffer at pH 8.0. After 6 hours of incubation at 37 °C, the hydrolysis was stopped by adding 3ml of 95 percent ethyl alcohol. The mixture was then titrated with 0.01N NaOH, with 1 percent phenolphthalein in ethanol serving as an indicator.

The same procedure was used for blank determination, except CE was added at the end of the incubation period after the addition of ethyl alcohol to the assay system. One unit of lipase activity was defined as the amount of 0.01N NaOH needed to neutralise the FFA formed during oil hydrolysis per milligramme of protein in extract.

RESULT

The quantitative and qualitative characteristics of LAB associated with fish waste FVW and NVW had LAB counts ranging from 4.2 to 5.9 log cfu/g and 8 to 9 log cfu/g, respectively (Table 1). The LAB count was found to be highest in Mrigal visceral waste (5.88 log cfu/g) and lowest in tilapia (4.22 log cfu/g). The proteolytic and lipolytic properties of LAB isolates from FVW and/or NVW are depicted in Figure 1. (Table 2).More than 100 discrete colonies were obtained from screening FVW and NVW samples for LAB. Thirty isolates with both lipolytic and proteolytic activity (16 from FVW and 14 from NVW) were chosen for further investigation. Out of the 30 isolates tested, 21 (16 from FVW and 5 from NVW) demonstrated higher lipolytic and proteolytic activities, which were coded and considered when selecting the best cultures (Table 1).Initial biochemical tests revealed that these 21 isolates belonged to the genus Pediococcus or Enterococcus (Table 2). Six isolates (LP3, MW2, FJ1, FJ4, FM37, and FD3) with antibacterial properties (against Listeria and/or Micrococcus) as well as being lipolytic and proteolytic were chosen for further characterization and identification (Table 2).Table 3 shows the morphological, biochemical, and physiological characteristics of these six isolates. The isolates LP3 and FM37 (both Pediococcus acidilactici) grew at 50°C, indicating their ability to tolerate higher temperatures; however, MW2 (Pediococcus acidilactici) grew in higher salt concentrations (9 percent).At 10 ^{o}C, none of the isolates grew. All of the isolates grew in the alkaline pH range (pH 7.5 to 8.5), but none grew at pH 4.2 (FJ1, MW2, FD3). Based on 16S rRNA homology, all six isolates, LP3, MW2, FJ1, FD3, FM37, and FJ4, were identified (data not shown). These were identified as Pediococcus acidilactici LP3, P. acidilactici MW2, and P. acidilactici LP3.acidilactici FM37, E. faecalis FJ1, E. faecalis FD3, and P. acidilactici FJ4, in that order. Based on these results, the isolates LP3, MW2, FM37, and FJ4 were identified as Pediococcus acidilactici, while FJ1 and FD3 were identified as Enterococcus faecalis (Table 3).

Table 1. Lactic acid bacteria (LAB) counts (log CFU/g) in visceral wastes of different freshwater fishes and isolates used for further characterization

Sample	Count, log CFU	Codes of isolates picked up
Rohu (*Labeo rohita*)	5.27 ± 0.25	LP1, LP4, LP5
Catla (*Cirrhinus mrigala*)	5.11 ± 0.05	FLB5, FS37, FS50, FS501
Common carp *(Catla catla)*	5.45 ± 0.17	FM38,FM371, FM50, FM501
Mrigal (*Cyprinsu carpio*)	5.78 ± 0.10	MW1, MW2, MW3, MW4
Tilapia (*Oreochromis mossambicus*)	4.21 ± 0.23	TL
Naturally fermented sample		
Jaggery	8.45 ± 0.07	FJ1,FJ4,
Dextrose	8.08 ± 8.08	F1,F2,F3

Values indicate the average counts of triplicate plates of MRS agar

CFU: colony forming units

Table 2. Antibacterial-, proteolytic- and lipolytic activity of LAB isolated from freshwater fish viscera and naturally fermented fish viscera

Fishes	Culture code	Identified* Genus	Antibacterial activity		Proteolytic activity	Lipase activity
			Listeria	*Micrococcus*		
Rohu	LP1	*Pediococcus*	+	++	+	-
	LP4*	*Pediococcus*	++	++	+	++
	LP5	*Pediococcus*	-	++	+	-
Catla	FLB5	*Enterococcus*	++	++	+	-
	FS37	*Enterococcus*	++	++	-	-
Common Carp	FM371	*Enterococcus*	++	++	-	-
	FM50	*Enterococcus*	++	++	-	-
	FM501	*Pediococcus*	-	++	-	-
Mrigal	MW1	*Enterococcus*	+	-	+	-
	MW2*	*Pediococcus*	++	++	+	+
	MW4	*Enterococcus*	++	++	+	+
Tilapia	TL	*Enterococcus*	+	-	++	-
Naturally fermented fish visceral waste						
Mixed waste	FJ1*	*Enterococcus*	++	++	++	++
	FJ4*	*Pediococcus*	-	++	++	++
	FM38*	*Pediococcus*	++	-	-	+
	F1	*Pediococcus*	-	++	+	++
	F2	*Enterococcus*	-	++	+	+
	F3*	*Enterococcus*	+	-	+	++

* Different biochemical tests are used to identify individuals (Grams staining, catalase test, Oxidase test, Growth at different pH, temperature and NaCl concentration, sugar fermentation test) +: moderate activity, ++: increased activity, -: negative activity

Table 3. Physiological and biochemical growth characterstics of LAB isolates from fresh water fish visecra

Biochemical Assay		LP4	FJ1	MW2	FM38	F3	FJ4
Morphology		cocci	cocci	cocci	cocci	Cocci	cocci
Gram Staining		+	+	+	+	+	+
Catalase / Oxidase		- / +	- / +	- / +	- / +	- / +	- / +
Growth temperature (°C)	10	-	-	-	-	-	-
	30	+	+	+	+	+	+
	50	+	-	-	+	-	-
Growth at NaCl (%)	3.0	+	+	+	+	+	+
	6.5	+	+	+	-	+	+
	9.0	-	-	+	-	-	-
Growth at pH	4.2	+	-	-	+	-	+

	7.5	+	+	+	+	+	+
	8.5	+	+	+	+	+	+
Casein utilization		+	+	+	+	+	+
Molecular identification		*P. acidilactici NCIM5368*	*E. faecalis NCIM 5367*	*P.acidilactici*	*P. acidilactici*	*E. faecalis*	*P. acidilactici*

LAB isolates' antibacterial activity and antibiotic sensitivity pattern

The antagonistic characteristics Table 4 shows the six different LAB isolates chosen for further research. The native LAB isolates from FVW showed antagonistic properties against the bacterial pathogens used in the study (Table 4), indicating a broad spectrum of activity against these pathogens. L. monocytogenes Scott A, on the other hand, was not inhibited by P. acidilactici FJ4, and Y. enterocolitica MTCC859 was not inhibited by P. acidilactici: FM37 and B. acidilactici: FM37.

P. acidilactici LP3 has no effect on cereus.Furthermore, antibiotic sensitivity testing revealed that the native LAB isolate was susceptible to a variety of antibiotics (Table 5). All native isolates were susceptible to cephalothin and clindamycin, but resistant to cotrimoxazole and vancomycin. Individually, P. acidilactici FM37, P. acidilactici MW2, and E. faecalis FD3 were erythromycin sensitive. Aside from that, the LAB isolates showed varying degrees of sensitivity to various antibiotics (Table 5).

Table 4. Antibacterial spectrum of four selected native isolates against different pathogens

Pathogens	Isolates					
	LP4	FJ1	MW2	FM38	F3	FJ4
L. monocytogenes Scott A	++	++	++	++	+	-
M. luteus	++	++	++	++	-	++
S .typhi FB231	+	++	++	++	+	++
S.itriditicus FB256	++	+++	+++	+++	++	+++
S. paratyphi FB254	++	++	+	++	++	+
Y. enterocolitica TCC859	+	+	++	-	+	+
E. coli MTCC118	++	+++	++	+++	++	+++
S. aureus FR1722	++	++	++	++	++	++
B. cereus F4433	-	++	++	++	+	+++

FJ1: Enterococcus faecalis, =LP4: Pediococcus acidilactici NCIM 5368 NCIM 5367, MW2: Pediococcus acidilactici, FM38: Pediococcus acidilactici, F3: Enterococcus faecalis, FJ4: Pediococcus acidilactici ('-'absence of zones, '+' 10mm diameter zones, '++' 16mm diameter zones, '+++' 16mm diameter zones greater than 16mm in diameter).

Table 5. Antibiotic sensitivity test for native LAB isolated from fresh water fish viscera

Antibiotics		Isolates					
	Concentration (µg)	LP4	FJ1	MW2	FM38	F3	FJ4
Cephalothin (Ch)	30	S	S	S	S	S	S
Clindamycin (cd)	2	S	S	S	S	S	S
CoTrimoxazole (co)	25	R	R	R	R	R	R
Erythromycin (E)	15	R	R	S	S	S	R
Gentamycin(G)	10	S	S	IM	R	IM	R
Ofloxacin (OF)	1	R	S	R	R	R	R
Pencillin-G (P)	10 units	IM	IM	IM	R	R	R
Vancomycin (Van)	30	R	R	R	R	R	R

S stands for sensitive, R stands for resistant, and IM stands for intermediate.Pediococcus acidilactici (LP3) NCIM 5367, MW2: Pediococcus acidilactici NCIM 5368, FJ1: Enterococcus faecalis Pediococcus acidilactici (FM38), Enterococcus faecalis (F3), and Pediococcus acidilactici (FJ4)

All six isolates were grown with or without a lipid substrate, and an in-vitro lipase assay was performed using CE, with the results shown in Figure 1. The results clearly showed that all of the isolates can produce extracellular lipase, with two isolates, LP4(P. acidilactici) and FJ1 (E. faecalis) exhibiting higher lipolytic activity. These characteristics indicated their potential for use in the reclamation of various biomolecules, particularly lipids and proteins from fish processing wastes. As a result, fromAmong the six identified LAB isolates, two isolates, P. acidilactici LP4 and E. faecalis FJ1, exhibited higher lipolytic (Figure 1; Table 2) and proteolytic activity, as well as being antagonistic to Listeria and/or Micrococcus (Table 2), and were deposited in the national repository of industrial microorganisms at the National Chemical Laboratory (NCL), Pune (http://www.ncl-india.org) (P. acidilactici LP4).

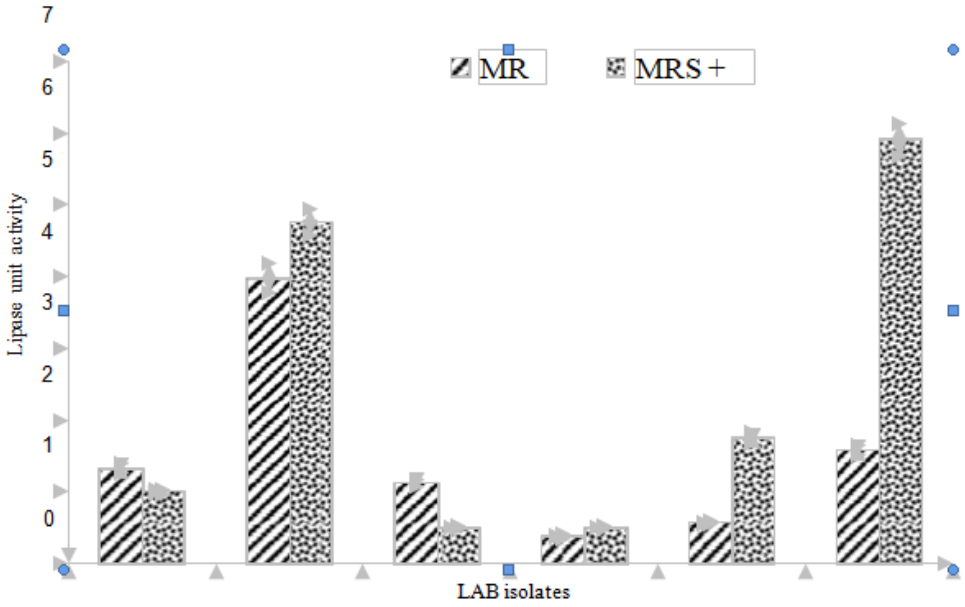

Figure 1. Lipolytic activity of LAB isolated from fresh fish viscera (n=3).
(MRS+T = MRS with 1% Tributyrin as lipid substrate)

CONCLUSION

Among the LAB isolated from FVW and/or NVW, two isolates, P. acidilactici LP4 and E. faecalis FJ1, demonstrated higher lipolytic and proteolytic activity, as well as antagonistic activity against bacterial pathogens. These were deposited in the national repository of industrial microorganisms under the accession numbers NCIM 5367 (E. faecalis FJ1) and NCIM 5368 (E. faecalis FJ2) (P. acidilactici LP4). Apart from in-situ enrichment of PUFA in the recovered fish oil, the production of lipase as a metabolite indicates the potential of these cultures for application in simultaneous recovery of both proteins and lipids. More research is needed to determine the effect of these native isolates on in-situ enrichment ofMore research is needed to determine the effect of these native isolates on the in-situ enrichment of PUFA in fish oil recovered from fish processing waste.

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