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INHIBITORY ACTIVITIES OF LACTIC ACID BACTERIA ON GRAM POSITIVE AND GRAM NEGATIVE FOOD BORNE PATHOGENS AND SPOILAGE ORGANISMS ASSOCIATED WITH FISH

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Abstract
This study aims to screen the inhibitory activity of lactic acid bacteria (LAB) isolated from idli batter against common food borne pathogens (FBP) and spoilage organisms (SO) associated with fish. *S. aureus, E. coli, S. typhi, B. cereus, P. vulgaris* and *P. aeruginosa* were isolated from ten fish samples and identified. Three LAB namely LAB-A, LAB-B and LAB-C exhibited the highest inhibitory activity, against all FBP and SO by agar well diffusion method, were selected and characterized morphologically and biochemically. The inhibitory activity of LAB was compared with indicator strain *L. acidophilus* MTCC 10307. 16S ribosomal RNA sequencing of isolates were done to identify the isolates. LAB-A was identified as *Pediococcus acidilactici* strain CSI29MX, LAB-B as *Pediococcus parvulus* strain MF233 while LAB-C as *Pediococcus pentosaceus* strain QN1D. All the three isolates exhibited the highest inhibitory activity, against Gram positive organism *S. aureus* and *B. cereus* with a Zone of Inhibition (ZOI) ranging from 14.9 mm to 15.9 mm and least for *P. vulgaris* with ZOI 12.8 mm to 13.6 mm. *L. acidophilus* MTCC 10307 showed lower activity against FBP and SO with ZOI of 11 $\pm$ 0.1 mm and 11 $\pm$ 0.2 mm against *P. aeruginosa* and *E. coli* respectively. The studies indicate that the LAB isolated from idli batter have highest potential of bacteriocin production than the indicator organism *L. acidophilus* MTCC 10307. Overall, the isolated LAB showed the remarkable inhibitory effect against both Gram positive and Gram negative FBP and SO. However, the spectrum of inhibition was different for the isolates tested. These results suggest that this potent isolates could be used as a natural biopreservatives in different food products.

Introduction:

Food processors run the risk of significant economic losses due to food spoilage resulting from microbial contamination. In industrialized countries, the percentage of the population suffering from food borne disease each year has been reported to be up to 30% (WHO, 2007). Although chemical preservatives may provide a solution, the use of such preservatives (nitrite) leads to negative consequences for human health (Mills et al., 2011).

The empirical use of microorganisms and their natural products for the preservation of foods (biopreservation) have been a common practice in the history of mankind (Galvez et al., 2007). Bacterial antagonism is of vital importance for the application of probiotic lactic acid bacteria (LAB). Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called "probiotics" in the intestinal tract (Salminen et al., 2004). The inhibitory spectrum of Lactic acid bacteria frequently includes spoilage and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* as well as Gram negative bacteria such as *E. coli* and *Salmonella* (Stevens et al., 1991). The anti-microbial properties of LAB have enabled the extension of the shelf life of many foods through fermentation processes. The inhibition of food spoilage microbes could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins (Atrih et al., 1993). A few strains are also known to produce active enzymes which inhibit other pathogenic bacteria (Lian et al., 2003).

Many species of *Lactobacillus*, used in the manufacture of fermented dairy products, inhibit the growth of other bacteria including the intestinal pathogens and spoilage organisms by producing anti-bacterial compounds or bacteriocins. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity against those bacteria which are closely related to the producer strain (Klaenhammer, 1988). The bacteriocins produced by Gram positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative and therapeutic potentials (Galvez et al., 2008 ; Jack et al., 1995). Considering this quality, there has been an increased concern in recent years on usage of bacteriocins due to the wide spread over-prescribing use of antibiotics and consequent increased development of antibiotic resistance.

Materials and Methods

The indicator organisms *L. acidophilus* MTCC 10307 was procured from Microbial Type Culture Collection (MTCC) Chandigarh, India. Bacteriocinogenic LAB were isolated from idli batter. Food borne pathogens isolated from fish

obtained from local market. Culture media and chemicals were obtained from HI media Mumbai.

Isolation of Lactic Acid Bacteria

The highest potential Bacteriocinogenic Lactic acid bacteria LAB-A, LAB-B and LAB-C were isolated from idli batter (Khandare and Patil 2014).16S ribosomal RNA sequencing of isolates were done to identify the isolates. LAB-A was identified as *Pediococcus acidilactici* strain CSI29MX, LAB-B as *Pediococcus parvulus* strain MF233 while LAB-C as *Pediococcus pentosaceus* strain QN1D.

Isolation of FBP and SO from Fish

Pathogens and spoilage organisms were isolated from perishable food fish. Samples were examined for initial pH, Total viable count, proteolytic organisms, FBP and SO.

10 gms of fish samples were homogenized with blender. 1:10 dilutions were prepared in 90 ml of 0.85% sterile physiological saline. Serial dilutions were made upto 10^{-7} to obtain different number of bacteria. The diluted samples from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were inoculated in differential and selective medias viz Eosine methylene blue agar, Cystine lactose electrolite deficient medium, Mannitol salt agar, Pseudomonas isolation agar, Salmonella Shigella agar and Mannitol egg yolk polymixin agar, and incubated at 37^{0}C for 24 h.

Identification of FBP and SO on the basis of biochemical tests

The colonies of FBP and SO obtained on selective and differential medias were identified on the basis of Gram staining , Sugar fermentation ,IMViC test , H_2S production, production of enzyme coagulase, urease and gelatinase. Christen Gram procedure was used for staining. The FBP and SO were tested for their carbohydrate fermentation ability by inoculating the LAB in glucose, lactose and mannitol broth. FBP and SO were tested for Indole ,methyl red, Voges Prousker ,citrate utilization and H_2S production similarly studied for production of enzyme, urease and gelatinase (Aneja, 2003) and coagulase (Ananthanarayan and Paniker 2013).

Inhibitory activity of bacteriocinogenic LAB against FBP and SO

The inhibitory activity of bacteriocinogenic LAB isolates and standard strain of *L. acidophilus* MTCC 10307 was tested against FBP and SO obtained from perishable food fish.

Cell free supernatant of LAB (CFS) was obtained by centrifugation at 12000 rpm, at 4^{0}C for 13 min. The possible inhibitory effect of either hydrogen peroxide or lactic acid on the indicator strain was eliminated following the procedure of Ogunbanwo et al. (2003). Supernatant was sterilized by filtration through 0.22 mm millipore membrane filter. 500 µl of 24 h broth culture of *S. aureus*, *E. coli. S. typhi. B. cereus P. vulgaris* and *P. aeruginosa* was seeded on Muller- Hinton agar plate and *L. acidophilus* MTCC 10307 on MRS agar. The wells of 2 mm were bored and to each well 20 µl of CFS was added and incubated at 37 ^{0}C for 24 h. Inhibitory activity was evaluated by observing zone of inhibition (Narayanapillai et al.,2012). The inhibitory activity sssof LAB isolates was compared with standard strain of *L. acidophilus* MTCC 10307 obtained from MTCC Chandigarh.

Results:

Ten fish samples were examined for initial pH, total viable count, proteolytic organisms, FBP and SO. The pH of the sample ranges from 6.8 -7.5. Total viable count was 5.4 to 12.8 x10^4 cfu / ml, proteolytic organisms were 2.2 to 6.4 x10^4 cfu /ml whereas FBP and SO were 0.3 to 4.0 x10^3 cfu/ml. (Table 1).

The morphological and biochemical characteristics of isolates of fish were studied. On Eosine Methylene Blue agar the colony with green metallic sheen was observed which showed Gram negative, sluggishly motile rods fermenting glucose, lactose and mannitol with production of acid and gas. Indole, methyl red tests were positive and Voges Proskauer, citrate and H_2S tests were negative.

The isolated green coloured colonies on Cystine lactose electrolyte deficient agar showed Gram negative, motile rods fermenting glucose and mannitol with production of acid and gas, lactose was not fermented. Indole, methyl red, citrate, H_2S and protease tests were positive while Voges Proskauer test was negative. On Mannitol salt agar pink coloured colonies were observed which showed Gram positive cocci, non motile, fermenting glucose, lactose and mannitol with production of only acid. Indole and citrate tests were negative and methyl red, Voges Proskauer, coagulase tests were positive.

Growth on Pseudomonas isolation agar, showed white greenish colony found to be Gram negative, actively motile rods, fermenting glucose with production of acid while lactose and mannitol were not fermented. Indole, methyl red, Voges Proskauer , H_2S test were negative, while citrate test was possitive. No enzyme production was observed. On Salmonella Shigella agar, black colony was observed which showed, Gram negative, actively motile, rod shaped organisms fermenting

glucose and mannitol with production of acid and gas but no lactose fermentation. Methyl red, citrate, H_2S test were positive, while indole,Voges Proskauer test were negative. On Mannitol egg yolk polymyxin agar the pink orange colonies was formed that showed Gram positive, motile rods which ferments glucose with production of only acid. Mannitol was not fermented. While gelatinase and Voges Proskauer test were positive (Table 2 ,Table 3).

The antimicrobial properties of bacteriocinogenic *Pediococcus acidilactici* CSI29MX, *Pediococcus parvulus* MF233 and *Pediococcus pentosaceus* strain QN1D were tested against six FBP and SO namely *E. coli, S. aureus, B. cereus, P. vulgaris, S. typhi, P. aeruginosa* and were compared with *L. acidophilus* MTCC 10307. The results of inhibitory activity demonstrated by the isolates in terms of diameter of the zone of inhibition (ZOI) were shown in Fig1. A diameter >1mm around the well was considered as a positive result. All the three isolates showed maximum ZOI against Gram positive organisms as compared to Gram negative organisms. Highest ZOI has been shown by *Pediococcus pentosaceus* QN1D viz 15 $\pm$ 0.9 mm against *B. cereus,* while 15$\pm$ 0.4mm against *S. aureus. Pediococcus acidilactici* CSI29MX demonstrated ZOI as 15 $\pm$ 0.6 mm and 15 $\pm$ 0.4 mm, where as *Pediococcus parvulus* MF 233 showed 14 $\pm$ 0.9 mm and 15 $\pm$ 0.2 mm against *B. cereus* and *S. aureus* respectively. *Pediococcus acidilactici* CSI29MX showed ZOI in the range of 12.8 mm to 14.4 mm, *Pediococcus parvulus* MF233 with 13.4 mm to 14.8 mm and *Pediococcus pentosaceus* QN1D with 13.6 mm to 14.4 mm against Gram negative organisms. *L. acidophilus* MTCC 10307 demonstrated 13.02 mm and 12.08 mm against *B. cereus* and *S. aureus* respectively while ZOI ranges from 10.04 mm to 12.5 mm against Gram negative organisms. The results indicates that the LAB isolated from idli batter have higher potential of bacteriocin production than the indicator strain *L. acidophilus* MTCC 10307 obtained from MTCC Chandigarh (Fig 1).

Table. 1- Physicochemical properties, FBP and SO obtained from fish

Sample	Consistency Colour odour	Initial pH	TVC x10^4 cfu/ml	*E. coli* x10^3 cfu /ml	*S. aureus* x10^3 cfu /ml	*S. typhi* x10^3 cfu/ml	*P. vulgaris* x10^3 cfu /ml	*P. aeruginosa* x10^3 cfu /ml	*B. cereus* x10^3 cfu /ml	Proteolytic organisms x10^4cfu /ml
F1	Brownish,musty odour,fresh	7.5	6.5		2.7	1.2	0.3		0.3	2.2
F2	Soft,Brown mudy odour	6.8	9.5					3.2		5.0
F3	Soft,Brown mudy odour	7	9.9	4.0		3.6	2.2			5.0
F4	Black seaweedy odour	7.4	12.8	3.4			2.7	1.4	1.0	6.1
F5	Brownish,musty odour,fresh	7.2	8.4		2.6				1.4	5.3
F6	Black seaweedy odour	7.0	10.6	2.9	2.0		1.6	1.4		6.4
F7	Brown seaweedy odour	7	5.4	2.0		1.8				3.0
F8	Soft,Brown mudy odour	7.4	7				2.9	3.2	1.0	3.5
F9	Black seaweedy odour	6.8	9		2.5			3.0		5.9
F10	Soft,Brown mudy odour	7	11.0	3.0			2.9			5.8

Table. 2 – Colony characteristics of organisms obtained from Fish

	Fish samples									
Media	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
EMB Agar			Green metallic sheen colony	Green metallic sheen colony		Green metallic sheen colony	Green metallic sheen colony			Green metallic sheen colony
CLED agar	Green colony		Green colony	Green colony		Green colony			Green colony	
MS agar	Small pink colony		Small pink colony			Small pink colony			Small pink colony	

PI agar		white greenish colony		Small white greenish colony		Small white greenish colony		white greenish colony	Small white greenish colony	
SS agar	Black centered colony		Black centered colony				Black centered colony	Black centered colony		
MYP agar	Pink orange colony				Pink orange colony			Pink orange Colony		

Table. 3- Biochemical characteristics of FBP and SO obtained from fish

Colony on	Gram staining	Motility	Sugar fermentation			Enzyme production	H_2S	Indole	M.R.Test	VP Test	Citrate Test
			Glucose	Lactose	Mannitol						
EMB Agar	Gram negative, rods	Sluggishly motile	AG	AG	AG	_	_	+	+	_	_
CLED Agar	Gram negative, rods	Highly motile	AG	_	AG	Protease	+	+	+	_	+
MS agar	Gram possitive cocci	Non motile	A	A	A	Coagulase	ND	_	+	+	_
PI agar	Gram negative, rods	Actively motile	A	_	_	_	_	_	_	_	+
SS agar	Gram negative ,rods	Highly motile	AG	_	AG	_	+	_	+	_	+
MYP agar	Gram positive, rods	Motile	A	ND	_	Gelatinase		ND	ND	+	ND

ND –Not determined , AG-Acid and gas production ,A-Acid production, + Positive , - Negative.

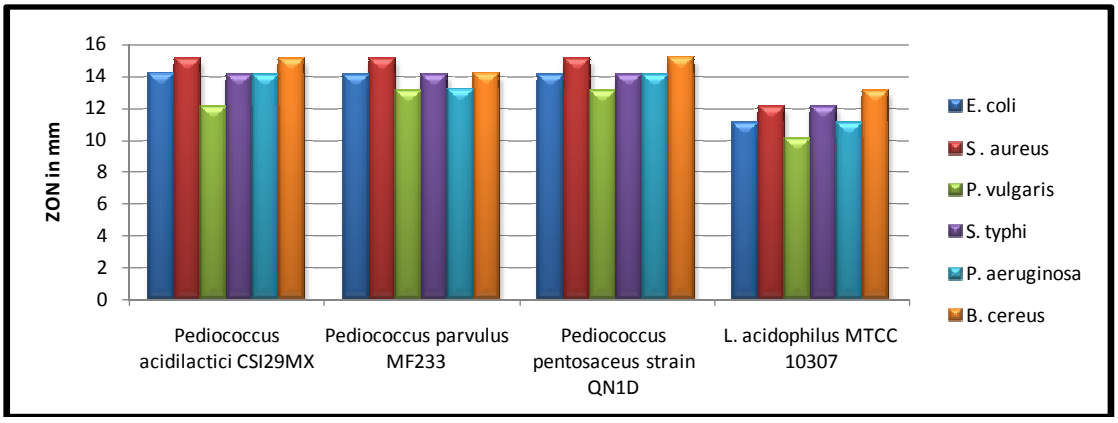

Figure. 1– Zone of inhibition shown by LAB isolates against FBP and SO

Discussion:

In industrialized countries, the percentage of the population suffering from food borne disease each year has been reported to be up to 30%. The epidemiology of food borne diseases has changed in the last two decades partly because newly recognized pathogens emerge and previously recognized pathogens increase in occurrence or become associated with food or new food vehicles (Meng, et al., 1997).

Bodhidatta et al., (2013) isolated food borne pathogens from raw chicken, pork and fish. In this study, only 97% of raw food samples were found to contain bacterial enteric pathogens. For Bacterial identification the bacteria was cultured on enrichment medium followed by conventional phenotypic tests. In our study 100 % food samples were found to contain pathogens and spoilage organisms, They were identified by morphology, cultural characteristics and phenotypic tests The method of identification of present study agreed with the

method of Bodhidatta used for identification of isolates.

Kazemipoor et al.,(2012) had earlier reported, greater activity of isolate MF15 against *E.coli* showed activity with a ZOI of 12 ± 0.8 mm while MF6 showed activity with a ZOI of 20 ± 1.3 mm. In the present study highest ZOI has been shown by *Pediococcus acidilactici* CSI29MX viz 15 ± 1.0 mm while *Pediococcus parvulus* MF233 and *Pediococcus pentosaceus* QN1D demonstrated 14 ± 0.8 mm ZOI against Gram negative pathogen *E. coli*. So the inhibitory spectra of *Pediococcus acidilactici* CSI29MX of present study was found to be more than isolate M6 while less than MF15.

Kivinac (2011), isolated the LAB *Leuconostoc citreum* KB2, from boza, a drink from Turkey, which demonstrate inhibitory activityagainst *B. cereus* 16.1 to 22 mm as ZOI, while another strain *Lactobacillus brevis* KB12 (1) showed 11.1 to 16 mm and 16.1 to 22 mm ZOI against *S aureus* and *B. subtilis* respectively. The *Leuconostoc citreum* KB2 and *Lactobacillus brevis* KB12 (1) strain was found to have more potential of bacteriocin production than our strains, since we got lower ZOI against *B. cereus* and *S. aureus* . The present results demonstrated that all the three isolates were equally antagonistic against both the Gram positive as well as Gram negative organisms. Also all the LAB isolates demonstrated higher inhibitory activityagainst pathogens and spoilage organisms compared with the activity of indicator strain *L. acidophilus* MTCC 10307.

Narayanapillai et al., (2012) isolated LAB from chick intestine showed production of antimicrobial compounds against *E. coli, P. aeruginosa, S. typhi, S. aureus, B. cereus, P. mirabilis* and *K. Pneumoniae*. This result showed similarity with the present study. The isolates of present study demonstrated the inhibitory activityagainst *E. coli, P. aeruginosa, S. typhi, S. aureus,* and *B. cereus*.

According to Zottola et al., (1994) the effect of nisin produced by LAB *in situ* against Gram positive microorganisms like *Listeria, Staphylococcus* and *Clostridium* was demonstrated and there are only few reports on Gram negatives. The effect of bacteriocin produced *in situ* against Gram negative is of greater importance since most of the food borne pathogens are Gram negatives. In the present study the LAB isolated showed inhibitory activityagainst Gram negative organism *E. coli., P. aeruginosa, P. vulgaris* and *S. typhi*. It is however difficult to comment on the reason for this variability in the antimicrobial property amongst the isolates since each one was different from the other.

Conclusion:

In conclusion the food grade *P. acidilactici* CSI29MX, *P.parvulus* MF 233 and *P. pentosaceus* QN1D isolated from idli batter produces a bacteriocin. The bacteriocin has potential of biopreservativation of perishable food like fish and can also be applied as a biopreservative to other perishable foods. The application of bioprotective cultures to ensure the hygienic quality of perishable foods is a promising tool in preservation of foods.

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