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ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM RAW AND FERMENTED PRODUCTS AND THEIR ANTIBACTERIAL ACTIVITY

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Abstract

Lactic acid bacteria (LAB) were isolated from raw and fermented products like milk, curd, idli batter and pickle. Out of 44 isolates, 16 species were identified. Among them *Enterococcus faecalis* was the dominant species (37.5%). *Streptococcus pyogenes*, *Lactobacillus casei* and *Streptococcus viridans* accounted for 18.75%, 18.75% and 6.25% respectively. The remaining 18.75% isolates of *Lactobacilli* were found to be *Leuconostoc* sp. Bacteriocin like inhibitory substance (BLIS) from the four isolates except *Leuconostoc* sp. were tested against selected pathogens of both gram positive and gram negative group. BLIS of *E. faecalis* in combination with 0.1% Ethylene diamine tetra acetic acid disodium salt (EDTA) showed the best antibacterial activity among all the isolates tested.

Keywords: Bacteriocin, Lactic acid bacteria, Antimicrobial, Well diffusion assay

Introduction

In recent years, extensive work has been carried out on bacteriocins and bacteriocin producing strains of Lactic acid bacteria (LAB) for their potential use as biopreservatives (Savadogo Aly et al., 2006). Several bacteriocin producing strains have been isolated from raw and fermented products (Rodriguez et al., 2000). The antimicrobial activity of LAB may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins (Remiger et al., 1999). Bacteriocins are ribosomally synthesized and cationic (Hurst, 1981) and have potential practical application in preservation of foods or to the prevention and treatment of bacterial infection (Jack et al., 1995). This paper reports on the spectrum of antimicrobial activity of BLIS from isolated LAB in combination with EDTA against pathogens.

Materials and Methods

Microorganisms

The indicator organisms namely *Bacillus cereus* MTCC-1305, *Staphylococcus aureus* MTCC-87, *Streptococcus sp.* MTCC-389, *E.coli* MTCC-40, *Klebsiella pneumoniae* MTCC-109 and *Pseudomonas aeruginosa* MTCC-424 were procured from Institute of Microbial Technology, Chandigarh, India.

Isolation and identification of LAB

Milk, curd, idli batter and pickle were procured from retail market in Bangalore, India. MRS agar and broth (HiMedia, Mumbai, India) were used for enumeration and culture of LAB (De Man et al., 1960). The samples were homogenized in a pestle and mortar using saline, serially diluted and pour plated on MRS agar plates. Inoculated MRS agar plates overlaid with MRS agar were incubated in an anaerobic jar at 37 °C for 48 h. Isolated colonies with typical characteristics of LAB were picked from each plate and transferred to MRS broth.

The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics up to genetic level (Harrigan and Margaret McCance, 1970; Sneath et al., 1986; Stiles Holzapfel, 1997). The biochemical tests used were Gram reaction; production of catalase and cytochrome oxidase; growth at 10°C, 45°C and 60°C for 1 week; growth at 10% NaCl, acid production from carbohydrates (1 % w/v) lactose, melebiose, raffinose; production of acid and gas from 1 % glucose; Hugh & Leifson (H&L) test in O/F medium; and production of ammonia from arginine.

Maintenance of microorganisms

All the LAB cultures were maintained at 4°C in MRS broth. Pathogenic organisms were maintained at 4°C on Brain Heart Infusion broth. All the bacterial cultures were sub-cultured at 15 days interval. Prior to their use in experiment, back cultures were sub-cultured in appropriate broth and LAB in MRS broth.

Production of bacteriocin like inhibitory substance (BLIS)

Isolated cultures maintained were inoculated in 100 ml of MRS broth and incubated anaerobically at

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37°C for 24 h. The cells were killed by heating at 80°C for 10 mins followed by centrifuging the broth at 10,000 rpm for 20 mins. The resulting cell debris that formed a pellet was discarded giving rise to a cell free supernatant. The pH of supernatant was adjusted to 5.0 with 1N NaOH, then concentrated to one tenth of the original volume by rotary flash evaporator and the solution thus obtained has been designated as BLIS. For synergistic activity BLIS was mixed with 1ml of 1% of EDTA and filter sterilized by 0.22µm membrane filter paper (Millipore, India) to carry out the anti-microbial activity by well diffusion assay (Vijai Pal et al., 2005).

Well diffusion assay method

0.1 ml of the 18 h old test cultures were inoculated onto previously poured and set nutrient agar plates by spread plate method. 4 wells of diameter 8 mm were made in each of the plates. These wells were filled with 100 µl concentrated BLIS with and without EDTA and the plates were incubated at 37° for 24hrs (Schillinger and Lucke, 1989). The inhibition zone was measured in millimeter using Antibiotic zone scale (HiMedia, Mumbai).

Results and Discussion

Out of 44 positive colonies picked from MRS agar only 16 were identified as LAB by biochemical tests (Table.1). Among 16 confirmed cultures 62.5% of the isolates were identified as gram-positive cocci, 18.75% were identified as gram-positive rods and remaining 18.75% were identified as *Leuconostoc* sp. (tests not shown). Under cocci, 6 isolates were identified as *E. faecalis* corresponding to 37.5% and were present in large numbers. 3 isolates corresponding to 18.75% were of *S. pyogenes* and 1 isolate corresponding to 6.25% of *S. viridans.* Under rods, 2 isolates corresponds to 18.75% of *L. casei* and 1 isolate corresponding to 6.25% *L. fermenti* (Table. 2). El-Shafei et al., 2000 in their studies, isolated, screened and characterized 100 strains of bacteriocin-producing lactic acid bacteria from traditional fermented foods and Renata Bromberg et al., 2004 have isolated *Lactococcus lactis* from meat and meat product.

Table 1. Biochemical test for the identification of isolated Lactic acid bacteria

No.	GM/ Mor	CAT	OXI	O/F	LAC	GLU	Growth at					NH_4 -arg	RAF	MEL	Organism Identified
							10°C	45° C	60°C	NaCl (10%)	pH 9.6				
1	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	*S. pyogenes*
2	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
3	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
4	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
5	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
6	+C	-	-	F	+	-	-	+	_	-	_	_	NA	NA	*S. viridans*
7	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	*S. pyogenes*
8	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
9	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	*E. faecalis*
10	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	*S. pyogenes*
11	+R	-	-	F	+	-	+	+	NA	NA	NA	-	-	-	*L. casei*
12	+R	-	-	F	+	-	+	+	NA	NA	NA	-	-	-	*L. casei*
13	+R	-	-	F	+	-	-	+	NA	NA	NA	+	+	+	*L. fermenti*

GM/Mor: Gram stain and morphology; LAC: Lactose utilization; RAF: Raffinose utilization; CAT: Catalase; GLU – Glucose utilization; MEL: Melibiose utilization; OXI: Oxidase; NH_4 – arg: Ammonia from arginine; +C: Gram positive cocci; O/F: Oxidative / fermentative; +R: Gram Positive rod; +: Positive reaction; -: Negative reaction; NA: Not applicable

Table 2. Number / percentage of lactic acid bacteria isolated from raw/fermented Food

Isolated / identified species	Number of isolates	Percentage	Source
Total number of catalase negative bacteria isolated	16		
Gram positive Cocci		**62.50**	
E. faecalis	6	37.50	Curd
S. pyogenes	3	18.75	Idli batter
S. viridans	1	6.25	Idli batter
Gram positive bacilli		**18.75**	
L. casei	2	12.50	Milk
L. fermenti	1	6.25	Curd
Others			
Leuconostoc sp.	3	18.75	Pickle

Antibacterial activity of BLIS

Four isolates of LAB namely, *E. faecalis, S. pyogenes, L. fermenti* and *L. casei* were selected for the study of the production of BLIS and their antibacterial activity against *E.coli, K. pneumoniae, P. aeruginosa, B. cereus, S. aureus* and *Streptococcus* sp. by well diffusion assay.

Table 3 shows the antibiogram of BLIS of isolated LAB species against above test organisms. *L. fermenti* showed good activity, S. *pyogenes* also showed activity except *S. aureus* and no activity was detected with *L. casei* against the entire test organisms. The BLIS of *E. faecalis* showed the highest inhibition against gram-positive bacteria namely *B. cereus* (19.0 mm) *S. aureus* (17.0 mm) and *Streptococcus* sp. (17.0 mm). The gram-negative organisms namely, *E.coli, K. pneumoniae P. aeruginosa* showed less inhibition compared to gram-positive organisms. This is in accordance with most of the reports (Jack et al., 1995), which showed that the bacteriocins of LAB were more active against gram-positive organisms compared to gram-negative organisms. The reason for the observed activity may be due to the gram negative organisms have an outer protective membrane, which covers the cytoplasmic membrane and peptidoglycan layer. EDTA, a metal chelating agent removes stabilizing cations from the outer membrane with result the lipopolysaccharide layer is partly lost and the membrane no longer functions as penetration barrier (Boziaris and Adams, 1999).

Table 3. Antibiogram (zone of inhibition in mm) of BLIS against selected pathogens

Spent culture broth	Gram negative bacteria			Gram positive bacteria		
	E.coli	*K. pneumoniae*	*P. aeruginosa*	*B.cereus*	*S.aureus*	*Streptococcus* sp
E. faecalis	15.0±0.20	13.0±0.02	13.0±0.20	19.0±0.70	17.0±0.70	17.0±0.20
S. pyogenes	11.0±0.25	9.0±0.20	10.0±0.20	12.0±0.30	NA	10.0±0.03
L. fermenti	13.0±0.30	11.0±0.25	10.0±0.20	15.0±0.02	13.0±0.03	13.0±0.20
L. casei	NA	NA	NA	NA	NA	NA

Values are Mean ±SD of 3 determinations; Values are excluding well; NA – No activity;

Table. 4 shows the antibacterial activity of EDTA alone and EDTA with BLIS of LAB against *E.coli, K. pneumoniae P. aeruginosa, B. cereus, S. aureus* and *Streptococcus* sp. The activity of EDTA alone as control against these bacteria was lower than the combination with BLIS.

Table 4. Antibiogram (zone of inhibition in mm) of BLIS with 0.1% EDTA against selected pathogens

Spent culture broth	Gram negative bacteria			Gram positive bacteria		
	E.coli	*K. pneumoniae*	*P. aeruginosa*	*B.cereus*	*S.aureus*	*Streptococcus* sp.
EDTA alone	11.0±0.10	10.0±0.20	8.0±0.10	12.0±0.10	11.0±0.20	ND
E. faecalis	23.0±0.05	21.0±0.02	20.0±0.05	27.0±0.03	24.0±0.20	24.0±0.20
S. pyogenes	14.0±0.20	13.0±0.30	13.0±0.20	14.0±0.25	10.0±0.05	14.0±0.70
L. fermenti	18.0±0.30	16.0±0.03	15.0±0.20	18.0±0.30	16.0±0.25	16.0±0.20
L. casei	12.0±0.05	10.0±0.02	9.0±0.10	12.0±0.02	12.0±0.10	NA

Values are Mean ±SD of 3 determinations; Values are excluding well; NA- No activity, ND-Not done

Of all the 4 BLIS of LAB tried in combination with EDTA against selected pathogens, the highest inhibition was shown by *E. faecalis* followed by *L. fermenti* and *S. pyogenes. L. casei* showed moderate inhibition against all the test organisms except *Streptococcus* sp. Kannappan et al, 2004 have also observed that BLIS of LAB with EDTA combination inhibited *Vibrio parahaemolyticus and E.coli.* Kelly et al., (1991) reported that nisin inhibited *Salmonella* sp. in combination with EDTA.

Therefore it is concluded that the BLIS produced by the isolates *E. faecalis, S. pyogenes, L. fermenti* in combination with EDTA were the effective inhibitor of all the pathogens tested as compared to *L. casei.* Further work on production and purification of bacteriocin are in progress.

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