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ISOLATION OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA FROM FISH AND ITS ANTIMICROBIAL ACTIVITY

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ABSTRACT

The objective of the present study was to isolate, characterize and identify the bacteriocin producing lactic acid bacteria from fish samples. Antimicrobial activity of the isolated strain was tested against spoilage pathogens. The extracted antimicrobial compound was also tested for some physiological conditions heat, pH and enzymes. The lactic acid bacteria strain was identified based on morphological, cultural, physiological and biochemical characters and carbohydrate utilization pattern identified as *Lactobacillus plantarum*. The extracted antimicrobial compound from the isolate exhibited no effect on the proteinase and pepsin. Although Trypsin has no effect on bacteriocin and active at 100°C. The effect of pH 2 to 12 on the crude protein extract was studied. The activity was not affected in pH 3 to 9.

INTRODUCTION

Bacteriocins are proteinaceous substance produced by many bacterial strains and exhibit bactericidal activity against the closely related organisms. They have been the subject of extensive studies in recent years because of their prospective use as natural food preservatives (Villiani *et al*., 2001). Lactic acid bacteria are widespread in nature. They have commercial applications as starter cultures in the dairy, baking, meat, fish and vegetable and alcoholic beverage industries.

LAB are known to produce bacteriocins and have great potential as food biopreservatives (Gilliand, 1990; Jamuna and Jeevaratnuam, 2004; Avant *et al*., 2004). During fermentation, the lactobacilli metabolize lactose to lactic acid. This lowers pH and creates an unfavourable environmental for pathogens and spoilage organisms (Aslim *et al*., 2004). In addition to acids, hydrogen peroxide, diacetyl and bacteriocins or bactericidal proteins produced during lactic fermentation may also have inhibitory roles against pathogenic microbes (Lindgren and Dobrogosz, 1990; Zhu *et al.*, 2000). A number of Lactobacilli including strains of *Lactobacillus acidophilus* produce bacteriocins. Most bacteriocins are heat stable, sensitive to certain proteolytic enzymes. LAB are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds.

Lactobacillus lactis and *Lactobacillus plantarum* were potent producers of bacteriocins. Bacteriocin produced by those *Lactobacillus* species had a large spectrum of inhibition of food spoilage microorganisms and various was carried out in order to investigate the inhibitory effect of bacteriocin a compound extracted from lactic acid bacteria from fish and its role in inhibition of food-borne pathogens.

MATERIALS AND METHODS

Isolation of bacteriocin producing microorganism

About 10 g of skin with muscle was cut aseptically from fish sample into a sterile sample dish. The sample was homogenized with 90 ml diluents (normal saline/NS) in a homogenizer and serially diluted to 10^2, 10^3, 10^4. About 1 ml of appropriate dilution of the sample was pipette into sterile petridishes. MRS agar media were poured and incubate at room temperature for 48 hrs.

The LAB was identified on the basis of growth on selective MRS agar (pH 5.2), cell morphology, gram staining, catalase activity and biochemical identification of LAB. Further identification of the species of these LAB was performed according to carbohydrate fermentation patterns and growth on MRS broth (HI Media) as described in Bergey's manual of systematic bacteriology. The isolated LAB were subcultured and the purified cultures maintained at MRS agar slants.

Test microorganisms

The pathogenic organisms were brought through the Institute of Microbial Technology (IMTECH), Chandigarh, India. They were maintained as pure cultures in TSB Agar slants with periodic subculturing every 4-8 days. The different pathogenic strains used in the present study are *Listeria monocytogenes* MTCC 657.

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Bacillus cereus MTCC 4079, *Escherichia coli* MTCC 2939, *Staphylococcus aureus* MTCC 3160.

Preparation of culture supernatants

The bacteriocin producing strain was grown in MRS broth (pH 5.5) at 37°C for 24-30 hrs. The isolated LAB culture centrifuged at 10000 rpm for 5 min. and then the supernatant was adjusted to pH 6.5-7.0 with 1 N NaOH (Anonymous, 1992).

Extraction of crude protein

The cell free supernatant from lactic acid bacterial culture were treated with solid ammonium sulphate to 40, 50 and 60 per cent saturation. The mixture was stirred for 2 h at 4°C and centrifuged at 20000xg for 1 h (4°C). The precipitate was resuspended in 5 ml of sodium phosphate buffer (50 mM; pH 7.0) then the crude extract was stored at -20°C.

Bacteriocin assay

The antimicrobial activity was determined by agar well diffusion method. BHI agar plates were overlaid with 10 ml BHI soft agar (0.75% agar) lawn containing an indicator bacterial strains. The indicator lawns prepared by adding 0.25 ml of a 10^{-1} dilution overnight cultures of test organisms. Wells 8 mm in diameter were cut into agar using sterile cork borer. Then 100 µl of culture supernatant fluids of LAB strains placed into each well. The plates were incubated at 37°C for 24 h and examined for zones of inhibition.

Sensitivity of crude protein to enzymes, heat and pH

The sensitivity of active substance to proteolytic and other enzymes were tested on crude protein (pH 7.0) of 24 h cultures incubated at 37°C. Samples of 100 µl were treated for 2 h with 1 mg ml^{-1} of proteinase, pepsin, trypsin, α-chymotrypsin, catalase, α-amylase and lipase. All the samples and controls were incubated at 37°C for 5 h and tested for activity. The sensitivity of active substance to different pH was estimated by adjusting the pH of LAB supernatant to pH 2, 3, 4, 6, 8, 10 and 12 with NaOH or HCl and testing against the indicator strain after 2 h incubation. The sensitivity to heat was tested by heating cell free supernatant samples to 37, 50, 70, 90, 100°C for 30 min. and 121°C for 10 min. and testing the residual activity after the treatment by well diffusion assay.

Optimum conditions for bacteriocin production

All the optimum conditions are tested in MRS medium which can replaced the different types of carbon and nitrogen sources. Production medium contains peptone – 10 g, carbon source – 20 g, potassium phosphate – 2 g, sodium acetate – 5 g, $MgSO_4$ – 0.2 g, $MnSO_4$ – 0.05 g with 1 litre distilled water.

Effect of carbon and nitrogen sources on bacteriocin production

The study of bacteriocin production with various carbon sources in the production medium supplemented with different types of carbon sources like lactose, starch, maltose and sucrose and nitrogen sources like peptone, tryptone, yeast extract. The sterilized medium flasks were inoculated with 0.1% inoculums and incubated on rotary shaker at 120 rpm for 2 days after the incubation period microbial cells were removed by centrifugation at 3000 rpm for 15 min. Antimicrobial activity in the supernatant was estimated by well diffusion method.

RESULTS AND DISCUSSION

Bacteriocins of lactic acid bacteria have the potential as food biopreservatives to control pathogenic and spoilage bacteria. In this study, lactic acid bacteria was isolated from fish. Microscopic identification of the isolate could determine the rod shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which indicated the typical basic characteristics of *Lactobacilli*. Based on the carbohydrate utilization pattern of bacterial isolates were identified as *Lactobacillus plantarum*. The results are tabulated in Table 1. Similar characters for lactic acid bacteria observed earlier by Kandler and Weiss (1986). Seema Nair and Surendran (2009) in their studies isolated and characterized the lactic acid bacteria from fish and prawn. The lactic acid bacterial isolate was tested for their inhibitory activity over some food borne pathogens *Listeria monocytogenes*, *Bacilus cereus*, *E. coli*, *S. aureu* and *S. typhi*. Almost all pathogens were inhibited by bacteriocin producer. The results are tabulated in Table 2.

Table 1 Characterization of lactic acid bacterial isolate

Morphological tests	$LABF_1$
Gram reaction	+
Shape	Rod
Colony morphology	Round
Colour	Creamy white
Biochemical characteristics	
Citrate utilization test	-
Indole production	-
Gelatin hydrolysis	-
Catalase reaction	-
Methyl red test	+
Voges-Proskauer test	-
Ammonia from arginine	-
Nitrate reduction	+
Gas production from glucose	-
Carbohydrate utilization	
Arabinose	-
Cellobiose	+
Fructose	+
Galactose	+
Glycerol	-
Lactose	+
Mannose	+
Mannitol	+
Ribose	+
Raffinose	+
Rhamnose	+
Sucrose	+
Sorbitol	+
Xylose	-
Identified as	*Lactobacillus plantarum*

(-) No growth/reaction; (+) Growth/reaction

Table 2 Inhibitory spectrum of crude protein extract of lactic acid bacteria against test organisms

S. No.	T est organisms	Inhibition zone (in mm)
1.	*Listeria monocytogenes* MTCC 657	15.0
2.	*Bacillus cereus* MTCC 4079	13.0
3.	*Escherichia coli* MTCC 2939	11.0
4.	*Staphylococcus aureus* MTCC 3216	10.00

The effect of various enzymes on the inhibitory agent was studied complete inactivation was observed after treatment with proteinase K, trypsin, pepsin which indicated the proteinaceous nature of agents. No reduction in the zone of inhibition was encountered when the bacteriocins treated with amylase, catalase and lipase (Table 3). The enzymes like amylase and lipase did not show any effect on the bacteriocins suggesting the absence of glycosylated and lipid in the bacteriocins.

Table 3 Effect of enzymes, pH and heat treatment on inhibitory activity of culture crude protein of LAB

S. No.	Treatment	Inhibition zone (in mm)
1.	Crude protein	7.5
2.	Catalase	9.0
3.	Trypsin	-
4.	Pepsin	-
5.	Proteinase	-
6.	α-amylase	12.0
7.	Lipase	9.5
	pH	**Inhibition zone (in mm)**
8.	2	3.70
9.	3	4.50
10.	4	5.20
11.	5	6.20
12.	6	9.30
13.	7	8.20
14.	8	6.80
15.	9	3.50
16.	10	3.0
	Heat	
17.	37℃	9.5
18.	50℃	8.8
19.	70℃	8.0
20.	90℃	7.6
21.	100℃	7.2
22.	121℃	7.0

The activity of bacteriocin elaborated by the test isolates was also pH dependent highest antimicrobial activity was exhibited in the pH range of 5 to 8 while the activation restricted at pH 2 and 9. The plantaricin produced by *Lactobacillus plantarum* was active in a pH range of 3 to 7 (Jimenez-Diaz *et al*., 1993). While the bacteriocin produced by the isolates was found to be stable at pH 2.0 to 6.0. Similar observations were made by many researchers (Talarico and Dobrogosz, 1989; Delmer *et al*., 2005; Abdelbasset and Kirana Djamita, 2008).

The antimicrobial substances produced by the isolates were relatively stable during heat treatments at 37, 50, 70, 90, 100°C for 30 min and 121°C for 10 min. Residual activity of bacteriocin did not show significant difference from the control. The bacteriocin produced by the isolate was considered to be most heat stable as the activity remain after heating at 121°C. The results were in accordance with Oganbanwo (2003) who observed that activity of bacteriocin produced by *L. brevis* remained after heat at 121°C for 16 min. The similar properties were reported for bacteriocins produced by other LAB such as pediocin A, Pediocin PAI, Lactacin and helverticin (Daeschel and Kalenhammer, 1985). Different carbon sources are used for the bacteriocin production when maltose was used as carbon source it shows maximum antimicrobial activity compared to remaining carbohydrates such as sucrose, lactose and starch respectively. The results are shown in Fig. 1. Similar results was observed by Todorov *et al*. (2005). Optimal bacteriocin production (12800 AU ml^{-1}) was recorded in the presence of maltose 20 g l^{-1}. However in the presence of glucose 20 g l^{-1}, sucrose 20 g l^{-1} or lactose 20 g l^{-1} as sole carbon source only 6400 AU ml^{-1} were recorded. From these results, it can be concluded that glucose is only stimulating when present as a disaccharide (maltose) and not when incombination with fructose (as in sucrose) or gluconate (as in lactose). In case of nitrogen sources, the maximum growth was observed in yeast extract compared to other sources (Fig. 2).

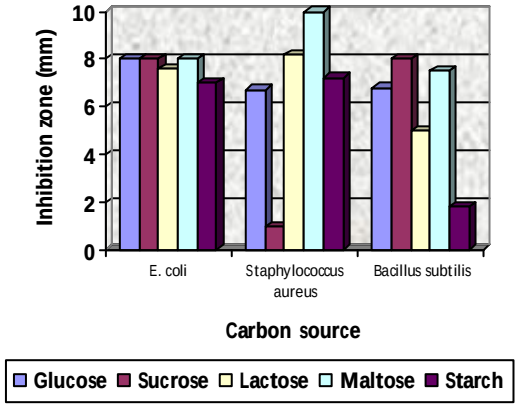

Fig. 1 Effect of carbon sources on bacteriocin production

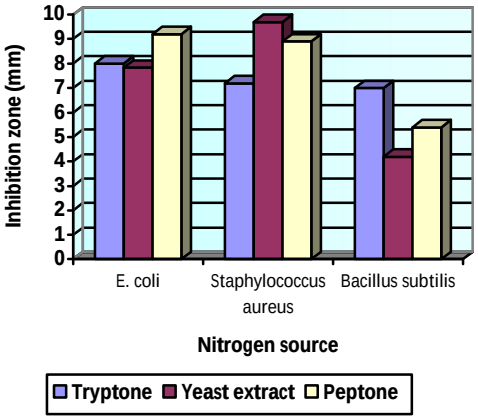

Fig. 2 Effect of nitrogen sources on bacteriocin production

The results obtained from this study the isolated lactic acid bacteria potent bacteriocin producer and

antimicrobial properties that exerts in the usage of this compound as a preservative for maintaining hygiene of fermented foods.

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