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Full Length Research Paper

Spectrum of antimicrobial activity of lactic acid bacteria (*Lactobacillus* KSBT 56) isolated from indigenous fermented products of Odisha

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The strain KSBT56 was isolated from a traditional fermented product '*dahi chhenna'* of Odisha by pour plate method on MRS agar. Biochemical and 16s rDNA sequencing confirmed KSBT 56 as genus *Lactobacillus*. The cell-free supernatants (CFS) of *Lactobacillus* KSBT56 demonstrated an antibacterial activity against a wide range of Gram-negative and Gram-positive pathogens (*Escherichia coli* MTCC 82*, Bacillus cereus* ATCC10702, *Salmonella* spp. (*Salmonella enteritidis* 125109*, Salmonella enterica* serovar *typhi* MTCC 3216, *Salmonella typhimurium* SB300), *Aeromonas hydrophila* ATCC 7966*,* and *Staphylococcus aureus* MTCC 96. In contrast, it did not inhibit any lactobacilli tested. The putative metabolite produced by this organism was heat stable, sensitive to various pH values, a low molecular mass (<3000 Da) and either insensitive or low level sensitive to enzymatic treatments and require an acidic condition to develop. The compound resembles defensin more closely rather than bacteriocin and microcin.

Key words: Lactic acid bacteria, antibacterial activity, indigenous product, bacteriocin, microcin, defensin.

INTRODUCTION

Lactic acid bacteria play an important role as starters in fermented dairy foods (Tripathy, 2006). These organisms constitute a heterogeneous group of microbes that normally includes members of the genus *Lactococcus, Lactobacillus,* and *Streptococcus* (Tripathy, 2006). Many species of LAB are used in combination as essential biocatalytic agents to carry out biochemical reactions under a variety of controlled conditions that result in formation of enormous fermented dairy foods. When present in such foods, LAB function as biopreservatives, preventing microbial spoilage and control pathogenic microorganisms through acidification, competition for essential nutrients, and/or production of a variety of other inhibitory metabolites (Tripathy, 2006). These organisms have been given the 'generally regarded as safe' (GRAS). In this paper we evaluate antimicrobial potential of indigenous *Lactobacillus* species followed by characterisation of the nature of antimicrobial compound produced by an indigenous isolate of *Lactobacillus* and tentative identification of the said culture.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study were listed in Table 1. Some reference *Lactobacillus* strains were kindly donated by Prof. Dr. K.J. Heller of Federal Research Institute for Nutrition and Food, Kiel, Germany. The *Lactobacillus* KSBT56 was isolated from a traditional indigenous fermented product '*Dahi Chhenna'* and maintained at KIIT School of Biotechnology culture collection (KSBT, Bhubaneswar, Odisha). This *Lactobacillus* culture collection from indigenous fermented product of Odisha is part of an ongoing project for the search of potential probiotic and bioactive peptide producing strain funded by Department of Science and Technology, Government of India. Preliminary characterization of KSBT56 isolate was performed as per Bergey's Manual (2009) and API 50 CH kit (BioMerieux). *Lactobacillus* strains were grown in

Table 1. Bacterial strains used in this study.

Bacterial species	Strain no	Culture provider	Origin
Lactobacillus cultures			
Lactobacillus casei	ATCC 9595	[a]ATCC	ATCC
Lactobacillus helveticus	MRI-Ki 92078	Dr.Knut Heller	Max-Rubner-Institut (MRI), Kiel. Germany
Lactobacillus johnsonii	MRI-Ki 92170	Dr.Knut Heller	Max-Rubner-Institute (MRI), Kiel. Germany
Lactobacillus gasseri	MRI-Ki 92272	Dr.Knut Heller	Max-Rubner-Institute, Kiel. Germany
Lactobacillus species	KSBT56	Isolated in this study	Dahi Chhenna (a traditional fermented product of Orissa)
Pathogens			
Escherichia coli	MTCC 82	[b]MTCC	MTCC
Staphylococcus aureus subsp.*aureus*	MTCC 96	MTCC	MTCC
Salmonella enterica serovar *typhi*	MTCC 3216	MTCC	MTCC
Aeromonas hydrophila	ATCC 7966	ATCC	ATCC
Bacillus cereus	ATCC 10702	ATCC	ATCC
Salmonella typhimurium (wild type)	SL1344 (SB300)	Dr. M.Suar	Institute of Microbiology, ETH Ho¨nggerberg, 8093 Zürich, Switzerland
Salmonella enteritidis	125109	Dr. M.Suar	Institute of Microbiology, ETH Ho¨nggerberg, 8093 Zürich, Switzerland

[a]ATCC: American Type Culture Collection, USA, [b]MTCC: Microbial Type Culture Collection, Chandigarh.

Lactobacillus MRS Broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) at 37°C for 24 and 48 h as per requirement. Indicator strains were grown in Luria Bertani broth (LB, Hi Media Laboratories Pvt. Ltd, Mumbai, India) at 37°C for 24 h. Stock cultures were stored in 50% glycerol, 50% MRS/LB at -80°C.

Physiological and molecular characterization of KSBT56

Sugar fermentation pattern were determined using API 50 CH kit from BioMereiux as per Manufacturer's instruction. The change of the indicator from purple to yellow was documented after 24 and 48 h. Only definitive changes were rated as positive results. These experiments were repeated three times. The formation of the lactate isomeres in the fermented broth was determined enzymatically using the DL-lactate test kit (Boehringer Mannheim, GmbH, Germany). Arginine hydrolysis was determined according the method as described by Briggs (1953). Cell morphology was studied using Phase-contrast microscopy. Gram staining was performed by using Gram staining Kit (Hi Media Laboratories Pvt. Ltd, Mumbai, India). For molecular characterisation (16s rDNA sequencing and phylogenetic tree) *Lactobacillus* KSBT 56 was sent to MTCC, Institute for Microbial Technology (IMTECH), Chandigarh. The 16s rDNA sequence was submitted at NCBI for getting accession no.

Preparation of cell-free supernatants

The *Lactobacillus* KSBT56 was incubated in MRS broth for 24 h at 37°C. Bacterial cells were removed by centrifuging the culture at 8000 rpm for 20 min at 5°C. The culture supernatants were filtered through a sterilized 0.22 µm MILLEX-GP filter, PES membrane (Millipore, Ireland) and stored at 4°C.

Antimicrobial assay

The antagonistic effects of culture supernatants of *Lactobacillus* KSBT56 isolate on indicator organisms like (*Escherichia coli* MTCC 82*, Bacillus cereus* ATCC10702, *Salmonella* spp. (*Salmonella enteritidis* 125109*, Salmonella enterica* serovar *typhi* MTCC 3216, *Salmonella typhimurium* SB300), *Aeromonas hydrophila* ATCC 7966 were tested by the agar-well-diffusion assay (Kaushik et al., 2009). These assays were performed in duplicate. Prepoured LB agar plates were overlaid with 10 ml of soft LB agar inoculated with 0.3 ml (~10^8 cfu/ml) of a 8-10 h grown culture of the indicator organism grown in LB broth at 37°C. Once solidified wells of 0.7 mm diameter were made with a sterile cork borer. 100 µl of the cell-free supernatant of KSBT56 was filled into each well. The plates were preincubated at 4°C to facilitate diffusion. Afterwards plates were kept aerobically at 37°C for 6 to 8 h in upright position and the diameter of the zone of inhibition was measured. The activity of the inhibitory substances was calculated by AU mL^{-1} (highest dilution that showed a distinct zone of inhibition × 1000 µl /volume (µl) used in the well).

Sensitivity to pH, heat, and proteolytic enzymes

The pH values of CFSs were adjusted to 4.0-6.5 by the addition of 0.1 N NaOH, filtered through a sterilized 0.22 µm cellulose acetate filter (Millipore, USA) and stored at 4°C. For heat inactivation, the CFS was heated at 100°C for 1 h. For the following experiments *S. typhimurium* SB300 was taken as indicator strain. To test the sensitivity of proteases, the CFS was incubated at 37°C for 1 h with and without pepsin (200 µg/ml), trypsin (200 µg/ml), chymotrypsin (200 µg/ml) and proteinase K (200 µg/ml) (Sigma, St. Louis, MO, USA). To determine whether the lactic acid in the KSBT56-CFS participates in the antimicrobial activity, the KSBT56-CFS was subjected to lactate dehydrogenase (LDH) treatment (250 µg/ml, 2 h at 37°C). Catalase (5 mg/ml) was added to the CFS to eliminate the inhibitory activity from the hydrogen peroxide if any. The remaining activity in both treated and untreated samples was determined by the agar well diffusion method (Kaushik et al., 2009).

RESULTS AND DISCUSSION

Cell morphology, physiological and biochemical properties

The KSBT56 isolate was obtained from a traditional

Table 2. Physiological and biochemical characteristics of *Lactobacillus* KSBT56.

Characteristic	*L. pentosus* ATCC 8041	*L. pentosus* (Bergey's Manual)$	*Lactobacillus* KSBT 56	*L. plantarum* MTCC 1407
Growth at 15°C/45°C	+/-	+/-	+/-	+/-
Amygdalin	+	+	+	+
DL-Arabinose	+	+	+	+
Cellobiose	+	+	+	d
Esculin	+	ND	+	+
Gluconate	+	+	+	+
Mannitol	+	+	+	+
Melezitose	+	d	+	+
Melibiose	+	+	+	+
Raffinose	+	+	+	+
Ribose	+	+	+	+
Sorbitol	+	+	+	+
Sucrose	+	+	+	+
Xylose	+	+	+	+

Symbols and abbreviations: +, 90% or more of strains are positive; -, 90% or more of strains are negative; d: 11-89% of strains are positive; ND: Data not available, $: Data are from Bergey's Manual (2009).

indigenous fermented product of Odisha '*Dahi Chhenna'*. On MRS (Pour plate), the colonies of KSBT56 appeared as yellowish, lens shaped and, after 3 days growth, they were 1 mm in diameter. On streak plate in MRS agar, the colonies were small, round and appeared whitish. The cells were Gram-positive, nonsporeforming, nonmotile very small rods (cocobacilli) that occurred singly or in pairs. They were facultatively heterofermentative, catalase negative. Characterization by 16s rDNA sequence analysis and phylogenetic tree (Figure 3), demonstrated 100% match with *Lactobacillus pentosus* and 99.9% with *Lactobacillus plantarum*. Further physiological and biochemical characteristics of KSBT56 strain was performed with API 50CH kit and results matched with 100% identity with both *L. plantarum* and *L. pentosus* reference strains (Table 2) (Bergey's manual of systematic bacteriology, 2009). The above result is not surprising because as reported by several authors (Osawa et al., 2000; Torriani et al., 2001) the species *L. plantarum, L. pentosus* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. Further confirmatory test by molecular typing method (Torriani et al., 2001; Van Reenen and Dicks, 1996) is required for species identification. Therefore at present we could not report exact species for KSBT 56. The 16s rDNA sequence has been deposited in the NCBI genebank database (Accession No. JQ669800).

Antimicrobial activity

The *Lactobacillus* KSBT56 exhibited antimicrobial activity against all the seven target organisms tested (Figure 1a and b). The KSBT56 isolate was obtained from '*Dahi chhenna'* (a traditional fermented product of Odisha). Table 3 depicts the antibacterial activity of filtered supernatant and heat inactivated supernatant of KSBT56. The diameters of inhibition zones were greater than 7.3 mm. This result was in agreement with several previous studies (Lin and Pan, 2002; Schillinger and Lucke, 1989; Coconnier et al., 1997). Schillinger and Lucke (1989) observed the antagonistic activity of *Lactobacillus sake* isolated from meat whereas Lin and Pan (2002) observed the same for *Lactobacillus paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU102 isolated from infant feces and home-made Korean-style cabbage pickles respectively. In India Kaushik et al. (2009) observed antimicrobial potential of *L. plantarum* 9 (isolated from buffalo milk of Karnal, Haryana) against several Gram positive and Gram negative pathogens. However we could not get any reference from Odisha regarding antimicrobial potential of *Lactobacilli*. Therefore, to our knowledge this is the first report demonstrating antimicrobial potential of lactobacillus species. The CFS of KSBT56 also demonstrated an antibacterial activity active *in vitro* against an enteroinvasive pathogen (*S. typhimurium*, wild type, SL1344 (SB300)) which adheres to and enters cultured human enterocytic cells (data not shown). Coconnier et al. (1997) inferred similar results of human *Lactobacillus acidophilus* Strain LB both *in vitro* and *in vivo*.

Sensitivity to pH, heat, and proteinase

The pH values of CFSs were adjusted to 4.5, 5.0, 5.5, 6.0, and 6.5 by the addition of 0.1 N NaOH solution. The inhibition zones of all the cultures measured were

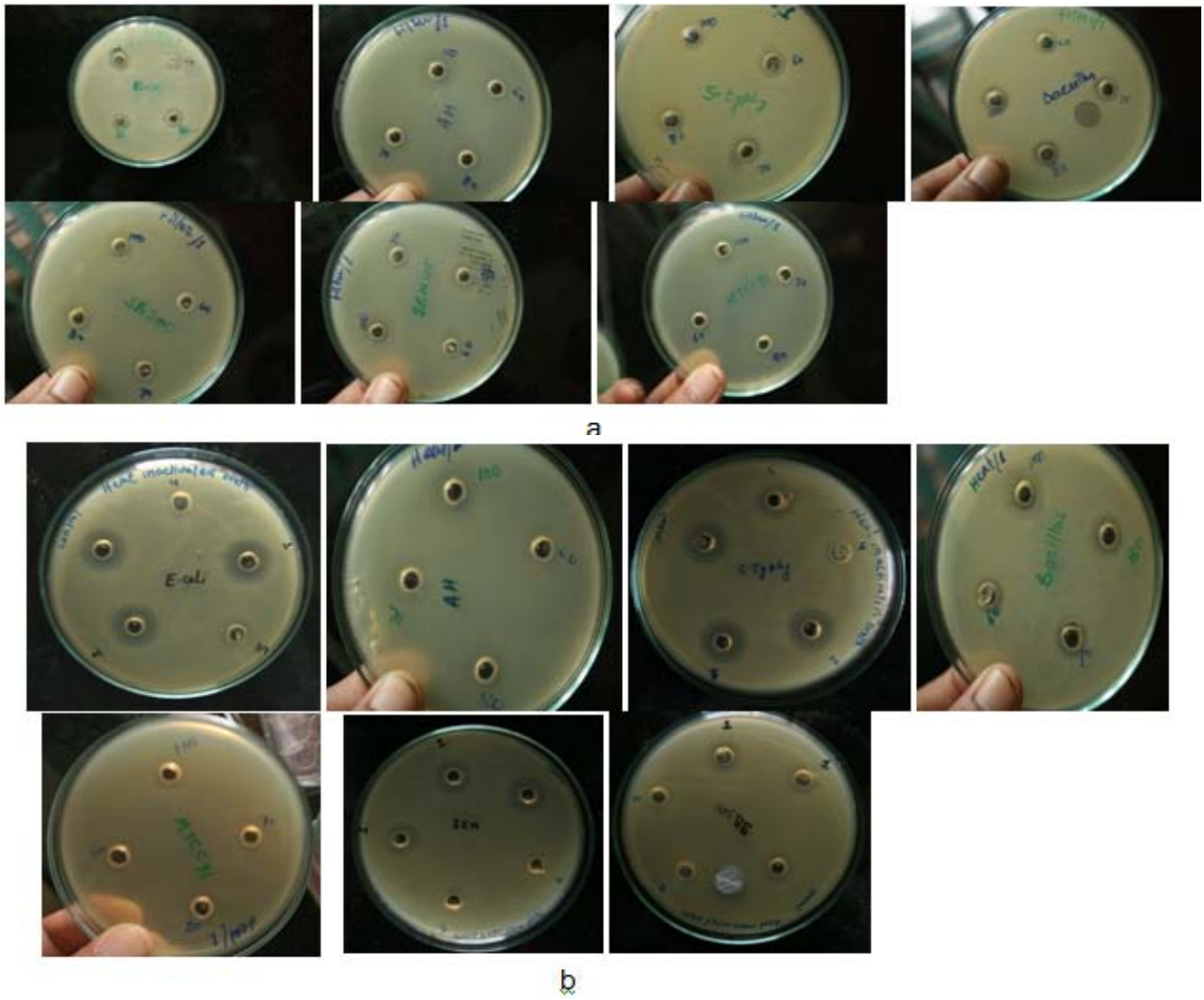

Figure 1. (a) Antibacterial activity of filter sterilized MRS broth supernatant of *Lactobacillus* KSBT56 against (i) *E. coli* MTCC 82 (ii) *Aeromonas hydrophila* ATCC 7966 (iii) *Salmonella enterica* serovar *typhi* MTCC 3216, (iv) *Bacillus cereus* ATCC10702, (v) *Salmonella typhimurium* SB300, (vi) *Salmonella enteritidis* 125109 and (g) *Staphylococcus aureus* subsp. *aureus* MTCC 96. The no. 1 represents strain *Lactobacillus* KSBT56. CFS was loaded at different concentrations (60, 70, 80 and 100 µl) in separate wells. (b) Antibacterial activity of heat inactivated MRS broth supernatant of *Lactobacillus* KSBT56 against (i) *E. coli* MTCC 82 (ii) *Aeromonas hydrophila* ATCC 7966 (iii) *Salmonella enterica* serovar *typhi* MTCC 3216, (iv) *Bacillus cereus* ATCC10702, (v) *Salmonella typhimurium* SB300, (vi) *Salmonella enteritidis* 125109 and (g) *Staphylococcus aureus* subsp. *aureus* MTCC 96. The no. 1 represents strain *Lactobacillus* KSBT56. CFS was loaded at different concentrations (60, 70, 80 and 100 µl) in separate wells. Control represents heat inactivated supernatant of *Lactobacillus casei* ATCC 9595. Well 4 and 5 represents only MRS broth.

decreased as the increase of pH and lost the inhibitory activity either at pH 5.0 or above (Table 4). These results indicate that the antimicrobial substances required an acidic environment to optimally develop its activity. Heat treatment at 100°C for 1 h did not lead to loss of inhibitory activity on indicator strains (Table 3 and Figure 1b) suggested that the antibacterial substance produced by *Lactobacillus* KSBT 56 was a heat stable component. The activity decreased slightly after enzymatic treatments with pepsin (20% decrease of activity) whereas treatment with trypsin, chymotrypsin, and proteinase K did not lead to loss of any inhibitory activity. We assumed that the CFSs produced by this strain might have some anti-bacterial activities. Lactate dehydrogenase (LDH) degrades lactic acid. Treatment with LDH also leads to no change of activity of the antibacterial substances indicating lactic acid may not participate in the study. Also treatments with DL-lactic acid at different concentrations (200 to 250 mM) demonstrated no activity upon the indicator strain (Figure 2). Lactic acid present in the CFS

Table 3. Spectrum of antibacterial activity of *Lactobacillus* sp. against different pathogens.

Supernatant (cell free and heat inactivated) of Lactobacillus	Indicator Organisms showing Inhibition zones (diameter in mm)						
	***B. cereus* ATCC 10702**	***S. typhi* MTCC 3216**	***S. aureus* MTCC 96**	***A. hydrophila* ATCC 7966**	***E. coli* MTCC 82**	***S. typhimurium* SB300**	***S. enteritidis* 125109**
Control (*L. casei* ATCC 9595)	20 ± 0.7	19 ± 0.0	17 ± 0.56	18± 0.14	18 ± 0.84	16 ± 0.7	17 ± 0.0
Lactobacillus KSBT 56 (unfiltered)	19.5 ± 0.35	16 ± 0.21	16 ± 0.0	22 ± 0.7	18 ± 0.0	16.5 ± 0.0	16.5 ± 0.35
Lactobacillus KSBT 56 (filtered, 0.22 µm)	17 ± 0.0	13 ± 0.84	16 ± 0.0	20 ± 0.0	16 ± 0.0	16 ± 0.7	16 ± 0.7
Heat inactivated	19 ± 0.7	18.5 ± 0.0	15 ± 0.0	20 ± 0.42	18.5 ± 0.35	14.5 ± 0.0	15.5 ± 0.49

*Inhibition zone (diameter in mm) includes the disc diameter (7 mm), the values are means ± standard deviations for duplicate. *L. casei* ATCC 9595 was taken as reference.

Table 4. Effect of pH on antimicrobial activity of cell-free supernatants of *Lactobacillus casei* ATCC 9595 (Control) and *Lactobacillus* KSBT 56.

	Inhibition zone (diameter in mm)*													
	Bacillus cereus ATCC 10702		*S. typhi* MTCC 3216		*S. aureus* MTCC 96		*Aeromonas hydrophila* ATCC 7966		*E. coli* MTCC 82		*S. typhimurium* SB300		*S. enteritidis* 125109	
	Control	KSBT 56 (filtered, 0.22 µm)	Control	KSBT 56 (Filtered, 0.22 µm)	Control (*L. casei* ATCC 9595)	KSBT 56 (filtered, 0.22 µm)	Control	KSBT 56 (filtered, 0.22 µm	Control	KSBT 56 (filtered, 0.22 µm	Control	KSBT 56 (filtered, 0.22 µm	Control	KSBT 56 (filtered, 0.22 µm)
Control (pH 4.0 to 4.05)	20 ± 0.7	19.5 ± 0.35	19±0.4	16±0.2	17±0.6	16±0.3	18±0.7	20±0.4	18±0.3	16±0.4	16±0.1	16±0.3	17±0.4	15.6±0.2
pH 4.5	18.6 ± 0.7	18.3± 0.35	17±0.5	16±0.3	17±0.5	16±0.2	18±0.2	20±0.3	17.8±0.1	16±0.3	15.5±0.3	15.3±0.4	14±0.1	15±0.2
pH 5.0	7.6±0.1	8.1±0.3	-	-	-	-	-	-	8.3±0.1	8.6±0.2	-	-	-	-
pH 5.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 6.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Inhibition zone (diameter in mm) includes the disc diameter (7 mm), the values are means ± standard deviations for duplicate.

were in the range of 55 to 140 mM at different time period which was less than the active concentration against different pathogens. These results revealed that the inhibitory agents in the CFSs were sensitive to various pH values, pepsin, but not to heat and other proteolytic enzymes. Lactic acid and hydrogen peroxide were not participating in the antagonistic activity. These results indicated that the metabolites produced by *Lactobacillus* KSBT56 had quite a few characteristics common with those of the antimicrobial substances produced by other lactobacilli, such as insensitive or low-level sensitivity to proteases, a low molecular mass (<3 kDa); active against both gram positive and gram-negative bacteria *in vitro* and require an acidic conditions to develop (Hamdan and Micolajcik, 1974; Scolari et al., 1993; Silva et al., 1987; Vescovo et al., 1993). Coconnier et al. (1997) observed that similar compound was produced by *L. acidophilus* strain LB and reported that the compound is similar to defensin (small cationic peptides). Defensins are known to develop activity *in vivo* against microbes ingested by neutrophils in the phagosomal vacuoles which present an acidic environment (Litteri and Romeo, 1993; Skerlavaj et al., 1990). Our isolate produced antibacterial activity at low pH and also observed loss of activity after the addition of pepsin. These results corroborated with the findings of Lin and Pan

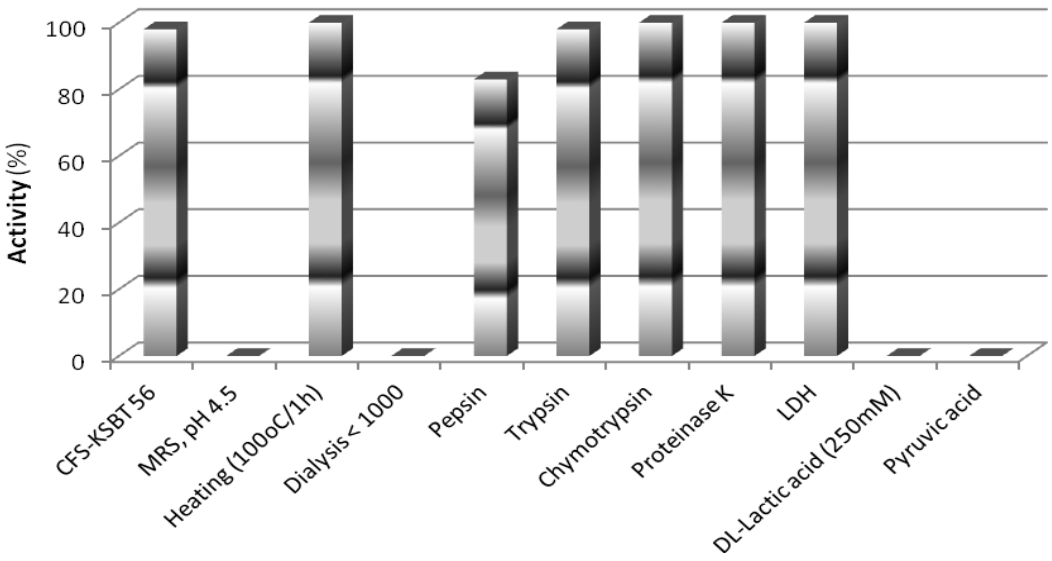

Figure 2. Effects of physical and chemical treatments on the antimicrobial activity of CFS of KSBT 56. *S. typhimurium* SB300 was taken as indicator strain. Experimental conditions are described in the text. Each value shown is the mean from three experiments. The variation in reproducibility was less than 5%. Dialysis with a molecular mass cut-off of 1,000 Da was used.

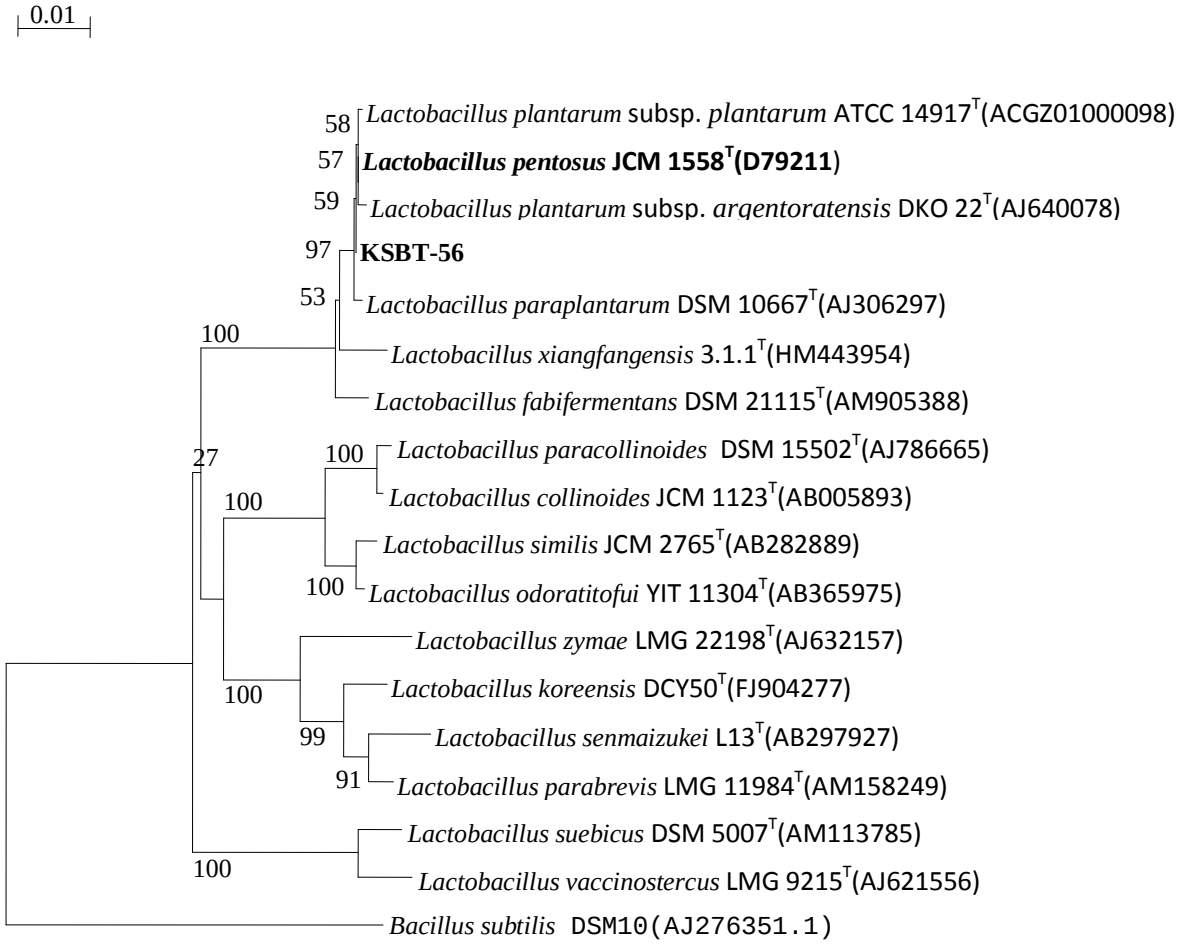

Figure 3. Phylogenetic tree constructed from 16s rRNA sequencing analysis of *Lactobacillus* KSBT 56 (Carried out At MTCC, Chandigarh).

(2002) and Batdorj et al. (2006). The isolate also did not show any activity against other lactobacillus culture tested. Thus, we assumed that the antibacterial substances produced by *Lactobacillus* KSBT 56 resembles closely todefensin rather than microcin and bacteriocin. The KSBT 56 also demonstrates as a good probiotic organism (Saini, 2011) and encourages us to isolate more such strains from traditional food product of Odisha.

Conclusion

Our results explained the in vitro antibacterial potential of indigenous Lactobacillus KSBT56 strain LB against both gram-negative and gram-positive pathogen including fish pathogen *Aeromonas hydrophila*. The antibacterial substance differs from both bacteriocin and microcin by definition and resembles more closely with defensin. Thus we believe that the components secreted by KSBT 56 could contain a novel peptide. Further purification and characterization of this substance is in progress in our laboratory.

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