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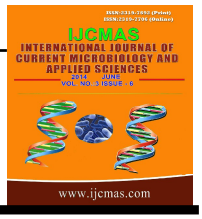


Original Research Article

Bioactive potential assessment of antibacterial peptide produced by *Lactobacillus* isolated from milk and milk products

D.P. Mohanty[1], P. Tripathy[1], S. Mohapatra[2] and D.P. Samantaray[2*]

[1]KIIT School of Biotechnology, KIIT University, Bhubaneswar, Odisha
[2]Department of Microbiology, OUAT, Bhubaneswar-3, Odisha, India
Corresponding author

Keywords

Antimicrobial; Bioactive; Probiotic and Peptide.

A B S T R A C T

Bioactive peptides are specific protein fragments present in milk which regulate the gastrointestinal development, infant development, immunological function and microbial activity including antibiotic and probiotic action. Research in this topic, not only to isolate and characterize a novel strain of *Lactobacillus*KSBT46 from milk and milk products but also to produce bioactive peptide by the native bacterial isolates. The cell free supernatant (CFSs) showed an antimicrobial activity against wide range of both Gram negative and Gram positive pathogenic bacteria such as *Escherichia coli* MTCC82, *Bacillus cereus* ATCC10702, *Salmonella enteritidis* 125109, *Salmonella typhi* MTCC3216, *Salmonella typhimurium* SB300, *Aeromonas hydrophila* ATCC7966 and *Staphylococcus aureus* MTCC96. The antimicrobial bioactive peptide was purified from milk by salt precipitation and ion exchange chromatography. This novel antimicrobial bioactive peptide may be used as chemotherapeutic agent or bio-preservatives in foods.

Introduction

Now a days, functional foods and bioactive components in foods have gained a lot of attention and interest of researchers and consumers. A functional food has various physiological (Schanbacher et al., 1998; Korhone n and Pihlanto-Leppala, 2004) benefits and can reduce the risk of chronic diseases. Recent studies (Gobbetti et al,. 2007) have shown that a broad range of biologically active compounds such as antibacterial peptides, immunoglobulin, antimicrobial proteins, oligosaccharides and lipids present in milk, that give protection against pathogens. Milk contains a wide range of bioactive peptides that have positive impact on body function and in due course influence health. Biologically active peptides derived from dairy products are initially found in inactive form within the sequence of the precursor protein but can be released by hydrolysis of the precursor molecules by digestive protease enzyme or proteolysis by proteolytic microbes. Once these bioactive peptides are released either by digestion or proteolysis, they may

impact beneficial effects on human health such asgastrointestinal development, infant development, immunological development and antimicrobial activity (Gobbetti et al., 2007).

Microbes compete for the limited space and nutrients present in natural ecological niches, therefore they have developed production of antimicrobial peptides especially bacteriocin (Heng et al., 2007) for their survival. Lactobacillus (LAB) is fermentative bacteria generally present in gastrointestinal tract of human and milkis now being increasingly studied for production of different antimicrobial peptides.

LAB strains are potentially promising because they generate bactericidal bioactive peptides (bacteriocins) and enzymes that are able to control biofilm formation and growth of pathogens (Millette et al., 2006). In this investigation an attempt has been made to explore a low molecular bioactive peptide from cow milk by using a novel *Lactobacillus* (KSBT46). This novel peptide maybe used as chemotherapeutic agent or bio-preservatives.

Materials and Methods

Sample Collection

Representative milk samples were collected aseptically in sealed, sterile containers from different places of Odisha, then the samples were transported and processed immediately in the laboratory for bacteriological analysis. All the culture media, chemicals and reagents used during the investigation were obtained from Hi-Media Laboratories Pvt. Ltd, Mumbai, India and were prepared as per the manufacturer's instruction by using distilled water.

Bacteriological analysis of sample

One ml of milk sample was tenfold serially diluted in 0.89% of normal saline solution and poured on Skim milk agar (SMA) plates and incubated at 37ºC for 24-48 hours. Single submerged colonies of different morphologies including branched, circular and rhizoidal forms were individually picked and sub cultured on SMA plates to obtain pure culture. Then the bacterial isolates were maintained on SMA slants and preserved for further use. Identification of bacterial isolates were done on the basis of their colony characteristics on the Gram's reaction, sugar utilization tests and enzymatic activities shown by the isolates. Gram staining followed by microscopic observation of all the bacterial isolates was done to know the Gram variability, morphology and arrangement of bacteria. Biochemical test of Gram-positive bacterial isolates was carried out by using API 50 CH kit (Biomeriux, France) as per manufacturer's instruction and following Bergey's manual of systematic bacteriology (1994).

Antagonistic profile of bacterial isolate

Antagonistic profile of bacterial isolates were studied in order to observe the inhibitory activity against standard pathogens such as *Escherichia coli* MTCC82, *Bacillus cereus* ATCC10702, *Salmonella enteritidis* 125109, *Salmonella typhi* MTCC3216, *Salmonella typhimurium* SB300, *Aeromonas hydrophila* ATCC7966 and *Staphylococcus aureus* MTCC96. For this qualitative study lawn culture of pathogenic bacterial isolates were prepared in LB agar plates then *Lactobacillus* bacterial isolates were inoculated to the well (Al-Allaf et al., 2009) and incubated at 37^0C for 24-48hrs. Finally the zone of inhibition was measured.

Antibacterial peptide assay

The isolate showing antibacterial activity inoculated in skimmed milk broth and incubated at 37°C for 24hrs. Then the sample was centrifuged at 8000 rpm/5°C for 20 minutes and supernatants were allowed to pass through 30kda centricon. Supernatant containing peptides of molecular weight less than 30kda were collected and sterilized by using membrane filter of 0.22 μm (MILLEX-GP filter). The filter sterilized supernatant was heat killed and subjected for assay. Fresh culture of standard pathogenic bacterial isolates were lawn cultured in LB agar plates and 100μl of filter sterilized supernatant was added into well then the plates were incubated in the incubator at 37^0C for 24hrs. Finally the zone of inhibition was measured.

Purification of antibacterial peptide

For purification of antibacterial peptide, supernatant containing peptide of molecular weight less than 30kda was precipitated by using 70% saturated ammonium sulphate and the suspension was incubated at 4°C for 24hrs with continuous stirring. Then the suspension was centrifuged at 10000g for 20 minute and pallet was dissolved in 10 ml phosphate buffer (pH 7.0). The suspension was desalted by dialysis with phosphate buffer at 4°C for 12hrs by using benzoylated membranes and the sample was subjected to column chromatography.20ml of sp-sephadex C50 resin was packed in column and equilibrated by 100-200ml 1XPBS pH-7.0. Sample was loaded and fractions were collected. Illusion of peptide was done with 0.01N, 0.05N, 0.1N, 0.5N and 1N NaCl, then all the fractions were tested for antibacterial activity against pathogens. The peptide fraction showing antimicrobial activity was determined by Bradford method. Then the low molecular weight peptide showed antibacterial activity was confirmed by Tricine SDS PAGE (16.5% separating gel and 4% stacking gel).

Results and Discussion

Bacteriological analysis

Fifty four bacteria were isolated from the collected milk sample by using the selective culture media skim milk agar. Gram staining followed by microscopic observation result indicated that, all the bacterial isolates are Gram positive and rod shaped. The primary identification result observed from catalase and oxidase test, which revealed that all the bacterial isolates are catalase negative, oxidase positive, nonmotile, non-spore forming and categorized in to one genera Lactobacillus. Then the antagonistic profile of 54 bacterial isolate were studied in order to observe the inhibitory activity against standard pathogens. However, the bacterial isolate showed highest antibacterial activity identified (Table 1) as *Lactobacillus* KSBT46 by using API 50CH kit and the result matched with Bergey's manual of systematic bacteriology. Similar result was also observed by Agrawall and Prakash (2013), who isolated 13 lactic acid bacteria (LAB) from dairy products and identified as *Lactococcus sp.*by biochemical characterization. Presence of LAB in milk and milk products was also observed by Yodoamijoyo et al., (1983) in Idonesie, Hamama (1992) in Morocco, Isono et al., (1994) in Tanzania, Beukes et al., (2001) in South Africa. The predominant bacteria of dairy & dairy products are different spices *Lactobacillus,* because it is the normal microflora of milk and the have

complex nutritional needs for different kinds of amino acids and vitamins for growth. It also requires sugar lactose for growth, which is rich in milk. The heterogeneous group of the lactic bacteria observed, would contribute to the hygienic quality and organoleptic of the milk fermented traditionally, that justifies the importance of his consumption

Antagonistic profile of LAB

Out of 54 species of Lactic acid bacteria tested, 16 bacterial isolates showed (fig.1) antibacterial activity. However *Lactobacillus* KSBT46 showed highest zone of inhibition (Table 2) against standard pathogens such as *Escherichia coli* MTCC 82 (18mm), *Bacillus cereus* ATCC10702 (15mm), *Salmonella enteritidis* 125109 (18mm), *Salmonella typhi* MTCC 3216 (16mm), *Salmonella typhimurium* SB 300 (17mm), *Aeromonas hydrophila* ATCC 7966 (16mm) and *Staphylococcus aureus* MTCC 96 (16mm) respectively. These results indicate that the lactic acid bacteria are capable of synthesizing substances inhibiting growth of pathogenic bacteria.This anti-bacterial substances produced by LAB generally protein in nature (Klaenhammer, 1993; Jimenez-Diaz et al., 1993; Vandenbergh, 1993). However the efficiency of the inhibitory substance effect varies according to serotypes of LAB (Savodogo et al., 2004).

Antibacterial peptide assay

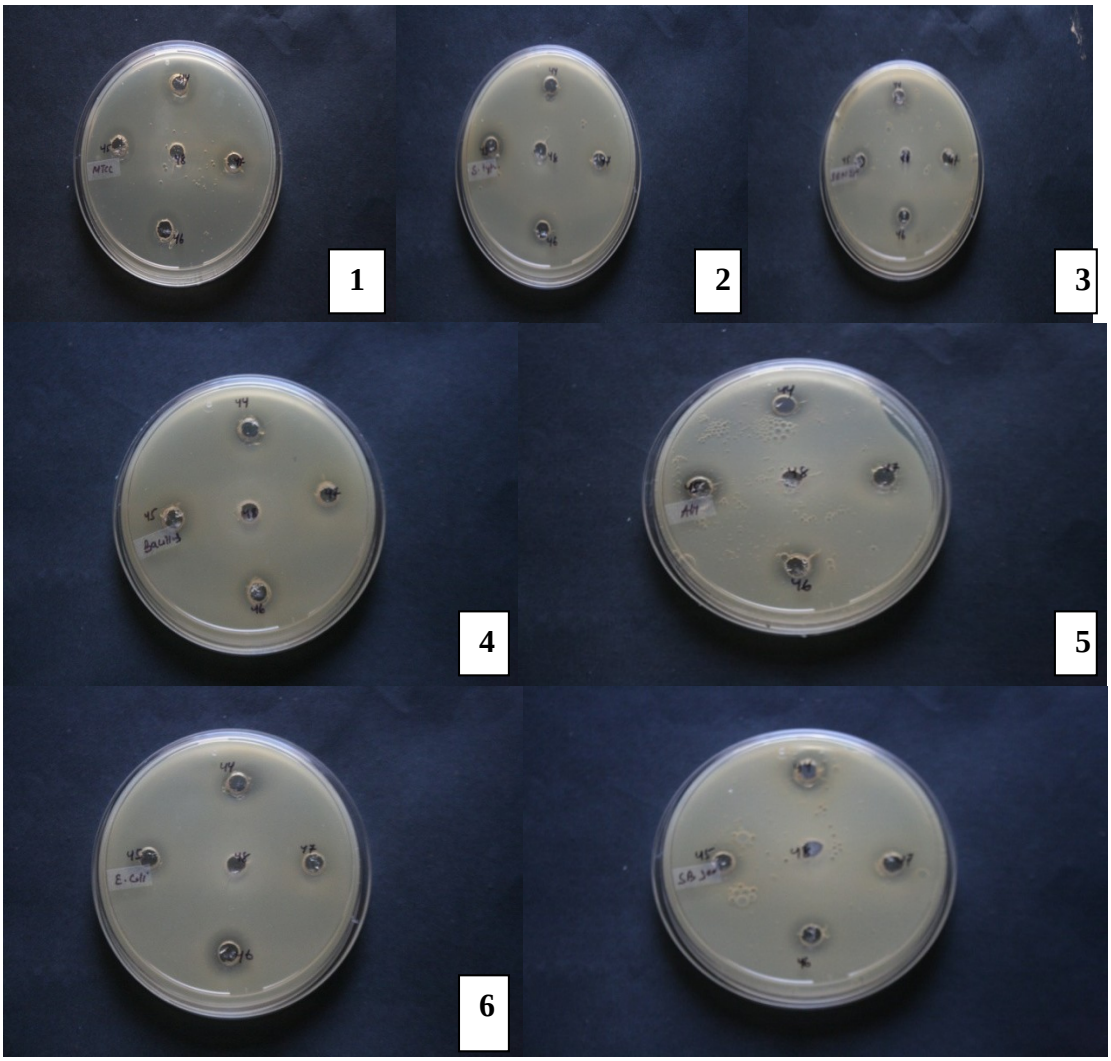
Ion-exchange chromatography data revealed that, out of the three fractions such as wash, elution 1, elution 2; elution1 of *Lactobacillus*KSBT46 showed zone of inhibition against all the tested pathogens as *E. coli* MTCC82 (16mm), *Aeromonas hydrophila* ATCC7966 (10mm), *Salmonella typhi* MTCC3216 (16mm), *Bacillus cereus* ATCC10702 (14mm), *Salmonella typhimurium* SB300 (16mm), *Salmonella enteritidis* 125109 (14mm), *Staphylococcus aureus* MTCC (13mm) respectively. However, no zone of inhibition was observed in case of elution 2. In SDS PAGE, the purified peptide fractions of *Lactobacillus* KSBT 46 was 570 µg/ml and showing low molecular weight (<30 kda). This low molecular weight peptide fraction showed antibacterial activity against the seven tested pathogens (Fig. 2). Similar result was also observed by Klaenhammer (1993) on *L. brevis* DSM9296 on one hand looked 9 mm of diameter of inhibition with *E. faecalis*, 16 mm of diameter of inhibition with *Streptococcus xylosus*; on the other hand on *L. lactis* 99 looked 15 mm of diameter of inhibition with *Bacillus linens* SR3, 10 mm of diameter of inhibition with *Streptococcus xylosus*, 4 mm on to *Staphylococcus aureus*. Thus it is concluded that the low molecular peptide present in milk and milk products showed antibacterial property against pathogenic microbes.

Biologically active peptides produced from milk and milk products have very healthy impact on human physiology and give protection against different type of pathogens. When milk derived proteins degrades due to some enzymatic activity (mostly the predominate lactobacillus) and forms peptides that shows multifunctional properties including antimicrobial, immune-modulatory, inhibition of angiotensin converting enzyme (ACE), antithrombotic, antioxidant, opioid agonistic and antagonistic activity. Milk is one of the rich sources of bioactive peptides which may influence immunological, gastrointestinal, hormonal, neurological, nutritional response.

Table.1 Biochemical characteristics of *Lactobacillus* KSBT46

Sl. No.	Biochemical Tests	Observation (24hr.)	Observation (48hr.)
1	Glycerol	-	-
2	Erythritol	-	-
3	D-arabinose	-	-
4	L-arabinose	-	-
5	D-ribose	+	+
6	D-xylose	-	-
7	L-xylose	-	-
8	D-adonitol	-	-
9	Mythyl-BD-xylopyranoside	-	-
10	D-galactose	+	+
11	D-glucose	+	+
12	D-fructose	+	+
13	D-mannose	+	+
14	L-sorbose	-	-
15	L-rhamnose	-	-
16	Dulcitol	-	-
17	Inositol	-	-
18	D-manitol	+	+
19	D-sorbitol	-	+
20	Methyl-aD-mannopyranoside	-	-
21	Methyl -aD-glucopyranoside	-	+
22	N-acetylglucosamine	+	+
23	Amygdalin	+	+
24	Arbutin	+	+
25	Esculin ferric citrate	-	-
26	Salicin	+	+
27	D-celiobiose	+	+
28	D-maltose	+	+
29	D-lactose(bovine origine)	+	+
30	D-melibiose	+	+
31	D-sachharose(sucrose)	+	+
32	D-trehalose	-	_
33	Inuline	-	_
34	D-melizitose	-	-
35	D-raffinose	-	+
36	Amidon(starch)	-	-
37	Glycogen	-	-
38	Xylitol	-	-
39	Gentibiose	+	+
40	D-turranose	+	+
41	D-lyxose	-	_
42	D-tagatose	-	-
43	d-fucose	-	-
44	L-fucose	-	-
45	D-arabitol	-	-
46	L-arabitol	-	-
47	Potassium gluconate	-	-
48	Potassium 2-ketogluconate	-	-
49	Potassium 5- ketogluconate	-	-

Fig.1 Antibacterial activity of Lactobacillus KSBT46



NB: Antibacterial activity of filter sterilized supernatant of *Lactobacillus* KSBT46 against (1) *E. coli*MTCC82 (2) *Aeromonas hydrophila* ATCC7966 (3) *Salmonella typhi* MTCC3216, (4) *Bacillus cereus* ATCC10702, (5) *Salmonella typhimurium* SB300, (6) *Salmonella enteritidis* 125109 (7) *Staphylococcus aureus* MTCC 96.

Table.2 Antagonistic activity of *Lactobacillus* KSBT46 against pathogens

Isolate No.	Zone of Inhibition in mm						
	E. coli MTCC 82	*B. cereus* ATCC 10702	*S. typhi* MTCC 3216	*S. typhimurium* SB 300	*S. enteritidis* 125109	*S. aureus* MTCC 96	*A. hydrophila* ATCC 7966
11	12	15	12	14	11	13	12
12	14	12	13	13	14	13	12
13	14	14	13	16	12	12	16
14	16	15	15	12	17	15	10
15	15	14	13	11	13	14	13
16	13	14	13	13	13	13	11
19	15	13	14	12	12	11	11
37	13	13	13	11	14	10	14
38	14	15	13	13	15	13	13
40	13	14	14	13	13	12	14
41	13	13	12	14	12	15	12
43	15	14	12	12	13	14	13
44	13	12	14	14	16	16	13
45	14	15	13	11	11	14	11
46	**18**	**15**	**16**	**17**	**18**	**16**	**16**
48	12	12	11	13	13	13	12

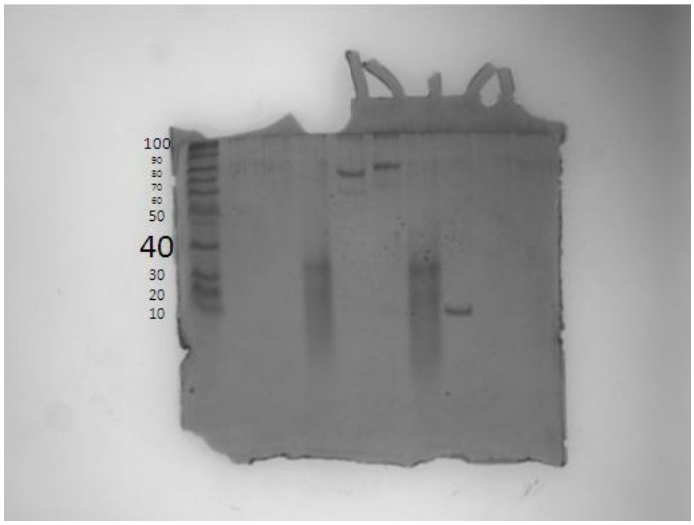


Fig.2 SDS-PAGE of supernatant and peptide fractions of Lactobacillus KSBT46

It may function as dietary and pharmaceutical supplements, healthcare products, chemotherapeutical or bio preservatives. The heterogeneous group of the lactic bacteria observed, would contribute to the hygienic quality and organoleptic of the milk fermented traditionally, that justifies the importance of his consumption.

So to carry-out this experiment we have isolated 54 bacterial isolates 16 isolates showed antimicrobial activity against standard pathogens. Then these 16 bacterial isolates were taken for anagonistic profiling where out of 16 bacterial isolates *Lactobacillus* KSBT46 showed highest antibacterial activity by using API 50CH kit *Escherichia coli* MTCC 82 (18mm), *Bacillus cereus* ATCC10702 (15mm), *Salmonella enteritidis* 125109 (18mm), *Salmonella typhi* MTCC 3216 (16mm),*Salmonella typhimurium* SB 300 (17mm), *Aeromonas hydrophila* ATCC 7966 (16mm) and *Staphylococcus aureus* MTCC 96 (16mm) This crude peptides were purified by ion-exchange chromatography and the anti bacterial peptide assay revealed that the highest inhibition was observed against *Salmonella sp.* and lowest inhibition was observed again*st Aeromona sp.* To ensure this potential eluted bioactive peptides SDS page carried out which specify it molecular weight is <30 kda (in 570 µg/ml).

The cell-free supernatants and peptide of *Lactobacillus* KSBT46 exhibited antibacterial activity. Lactic acid bacteria have an essential role in most foodand beverage fermentation processes, one of theearliest known food preservation of fermented foods andbeverage.The bacterial isolate can positively have impact ontheir use as starter cultures for traditional fermentedfoods, with a view to improving the hygiene and safety offermented milk so produced. Antibacterial compounds produced by LAB have provided the bacteria with a competitive advantageover other bacterial isolate. Thus, further studies will be focused on thecharacterization and other applications of peptide.

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