Therapeutic intervention and molecular characterizations of bacteriocin producing *Lysinibacillus* sp., nov., isolated from food sample

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Abstract: Many bacteriocins from *Lactobacilli* have been reported as immunostimulatory, preservatives, anticancerous and biocontroling. However, antimicrobial potential of *Lysinibacillus* is not much reported. In this study, an attempt was made to isolate and anticipate therapeutic potential of *Lysinibacillus* from spoiled food sample. We screened 125 *Lactobacilli* for their antagonistic nature against food borne and disease causing bacterial and fungal pathogens. Among them, one *Bacillus* was phenotypically, and 16S rRNA based, molecularly identified as *Lysinibacillus* species given with accession numbers JX416855 in NCBI. The type strain JX416855 has shown the 99% identity with the *Lysinibacillus fusiformis, Lysinibacillus sphaericus and Lysinibacillus xylanilyticus.* It was amylase, protease, gelatinase, nitrate reductase and urease negative and catalase positive. The growth conditions and bacteriocin activity were found optimum with MRS media at pH 7-10, Temp-35-40^oC and salt tolerance at 1-3% which was optimized with MRS broth at pH 7.4, 37 $^{\circ}C$, 1.5% NaCl for 48 h in shaking conditions @ 100 rpm. The isolate showed broad-spectrum antibacterial activity against gram positive (10-13mm) and gram-negative (20mm) bacteria. It also strongly inhibited to fungus *Aspergillus*, *Fusarium* and *Trichoderma*. Bacteriocin from 60% ammonium sulphate fraction strongly inhibited to gram-negative *R. planticola* and *Pseudomonas aeruginosa,* which showed three protein bands of high molecular weight (nearly 40-70kD) by SDS-PAGE analysis.

Keywords: *Lysinibacillus*; Bacteriocin; 16S r RNA gene; Phylogenetic analysis

INTRODUCTION

Lactic acid bacteria are generally recognized as safe. They play a significant role to antagonize microbial pathogens causing diseases and deterioration of food. The inhibitory effect exerted by LAB is mainly due to production of signaling and protective molecules like organic acids, hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas *et al*., 2001). Bacteriocin from lactic acid bacteria have been studied extensively as therapeutic and food preservative agents (Cotter *et al*., 2005; Jack *et al*, 1995; Tagg *et al*., 1976). It is ribosomally synthesized antimicrobial protein that are functionally diverse in terms of molecular size, mode of action, biosynthesis, self protection mechanisms, antimicrobial potential and spectrum (Quadri *et al*., 2003). Most bacteriocins are classified into four major classes, Class -1 bacteriocins or lantibiotics are extensively post translationally modified by enzymes, necessary for antimicrobial activity of peptide. The class II bacteriocins are small (10kd), non lanthionine, heat stable unmodified peptides that do not require post-translational modification for antimicrobial activity, class III bacteriocins, large (>30 kDa), heat labile proteins and class IV, complex bacteriocins containing lipid or carbohydrate moieties. The class II bacteriocins are further subdivided into Listeria-active peptides with the N-terminal consensus sequence of YGNGVXC (class IIa), (Ennahar *et al*., 200; Nes and Holo, 2000; Sablon *et al*., 2000; Lade *et al* 2006). Bacteriocin as a lethal agent exerts its quantal killing action through adsorption and translocation. It also inhibits protein & DNA synthesis and induces leakage of ions through pores in cell membranes (Schein *et al*., 1978; Schwartz *et al*., 1971). In recent years, a number of different molecular techniques for the identification and characterization of lactic acid bacteria has been developed. More recently, use of 16S rRNA has also been proposed for the identification of unknown bacteria. The 16S rRNA gene is highly conserved among bacterial species, the presence of some variable zones that can be used for identification purposes. These zones can be amplified by PCR with specific primers and the sequence can be introduced for the similarity search, available on-line data bases (Moreno-Arribas and Polo, 2008; Du Plessis *et al*., 2004). *Lysinibacillus* is a Gram-positive, rod-shaped, and round-spore forming bacterium in the family *Bacillaceae* of phylum fermicutes. *Lysinibacillus* is commonly found in soil, plants and animals (Hayat *et al*., 2013). The genome of *Lysinibacillus sphaericus* strain C3-41 was the first strain in the genus *Lysinibacillus* which was polyphasic cell wall peptidoglycan based taxonomically classified (Ahmed *et al*., 2007)**.** The biotechnological potential of *Lysinibacillus* also has been described (Lucıa and Dussan, 2013; Hu *et al*., 2008). The abusive use of chemicals and emergence of antibiotic and multi drug resistant

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pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE) has become a serious problem in public health worldwide (Cetinkaya *et al*., 2000). Therefore, new strategies for controlling these pathogenic bacteria are urgently needed (Papagianni, 2003).

The aim of this study was to isolate and screen bacteriocin producing *Bacilli* from spoiled food samples and to select isolates with high and broad spectrum antimicrobial activity against microbial pathogens. Among selected isolates one bacteriocin producing *Lysinibacillus* was phenotypically, molecularly characterized and effect of culture conditions on production of its bacteriocin was also optimized. The new isolates and relevant bacteriocins can be potentially useful in pharmaceuticals, food and feed application.

MATERIALS AND METHODS

Isolation of microorganism

The selected isolates for this study were isolated from spoiled food samples, collected from local fruits and vegetables market, Lucknow U.P., India. The isolation was done on MRS (de Man, Rogosa and Sharpe) agar medium seeded with serially diluted spoiled food sample and incubated at 37 °C for 24hours. The colonies were randomly selected, screened for bacteriocin activity and stored at -20 °C in 30% glycerol on MRS.

Microbial indicator strains

Gram positive and Gram-negative bacterial indicator strains were used to evaluate antimicrobial activity of isolates. These were *Bacillus pumilus (MTCC160), Bacillus subtilis (MTCC441), Staphylococcus epidermidis (ATCC12228), Salmonella abony (NCTC6017), Pseudomonas aeruginosa (ATCC9027), Staphylococcus aureus (ATCC6538), Vibrio cholera (ATCC 14033), Mycobacterium luteus (10240), Listeria monocytogenes (ATCC19115), Bacillus cereus (ATCC 14579) and Raoultellaplanticola (MTCC530). Lysinibacillus isolate* was also tested against fungal strains *Aspergillus niger (ATCC4695), Aspergillus flavus (ATCC 16872), Fusarium oxysporum (ATCC 1198)* and *Trichoderma viridae (52438).* The microbial strains were obtained from the NCIM (National Collection of Industrial Microorganisms), NCL Pune, India. Bacterial strains were maintained at -20^{0}C in 30% glycerol and subcultured on nutrient agar while fungal strains were maintained on PDA slants.

Assay for bacteriocin activity against bacterial and fungal indicators

An agar well diffusion assay was used for detection of antibacterial activity of *Lysinibacillus* isolate. Nutrient agar plates were inoculated with 100µl ((2x10^{8}cfu/ml) of each bacterial indicator strain (Iqbal, 1998). 100µl filter sterilized (0.22µm), neutralized cell-free supernatant (CFS) of bacteriocin producing isolate grown in MRS broth at 37 °C for 48 hours was filled into wells of agar plates (6 mm in diameter). Plates were incubated at 37 °C for 24h and antimicrobial activity was determined by measuring diameter of inhibition zone around the wells (Tagg and McGiven, 1971). Antifungal activity was evaluated with *Lysinibacillus* isolate by dual culturing method and dilution methods. Fungal cultures were inoculated on one side of the PDA medium and another side streaking of bacterial strain to be tested for bacteriocin activity, was done at approximately 3.5cm from the fungal margin and incubated at the 25 °C for the seven days and inhibition was recorded (Wang *et al*., 2011).

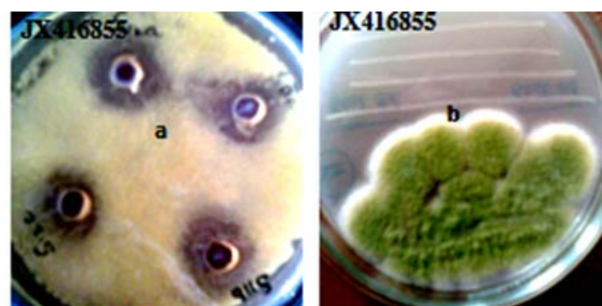

Fig. 1: Showing antimicrobial effect of *Lysinibacillus* isolates JX416855 on bacteria and fungi. a: Activity of *Lysinibacillus* against bacterial indicator *R. planticola*, in b: Activity of isolate JX416855 against *Aspergillus flavus*.

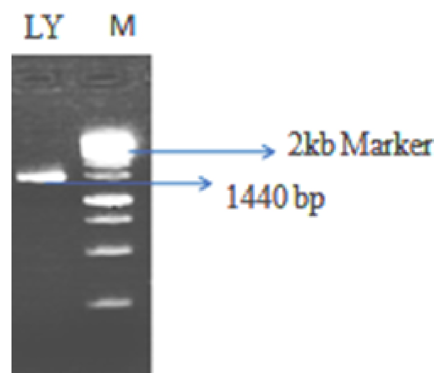

Fig. 2: Agarose gel electrophoresis of 16S rRNA amplified genes from *Lysinibacillus*JX416855. In lane M-2kb Ladder. Lane-LY. 1440bp amplified product of 16S rRNA gene.

Bacteriocin production and effect of physical parameters

Bacteriocin from *Lysinibacillus* JX416855 was produced in sterilized MRS broth contained-protease peptone 10.0g/L, Beef extract 10.0g/L, Yeast extract 5.0g/L, Dextrose 20.0g/L, Poly sorbate-80 1.0g/L, Ammonium citrate 2.0g/L, Sodium acetate 5.0g/L, Magnesium Sulphate 0.10 g/L, Manganese Sulphate 0.05g/L, Di

potassium phosphate 2.0g/L at pH 7.4. Inoculum (10ml) was grown in the medium at 37 ºC for 48 hours and was transferred to 90ml of fermentation medium and incubated aerobically at 37ºC for 48 hours with an agitation of 100 rpm. Cells were harvested by centrifugation at 12000 rpm for 20 minutes at 4 °C and cell free supernatant was filter sterilized (0.22µm) and stored at -20 °C for further studies. Different physical parameters like effect of media, pH, temp., time and dextrose, peptone, yeast extract conc. on bacteriocin production was optimized (Barros *et al*., 2001; Cladera-Olivera *et al*., 2004).

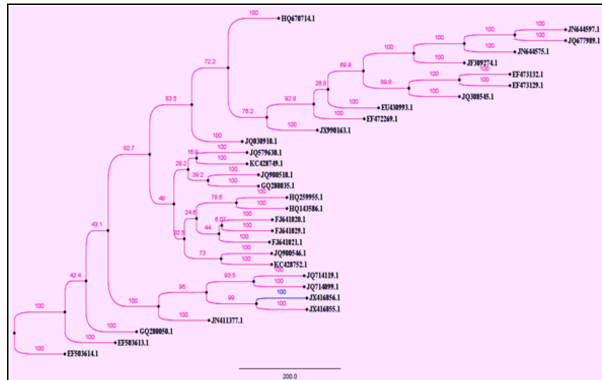

Fig. 3: Neighbour-Joining phylogenetic relationship of isolate JX416855 with closely related species of *Lysinibacillus* and other related genera. Bootstrap values Based on 100 replication are shown at branch nodes.

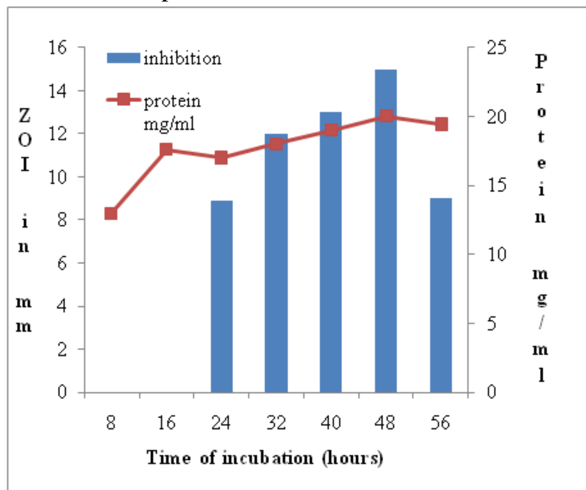

Fig. 4: Production of bacteriocin at different time intervals.

Isolation, Confirmation and partial characterization of bacteriocin.

Bacteriocin from CFS of *Lysinibacillus* was primarily extracted with butanol and DMSO reconstitute of it was tested for antimicrobial activity against indicator strains (Abo-Amer, 2007). The protein nature of bacteriocin in CFS and butanolic extract were qualitatively confirmed by Biuret, Ninhydrin and Lowry test and by TLC. Further, bacteriocin was isolated with 20%, 40%, 60% and 80% Ammonium sulphate precipitation and each fraction of bacteriocin was tested for antimicrobial activity. The active fraction of bacteriocin was analyzed by SDS-PAGE using 5% stacking gel and 12% resolving gel.

Taxonomic characterization of isolate

Taxonomic identification of isolate was based on morphological, biochemicals and molecular. Cell morphology, gram pattern and motility were analyzed by light microscope. Carbohydrate fermentation, gelatinase, amylase, catalase, protease nitrate reductase and drug susceptibility reactions were determined as describe by Lee *et al,* (2013).

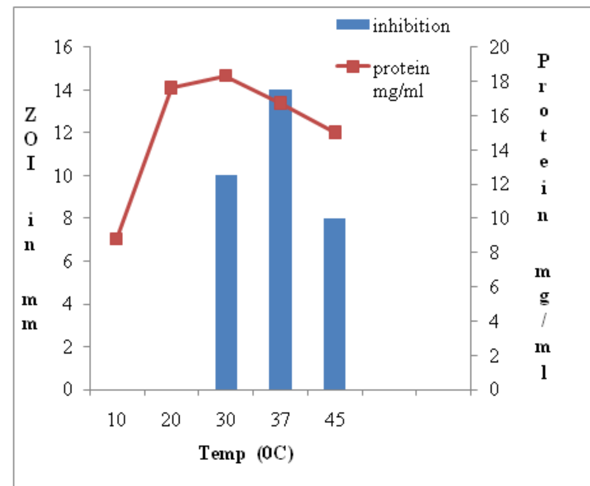

Fig. 5: Production of bacteriocin at different Temperature.

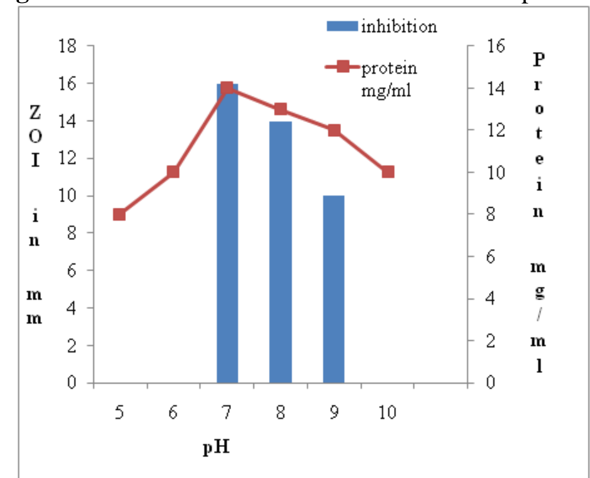

Fig. 6: Production of bacteriocin at different pH values.

Fatty acid methyl ester (FAME) Analysis

Fatty acid methyl esters were prepared from biomass of each *Lysinibacillus* grown at 37 °C on MRS for 48 h. The extraction was done according to Sasser (1990). Fatty acid ester was analyzed by Advanced Instrumentation Research Facility (AIRF) JNU, New Delhi, India, with gas chromatography using FAME mix 2mg-ml. The cellular fatty acids were identified by equivalent chain length of each compound to a peak naming that contains over known standard (Hayat *et al*., 2013).

Table 1: Showing antimicrobial effects of bacteriocin producing *Lysinibacillus*JX416855

Isolates	Zone of inhibition (mm)										
	*B.*pum	*B.sa*	*S.epi*	*S.abo*	*R.pl*	*S.aur*	*V.cho*	*M.lut*	*B.cer*	*P.aer*	*L.mon*
CFS	14	13	R	10	20	16	R	R	15	20	13
DMSO*	12	11	R	9	18	14	R	R	13	17	9
	A. flavus			*A. niger*		*F. Oxysporum*			*Trichodermaviridae*		
D-C*	(+++)			(+++)		(+++)			(+++)		
CFS	85%			90%		80%			60%		
	R_f (mm) Value of bacteriocin bands in Butanolic extraction										
TLC	48			67		67			84		
	Drug susceptibility of isolate JX416855										
Drug	T	P	E	AMP	OFL	CIP	K	VAN	CE	DOX	NF
ZOI	R	R	18	R	14	21	17	18	R	R	7

CFS Cell free supernatant; DMSO reconstitute; D-C* dual culturing effect; (+++) strong inhibition (>70%); T =Tetracycline, P= Penicillin, E=Erythromycin, AMP= Ampicillin, OFL= Ofloxacin, CIP= Ciprofloxacin, K= Kanamycin, V= Vancomycin, CE= Cephalotoxime, DOX= Doxycyclin, NF= Nitrofurantoin

Table 2: Physico-biochemical characterstics of *Lysinibacillus* JX416855

Morphological characters		Enzymatic characterization	
Colony	White round	Amylase	Negative
Morphology	*Bacillus*	Protease	Negative
Gram stain	Positive	Catalase	Positive
Motality	Motile	Gelatinase	Negative
Endospore	Positive	Urease	Negative
		Nitrate reductase	Negative
		MRVP	Negative
Growth Characters		Carbohydrate Fermentation	
MRS	+++	Gelatin	Negative
LB	+++	Starch	Positive- no gas
GB	+	Maltose	Positive -with gas
NaCl-0.5%	+	D-glucose	Positive- with gas
NaCl-1%	+	Dextrin	Negative- no gas
NaCl-1.5%	+++	D-glucose	Positive- no gas
NaCl-3%	+++	Ribose	Positive- no gas
NaCl-5%	+	Lactose	Positive- no gas
pH-4	+	Fructose	Positive- no gas
pH-7	+++	Sucrose	Positive- no gas
pH-10	+++	Manitol	Positive- no gas
pH-12	+		
T-10 °C	+		
T-20°C	++		
T-30°C	+++		
T-40°C	++		

Molecular characterization and phylogenetic analysis

The DNA extraction from *Lysinibacillus* was done (Sambrook and Russell, 2001), with 1.5ml MRS broth culture of *Lysinibacillus* which was incubated overnight. The purified DNA used for 16S rRNA gene amplification by PCR. The universal oligonucleotide primers 16SF(5´-AGAGTTTGATCCTGGCTCAG-3´) and 16SR (5´-GGTTACCTTGTTACGACTT-3´) were purchased from Ocimum Biosolution, Hyderbaad, India. 16S rRNA gene amplification was performed with 50µl reaction mixture using thermal cycler (Biored). The amplification program was as: preheating at 95 °C for 5 min of 1 cycle, followed by 30 cycles at 94 °C for 1min, 55 °C for 1 min and 72 °C for 1 min. After these cycles, the reaction was maintained at 72 °C for 10min and then cooled to 4°C. 5µl of the PCR product was analyzed on 1% agarose gel using 2 Kb molecular weight markers. The sequencing of the target gene was done using Big Dye Chemistry and performed as per the manufacturer's protocols (Applied Biosystems 3730 XL DNA Analyzer (Ocimum Biosolution

Table 3: Cellular fatty acid composition of *Lysinibacillus* JX416855 and the type strains of species of genus *Lysinibacillus.*

Fatty acids	1	3	4	5	6
14:0 ISO	1.31	1.58	1.87	1.25	3.66
14:0	1.6	-	1.01	-	-
15:0 ISO	48.65	30.29	49.01	48.58	49.85
15:0 ANTEISO	7.85	1.82	9.25	9.27	3.34
15:0	1.2	-	-	-	-
16:1 w7c alcohol	8.14	16.24	7.93	8.29	14.44
16:0 ISO	4.48	25.59	5.51	5.15	12.12
16:1 w11c	4.03	4.25	2.2	3.54	1.72
16:0	1.56	2.7	-	-	-
ISO 17:1 w10c	4.27	4.43	5.87	5.92	3.23
17:0 ISO	6.04	10.59	7.22	4.7	8.07
17:0 ANTEISO	3.85	1.6	4.81	3.38	1.07
18:n9c	-	-	-	-	-
20:0				6.7	-
21:0	0.51	-	-	-	-
22:2	-	-	-	-	-
Summed features 4	1	1	3.21	3.61	1

*Strains: 1. *Lysinibacillus* JX416855; 2. *L. pakiastanensis* NCCP-54; 3. *L. xylanilyticus* KCTC13433; 4. *L.fusiformis* KCTC3454; 5. *L. sphaericus* KCTC3346. *Data for texa 1 from this study; data for texon 2-5 are reference strains (Hayat *et al*., 2013; Lee *et al*., 2013)

Hyderbaad, India). Software is used for sequence alignment and comparisons were done by CLUSTAL X Version1. 8msw; (Thompson *et al*., 1997) and Chromas Pro 1.7.5 Sequencing Analysis software. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

RESULTS

Isolation, screening and characterization of LysinibacillusJX416855

The isolate was phenotypically characterized by colony characteristics, gram staining and physico biochemically. Colonies were seen in whitish cream, round and raised in appearance with entire margin. Microscopically it was found to be gram-positive rods. Isolate potentially inhibited to gram positive and gram-negative foodborne pathogenic bacteria as well as fungi (table 1). The optimum condition for growth of isolate was best observed in MRS medium and moderately was seen in LB broth followed by Nutrient broth. The salt conc. was found to be optimum at 1.5%-3% NaCl (Adiguzel and Atasever, 2009). The optimum pH range was found to be 6-10 and optimum temperature for growth was observed to be at 30-40°C. Enzymatically, it was observed negative with amylase, protease, gelatinase, nitrate reductase and urease but positive for catalase (table 1).

Drug susceptibility of isolate

The cells were also characterized by drug sensitivity test carried out by disc diffusion method, using disc of standard drugs. It was found sensitive to the most of the drugs used in this study but also showed different profile of drug resistant that indicating variable nature of plasmid encoding gene, providing the resistance to isolates for survival in ecological niche. *Lysinibacillus* JX416855 was found sensitive to E, OFL, CIP K, and VAN. and resistant to CEPH, DOXT, P and T (table 1).

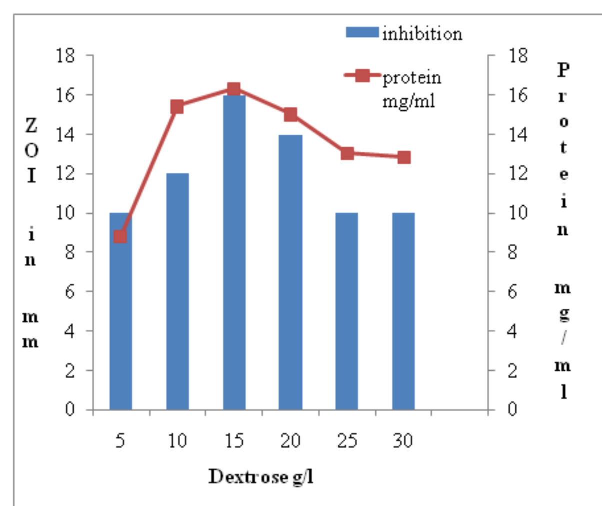

Fig. 7: Production of bacteriocin at different dextrose conc.

Fatty acid methyl ester (FAME) Analysis

The cellular fatty acid methyl esters profile of type strain JX416855 is shown in table 3 with reference to *L. pakiastanensis* NCCP-54; *L. xylanilyticus* KCTC13433; *L. fusiformis* KCTC3454; *L. sphaericus* KCTC3346, (Hayat *et al.,* 2013). The major fatty acids were $C_{15:0}$ ISO (48.65), $C_{16:1}$ w7c alcohol (8.14), $C_{15:0}$ ANTEISO (7.85). The specific diagnostic acidC_{18}: n9c (12.08%) was observed. The results of type strain are shown in table 3.

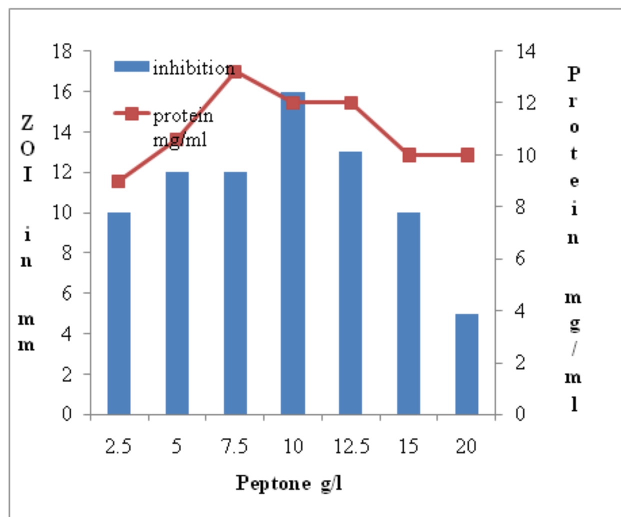

Fig. 8: Production of bacteriocin at different peptone conc.

Molecular characterization of Lysinibacillus

The 16S rRNA gene was amplified and given length was found to be 1440 bp for *Lysinibacillus* JX416855 (fig2). Evolutionary analysis has shown that it is close to *Lysinibacillus fusiformis BJ-40* (JQ 280050) and *Bacillaceae* bacterium (JQ714099, JQ714119*).* The phylogenetic analysis of *Lysinibacilli* isolates has shown in fig 3.

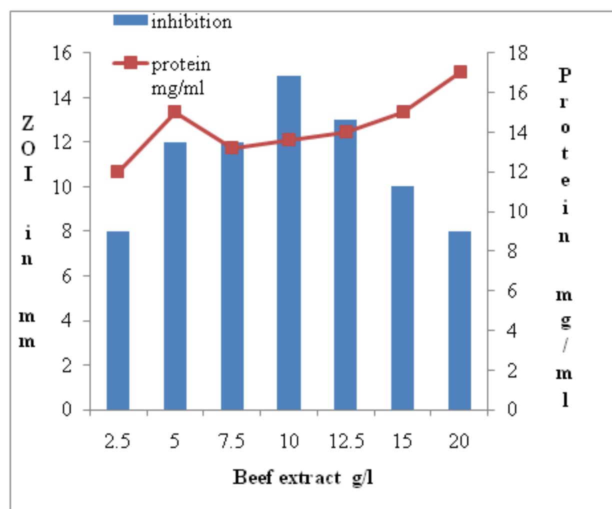

Fig. 9: Production of bacteriocin at different beef extract conc.

Bacteriocinproduction, isolation and analysis

Bacteriocin production from *Lysinibacillus* was carried out in MRS broth (pH 7.4, T 37°C, 48 h at 100rpm). The effects of different physical parameters are shown in (fig 4-9). The optimum conditions for bacteriocin production were found in MRS broth at pH 7.4, incubated aerobically at T 37°C for 48 h with shaking condition at 100rpm (fig 4-6). Further, effect of dextrose (15 g/l), beef extract (10 g/l) and peptone (10g/l) were found optimum for bacteriocin production. (fig. 7-9) bacteriocin extraction and isolation was done with butanol and ammonium sulphate. We analyzed nearly similar antimicrobial profile from DMSO reconstitute of butanolic extraction as in CFS. The activity of different ammonium sulphate fraction was tested on indicators strains *R. planticola* and *Pseudomonas aeruginosa*. The active fraction of bacteriocin was retained at 60% ammonium sulphate saturation, which was further analyzed by SDS-PAGE (3 bands of high mol weight) and confirmed the presence of antimicrobial substance as protein (fig 10).

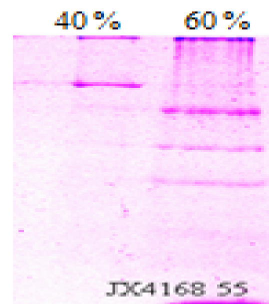

Fig. 10: SDS-PAGE analysis of bacteriocin from ammonium sulphate precipitated fraction; in 40% fraction (inactive) a single band while in 60% fraction (active) three bands were analyzed.

DISCUSSION

A number of bacteriocins have been proved very effective against gram negative and gram-positive bacteria (Abriouel *et al*., 2011). In the same view, *Lysinibacillus* has emerged as an interesting microbe in the field of bacteriocin because inhibitory potential and protease production from *Lysinibacillus* has also been recently described (Ahmad *et al*., 2014). In this study, twelve *lactobacilli* were selected from 125 isolates of *Lactobacilli,* isolated from spoiled food sample. *Lactobacilli* are found in a variable habitat such as food, marine, sewage and also in association of animals (Falsen *et al*., 1999; Sarmiento-Rubiano *et al*., 2010). It was analyzed from the conducted study that strain JX416855 has shown a significant antibacterial as well as antifungal activity (table 1; fig 1). It has been shown selective and greater inhibitory effect against gram positive as well as gram-negative pathogenic indicators (table 1). CSF of *Lysinibacillus* JX416855 inhibited to gram-positive pathogens *B. pumilus* (14mm), *B. subtilis* (13mm), *S.*

aureus (16mm), *B. cereus* (15mm) and *L.monocytogenes* (13mm) and gram negative *R. planticola and P.aeruginosa*. These results confirmed that *Lysinibacillus* JX416855 found very effective against gram negative. *Lysinibacillus* isolate has also been found potential fungal antagonistic against *Aspergillus niger, Aspergillus flavus, F.oxysporium* and *Trichoderma spp.* (table 1; fig 1). Biocontrolling (ahmad *et al*., 2014) and insecticidial nature of *Lysinibacillus* has been described (Hu *et al*., 2008; Melnick *et al*., 2011). Further, the industrial protease potential of lysinibacillus recently has been described (ahmad *et al*., 2014). Bacteriocins, like acidocin have been extracted with butanol and tested against fungi (Abo-Amer, 2007). The butanolic DMSO reconstitute shown significant antimicrobial activity with similar antimicrobial spectrum against used indicators as was observed with CFS (table 1). Further, active bacteriocin was isolated at 60% ammonium sulphate saturation. The proteinacious ammonium sulphate active fraction of bacteriocin from *Lysinibacillus* JX416855 has also been analyzed three bands of high molecular weight by SDS - PAGE.

The physico-biochemical parameters and molecular identification are the basis for differentiating two closely related species. However, the phenotypicals and genotypicals results concerning carbon and nitrogen source utilization, antibiogram, salt tolerance, maximum growth, pH, Temp., enzymatic and 16S rRNA gene sequences as analyzed, may give strong support about the taxonomic position of this isolate as *Lysinibacillus*. The specific fermentation pattern, specific antibiogram, presence of specific fatty acid methyl ester and bacteriocin production differentiate it from previously described *Lysinibacillus* (Hayat *et al*., 2013; Lee *et al*., 2013) and supports the novelty of this *Lysinibacillus.* The description of this potential inhibitor of gram negative *Lysinibacillus* isolate was further concluded as gram positive, motile, endospore forming *bacillus.* The colonies on MRS agar were white creamish circular with entire margin. The growth conditions were optimized with MRS media at pH 7-10, Temp. 35-40°C and salt tolerance at 1-3%. They were characterized biochemically as amylase, protease, nitrate reductase, gelatinase and urease negative with a specific carbohydrate fermentation pattern and diagnostic (C_{18}: n9c 12.08%) fatty acid methyl ester. 16S rRNA, NCBI gene bank accession number of this novel strains is JX416855 and might be designated as *Lysinibacillus JX416855.*

CONCLUSION

The present study was done to explore the antimicrobial potential of *Lysinibacillus.* In this study we, isolated, characterized and optimized bacteriocin production, strongly inhibiting to microbial pathogens. The novel isolate was identified as *Lysinibacillus*JX416855. Additional research work on these bacteriocins would be necessary to isolate, purify, characterize and to explore the therapeutic significance of these bacteriocins from *Lysinibaillus,* that can be used to combat drug resistant or MDR pathogens. The purified bacteriocin from *Lysinibacillus* isolate might be used as an alternative therapeutic agent to control microbial pathogen.

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