CHAPTER 5

OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF BACTERIOCIN-LIKE SUBSTANCE (BLIS) BY *BACILLUS* SP. NK7 ISOLATED FROM *KHAROLI* FROM ASSAM, INDIA

Optimization of culture conditions for the production of bacteriocin- like substance (BLIS) by *Bacillus* sp. NK7 isolated from *kharoli* from Assam, India

5.1. Abstract

In the wake of immergence of antibiotic resistant microorganisms, food preservation strategies have been reassessed. The study was aimed at the evaluation of antimicrobial properties of strains isolated from an alkaline fermented food *kharoli*, and the optimization of culture conditions for maximum antimicrobial substance production. It was found that the strain *Bacillus* sp. NK7, as identified using 16S rDNA sequencing, showed antimicrobial activities against different pathogenic indicator strains. The optimized culture conditions for the antimicrobial substance production were found to be pH 7.92, carbon source (fructose) 40 g/l and nitrogen source (ammonium citrate) 35.61 g/l. Under these conditions, the strain showed maximum activity of 24.10 mm (zone of inhibition) against *Bacillus cereus*, which is a major food- borne pathogen. The antimicrobial substance was characterized and found to be a bacteriocin like substance (BLIS) with an apparent size of 20 KDa. The BLIS was stable at a wide range of temperature and pH and therefore it has a potential to be used as a biopreservative in the food industry.

5.2. Introduction

Bactericins are proteinaceus compounds produced by bacteria to inhibit another bacteria present in its proximity. Bacteriocins have received a very important position among biopreservatives due to their Generally Regarded as Safe (GRAS) status and their suitability for the application in food preservation because of their digestible proteinaceus nature [1]. Bacteriocins are active against many food contaminating microorganisms such as *Listeria monocytogenes, Bacillus cereus, Salmonella typhemurium, Campylobacter jejuni* etc which cause food borne diseases. Food preservation by bacteriocin is well- reported and most of the emphasis is given on bacteriocins produced by the lactic acid bacteria. Class I bacteriocins or the lantibiotics are the most well- studied bacteriocins and are exploited industrially [2]. However, lactic acid bacteria are not the sole producers of bacteriocins, many gram positive and negative bacteria are also capable of producing wide array of bacteriocins. Abriouel et al. [3] proposed a different scheme of classification for the

bacteriocins produced by *Bacillus* strains which is independent of the conventional classification of bacteriocins produced by lactic acid bacteria. According to this classification, there are three types of bacteriocins produced by *Bacillus* strains, namely class I which contains post- translationally modified bacteriocins, class II which contains unmodified bacteriocins and class III which contains large proteins. These groups are analogous to the class I lantibiotics, class II small, linear peptides and class III large heat labile bacteriocins produced by lactic acid bacteria.

The presence of *Bacillus* strains is quite common in many alkaline fermented foods prepared in South- East Asia and African countries which are not well- known and less studied compared to the acidic fermented foods [3]. The famous examples of alkaline fermented foods are Japanese fermented soybean *Natto,* Thai food *Thua nao,* and *Kinema* from Nepal which are *Bacillus*- fermented soybean products having multiple health beneficial properties [4**,**5]. *Kharoli* is a fermented product of mustard seeds (*Brassica juncea* or *Brassica nigra*) consumed as a condiment in Assam, a North- Eastern state of India. It is prepared by grinding of mustard seeds and sieving, followed by the addition of a little salt and some traditionally prepared soda water known as *kolakhar* [6]. The mixture is then kneaded into dough, wrapped with banana leaves and kept for spontaneous fermentation. In the present study, bacteriocin producing strain *Bacillus* sp. NK7 was isolated from *kharoli* and its probiotic properties were investigated. The culture parameters for the maximum production of antimicrobial substances were optimized using response surface methodology (RSM) utilizing Box- Behnken design.

5.3. Materials and methods

5.3.1. Selection of microorganisms and growth conditions

Indicator strains *Bacillus cereus* (MTCC 430), *Salmonella enterica typhimurium* (MTCC 1252), *Yersinia enterocolitica* (MTCC 859), *Pseudomonas duminita* (MTCC 3361), *Listeria monocytogenes* (MTCC 839), *Staphylococcus aureus* (MTCC 3160), *Pseudomonas aeruginosa* (MTCC 3541), *Shigella boydii* (MTCC 11947), *Shigella flexneri* (MTCC 1457), *Providencia rettgeri* MTCC 8929, *Candida albicans* (MTCC 183), *Aspergillus niger* (MTCC 281) and *Aspergillus flavus* (MTCC 277) were purchased from Microbial Type Culture Collections (MTCC), IMTECH, India.

Pediococcus pentosaceus NCDC 273 was purchased from National Collection of Dairy Cultures, NDRI, India. *Bacillus cereus* AMDK1, *Listeria monocytogenes* AMDK2 and *Candida tropicalis* BSS7 were isolated from spoiled foodstuff. Bacterial strains were cultured in nutrient broth, tryptic soy broth, BHI and MRS media at 37 °C. Fungal strains were cultured in potato dextrose broth at 28 °C. Microorganisms were isolated from *Kharoli* samples collected from different places of Assam and screened for antimicrobial activities. For this, neutralized (pH 7) cell free supernatant of overnight grown cultures of different isolates were filter sterilized with 0.22 µm syringe filters and assessed for antimicrobial activities using agar well diffusion method.

5.3.2. Molecular identification of bacterial strain

Molecular identification was carried out using 16s rRNA gene sequencing followed by phylogenetic analysis. 16s rDNA region was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′). DNA amplification was carried out using colony PCR which was performed by taking a colony of the isolate into a lysis solution containing 5 µl of 0.5 M NaOH and 95 µl of Milli Q water followed by heating the mixture at 95 °C for 5 min. After heating, the solution was centrifuged at 10,000 rpm for 10 min and the supernatant was store at -20 °C as template DNA. The PCR reaction mixture consisted of 12.5 µl of PCR Master Mix (2X, Thermo Scientific), 9.5 µl of MilliQ water, 1 µl each of the primers and 1 µl of template DNA. The PCR conditions were as follows: 95 °C for 5 min (initial denaturation), 30 cycles of 95 °C 30 sec (final denaturation), 55 °C for 30 sec (annealing), 72 °C for 1.30 sec (initial extension) and 72 °C for 10 min (final extension).The amplified products were visualized using agarose gel electrophoresis. The PCR product was purified using QIAQuick Gel Extraction Kit (Quagen) and subjected to automated DNA sequencing in 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence was submitted to BLAST search tool (www.blast.ncbi.nlm.nih.gov/Blast.cgi) for homologous sequence and phylogenetic tree was constructed using MEGA 5.05 software [7, 8, 9].

5.3.3. Assessment of probiotic properties of the isolates

5.3.3.1. *In vitro* gastrointestinal stress tolerance

The tolerance of the isolates to simulated gastric and intestinal conditions was evaluated using a protocol reported by Maragkoudakis et al. [10] with some modifications. Simulated gastric juice (SGF) was prepared by adding filter- sterilized 0.3 mg/ml pepsin to PBS with pH adjusted to 2, 3 and 4. Simulated intestinal fluid (SIF) was prepared by adding 0.1mg/ml pancreatin and 0.3% (w/v) bile salt to PBS adjusted to pH 6.8 and 8. Isolates were cultured in their respective media and overnight cultures were harvested by centrifugation at 6000 X g and the cells were washed with phosphate buffered saline (PBS, pH 7.4). Cell suspension in PBS (pH 7.4) was added to PBS solutions with different pH values prepared beforehand at a final concentration of ~10^8 CFU/ml. For SGF, cells were incubated for 1, 2 and 3 h and for SIF incubation was done for 1, 2, 3 and 4 h. Viability was checked after 24 h incubation at 37 °C by plate count method.

5.3.3.2. Adhesion to Caco-2 cell line

The human colorectal adenocarcinoma Caco-2 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. The cell line was routinely grown and maintained in minimal essential medium (MEM) supplemented with 20% heat- inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, and grown at 37 ºC in humified atmosphere in presence of 5% (v/v) CO_2. Cells were supplemented with fresh MEM every 2-3 days.

Adhesion assay was performed using a method described by García-Cayuela et al. [11]. Briefly, Caco-2 cells were seeded onto 24 well tissue culture plates at a concentration of 10^4 cells per ml and incubated in humified atmosphere containing 5% CO_2 at 37 °C. Media was changed in every two days until cells become 80% confluent. After cells attain confluence, they were washed with phosphate buffered saline (PBS, pH 7.4) and replenished with new media containing no antibiotics. Now the monolayer cells were inoculated with probiotics (10:1 ratio of probiotics to cells) and incubated for 1 h. After the incubation period was over, media was discarded and the wells were washed with PBS to remove the non-adhered cells. Some wells were

stained with crystal violet and observed under microscope (100X). The other wells were trypsinized with 0.25% trypsin-EDTA solution and viable bacterial count was determined using plate count on MRS agar.

5.3.4. Single factor experiments for the determination of factors influencing BLIS production in culture media

To study the effects of different factors on antimicrobial activity, six different factors, i.e. culture time, temperature, initial pH, inoculum size, carbon and nitrogen source were selected [12]. The basal media used was Luria Bertani (LB) broth. Antimicrobial activity was expressed as zone of inhibition in mm [13]. *Bacillus cereus* MTCC 430 was used as an indicator strain. The individual effects of different factors on antimicrobial activity were determined by a step by step manner as described below:

Step 1: Optimization of culture time: For the determination of the effect of culture time, antimicrobial activity was determined at various time intervals, viz. 0, 6, 12, 18, 24, 36 and 48 h using the basal media.

Step 2: Initial pH: The pH of the basal media was adjusted to 3, 5, 7, 8, 9 and 10 and antimicrobial activities were determined after incubation for the optimized time as obtained from the previous step.

Step 3: Culture temperature: The culture temperature was maintained at 20, 25, 30, 37, 40, 45 and 50 °C with time and pH values optimized from the previous step.

Step 4: Inoculum volume: The culture media maintained at optimized culture time, pH and temperature were inoculated with inoculums of 1, 3, 5, 9 and 11% (v/v) adjusted to $O.D._{600}$ at 0.2.

Step 5: Carbon source: Different carbon sources such as glucose, sucrose, fructose, mannose, starch and lactose were added to the basal media at a concentration of 2% (w/v) and activities were measured after maintaining the culture conditions optimized in the previous steps.

Step 6: Nitrogen source: Ammonium citrate, ammonium sulphate, ammonium chloride and peptone were used as nitrogen sources and added to the basal media at a

concentration of 2% (w/v) and the optimized conditions obtained from the previous steps were maintained.

5.3.5. Statistical analysis for the optimization of factors using response surface methodology:

The factors which were found to be responsible for significant increase in antimicrobial activity which were determined during the single factor experiment were chosen as independent variables for the experiment design. Box- Behnken model in RSM (Design- Expert version 6.0.2, Stat-Ease Inc., Minneapolis, MN, USA) was applied for designing the experiment. Data obtained were fitted in to a quadratic polynomial equation as given below:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X^2_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the response (antimicrobial activity), β_0 is a constant, β_i, β_{ii} and $\beta_i\beta_j$ are the coefficients of linear, quadratic and interaction terms respectively. After fitting the model, multiresponse analysis was performed for optimizing the factors in order to achieve maximum response using desirability approach. The desired goal for the response was set as maximum. After obtaining the values for the optimized factors, validation of the optimized factors was done by determining antimicrobial activity at the optimized conditions.

5.3.6. Cell lysis:

For the determination of the mode of action of BLIS, a method developed by De Kwaadsteniet et al. [13] was employed with some modifications. Briefly, overnight culture grown in optimized media was harvested and the cell free supernatant was filter-sterilized, adjusted to pH 6. 20 ml of the supernatant was added to 100 ml culture of *B. cereus* MTCC 430 at the onset of growth (0 h) and at the early-exponential phase of growth ($O.D._{600} \sim 0.1$). The bacterial growth was observed at $O.D._{600}$ until stationary phase was observed.

5.3.7. Partial purification of BLIS:

The strain producing antimicrobial substance was inoculated to 1 L of the optimized media and incubated at the conditions showing optimum activity as determined in the

previous section. After incubation was over, cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 ℃. The proteinaceous substance was precipitated from the supernatant using 60% ammonium sulphate at 4 ℃. The precipitate was collected by centrifugation at 12,000 rpm for 15 min and dissolved in 0.01 M phosphate buffer (pH 7.0), and dialyzed using a 3 KDa cutoff membrane against the same buffer at 4℃ overnight. The dialysate was fractionated using Amicon Ultra-15 centrifugal filter device with 10 KDa filter. For the determination of molecular weight of the bacteriocin, tricin SDS- PAGE was performed [14] followed by in gel bacteriocin activity. For this, the gel was cut into two parts, one part is stained with Coomassie brilliant blue G-250 and the other part is washed with 0.01 M PBS for 3 h and overlaid with soft agar mixed with overnight culture of *B. cereus* MTCC 430. In gel activity was observed after 24 h incubation [15].

5.3.8. Effect of enzymes, pH, and temperature on BLIS activity

The partially purified bacteriocin was treated with proteolytic enzymes such as trypsin, proteinase K and pepsin in their respective buffers (0.05 M Tris hydrochloride, pH 8.0 and 1 N NaOH, pH 6.5) and non- proteolytic enzyme catalase. [16].The final concentration of the enzymes was maintained at 1 mg/ml and incubated at 37 ℃ for 2h. pH stability was assessed by adjusting the pH values in a range from 2 to 10 at 37 ℃ for 3 h. For the thermal stability test, the bacteriocin was exposed to different temperature such at 60, 80 and 100 ℃ for 30 min in water bath and 121 ℃ in an autoclave. The residual activities were determined by agar well diffusion method and keeping untreated bacteriocin as control.

5.3.9. Safety evaluation of BLIS producing strain

5.3.9.1. Assessment of antibiotic susceptibility

The antibiotic susceptibility was performed using Bauer–Kirby method [17]. Different standard antibiotics as provided for Hexa discs (Himedia laboratories) were used for the susceptibility testing.

5.3.9.2. Assessment for haemolysis

Hemolytic activity of the strains was performed applying a method reported by Anand et al. [18]. Briefly, Columbia blood agar plates were prepared containing 5% (v/v) goat blood and bacterial strains were streaked onto it. After 24 h incubation at 37 °C, plates were observed for haemolytic zones.

5.4. Results and discussions

5.4.1. Identification of BLIS producing bacteria

Total 48 different strains were isolated from *kharoli* samples and the strain NK7 showed antimicrobial activity against a number of gram positive and negative bacteria (Table 5.1.) and maximum activity was shown against *Bacillus cereus* MTCC 430 (zone of inhibition 16.5 ± 0.70 mm).This strain was further subjected to 16S rRNA gene sequencing. It was identified as *Bacillus* sp. after the sequence obtained was further searched for homologous sequences and from the phylogenetic analysis it was found that the strain shared close relationship with *Bacillus sp.* B210 (B) as shown in the Fig. 5.1. The sequence after submitted to NCBI Bankit (https://www.ncbi.nlm.nih.gov/BankIt/) an accession number was obtained (KY923226).

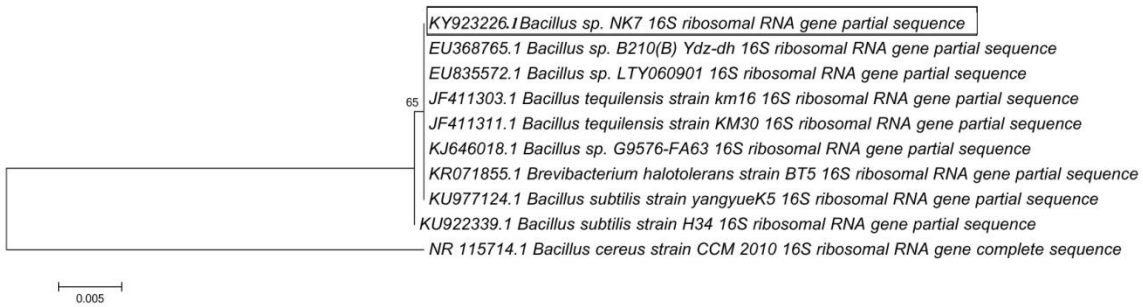

Fig. 5.1. Phylogenetic tree showing *Bacillus* sp.NK7 with closely related species based upon 16S rRNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes.

Table 5.1. Antimicrobial spectrum of the strain *Bacillus sp*. NK7

	Collection	**Growth conditions**	**Activity (Zone of inhibition in mm)**
Gram positive bacteria			
Bacillus cereus	MTCC 430	NA, 37 °C	16.5 ± 0.70
Bacillus cereus AMDK1	Genbank Accession No. KC683896	NA, 37 °C	13 ± 1.41
Listeria monocytogenes	MTCC 839	TSA, 37 °C	11±1.41
Listeria monocytogenes AMDK2	Genbank Accession No. KF894986	TSA, 37 °C	9±1.41
Staphylococcus aureus	MTCC 3160	NA, 37 °C	12.5±2.12
Pediococcus pentosaceus	NCDC 273	MRS, 37 °C	0
Lactobacillus plantarum	MTCC 1407	MRS, 37 °C	0
Gram negative bacteria			
Salmonella enterica typhimurium	MTCC 1252	TSA, 37 °C	13±1.41
Yersinia enterocolitica	MTCC 859	TSA, 37 °C	0
Pseudomonas duminuta	MTCC 3361	NA, 37 °C	0
Shigella boydii	MTCC 11947	TSA, 37 °C	0
Shigella flexneri	MTCC 1457	TSA, 37 °C	0
Providencia rettgeri	MTCC 8929	TSA, 37 °C	0
Yeasts and fungi			
Candida albicans	MTCC 183	PDA, 28 °C	0
*Candida tropicalis*BSS7	GenBank Accession No. KT387283	PDA, 28 °C	0
Aspergillus niger	MTCC 281	PDA, 28 °C	0
Aspergillus flavus	MTCC 277	PDA, 28 °C	0

5.4.2. Probiotic characterization

5.4.2.1. Tolerance to gastrointestinal stress conditions

Tolerance to gastrointestinal stress conditions is an intrinsic property of all gut microbiota and this property is also expected to be inherited by probiotics for better survivability under such environment. The viability of the strains under simulated gastrointestinal conditions is depicted in the table 5.2. It was observed that both the strains showed viability more than 80% under simulated gastric and intestinal conditions. The stomach pH varies between 1.5 and 3.5. At pH 2 and 3, viability of both the strains decreased only by 10-20% and at pH 4 viability was observed to be more than 95%. These results are in accordance with the previously published reports [19, 20].

Table 5.2. Simulated gastrointestinal tolerance of probiotics; (A) simulated gastric fluid tolerance and (B) simulated intestinal fluid tolerance

(A) SGF tolerance (viability in %)						
	pH 2		pH 3		pH 4	
Time (h)	NK7	MTCC 1407	NK7	MTCC 1407	NK7	MTCC 1407
1	85.67±1.98	88.46±0.497	89.9±3.59	88.52±0.113	98.55±3.48	95.99±1.202
2	87.46±0.948	84.4±0.402	88.9±1.517	84.73±0.475	100.76±3.43	99.5±1.69
3	86.83±0.072	84.61±4.948	91.92±0.736	84.36±1.84	102.03±3.34	98.44±1.29
(B) SIF tolerance (viability in %)						
Time (h)	pH 6.8		pH 8			
	NK7	MTCC 1407	NK7	MTCC 1407		
1	101.03±0.441	95.96±2.242	102.09±1.236	96.38±1.041		
2	101.42±2.549	99.57±2.084	103.29±0.71	90.53±0.19		
3	102.17±1.158	96.43±1.71	102.52±0.793	93.36±3.22		
4	99.77±0.618	98.23±0.976	105.87±0.051	96.88±0.962		

Values are represented as mean ± SD., n=6. Means with different letters in the same row are significantly different ($P<0.05$), checked by Tukey's multiple comparison test, GraphPad prism, ver. 5.0

Abbreviations: NK7- *Bacillus* sp. NK7, MTCC 1407- *Lactobacillus plantarum* MTCC 1407

5.4.2.2. Adhesion to Caco-2 cell line

The adhesion of probiotics to the intestinal epithelium mediates the probiotics to impart their health- beneficial effects. Caco-2 cells are used as a model for probiotic adhesion experiments since they resemble typical enterocytes covered with brush border microvilli upon confluence [21]. In our experiment, *Bacillus* sp. NK7 was assessed for its adhesion abilities towards Caco-2 cells by taking *Lactobacillus plantarum* MTCC 1407 as a reference strain. The microscopic studies showed adhesion towards the cell line (Fig. 5.2.). The cell count method for the quantitative determination of adhesion proved that *Bacillus* sp. NK7 showed 17.82% adhesion which was not significantly different from that of *L. plantarum* MTCC 1407. The adhesion capability of *Bacillus* sp. to Caco-2 cells was previously reported [22].

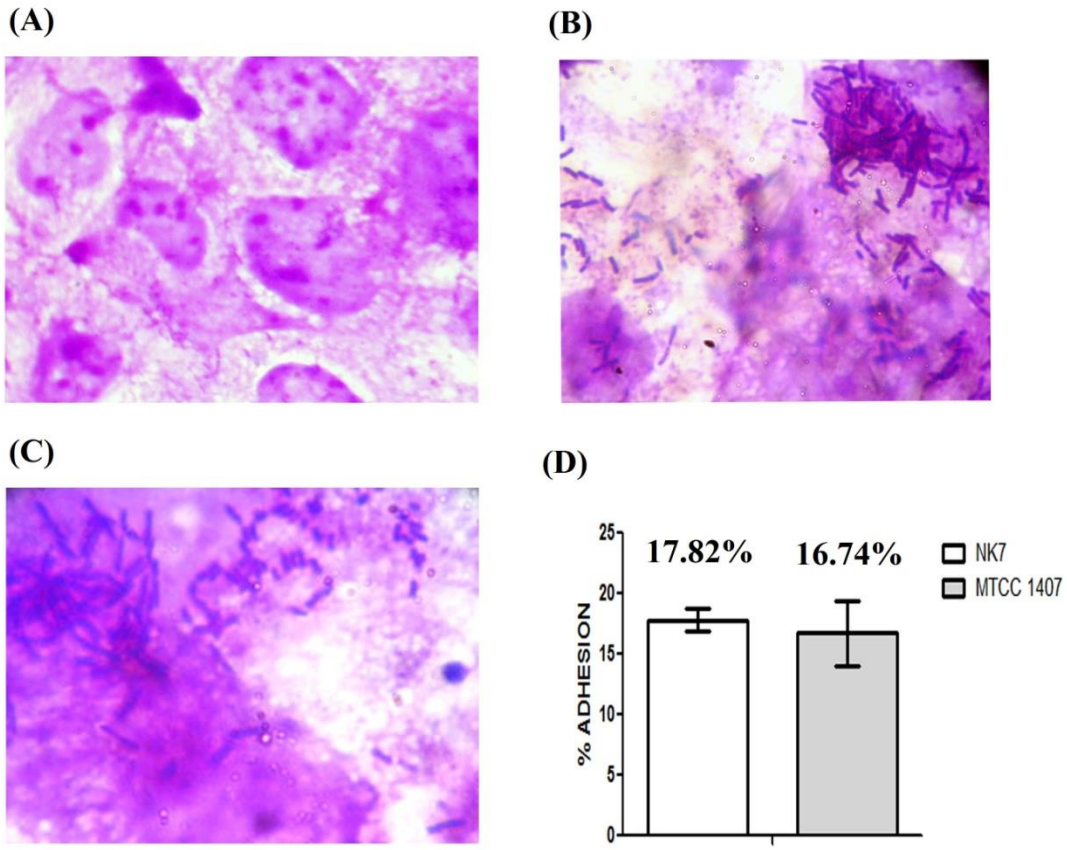

Fig. 5.2. Probiotic adhesion to Caco-2 cell line; (A) control Caco-2 cell, Gram staining depicting adhesion of (B) *Bacillus* sp. NK7 and (C) *Lactobacillus plantarum* MTCC 1407 observed under 100X, (D) Quantitative representation of adhesion showing percentage adhesion expressed as bar diagram.

5.4.3. Single factor experiments for the determination of factors influencing BLIS production

The effect of different factors for bacteriocin production is shown in the Fig. 5.3. The conditions for the maximum activity were found to be 18 h of incubation time, 37 °C growth temperature, initial pH of 8, inoculums of 5% (v/v), carbon source fructose and nitrogen source ammonium citrate. From the ANNOVA analysis it was found that the values of the initial pH, carbon source and nitrogen source showed significant effect on

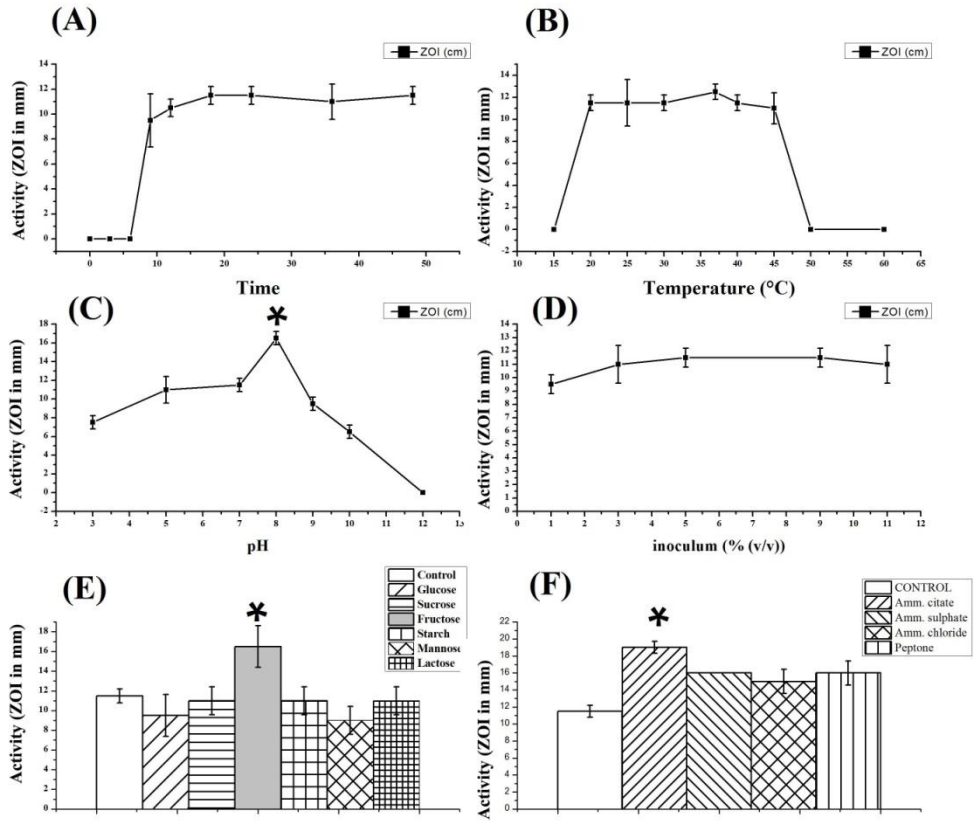

Fig 5.3. Single factor experiments showing effect of different factors on antimicrobial activity against *Bacillus cereus* 430; effect of (A) time, (B) Temperature, (C) pH, (D) inoculum size, (E) carbon source and (F) nitrogen source.
*indicates statistical difference at $P < 0.05$

the activity ($P < 0.05$). However, the other factors, i.e. time, temperature and inoculums volume did not significantly affect the activity. The strain *Bacillus* sp. NK1 was isolated from an alkaline fermented food, which might be the reason for the higher activity at alkaline pH 8. Moreover, bacterial growth rate was also found to be maximum at pH 8. Antimicrobial activity in presence of sugar other than glucose was found to be more in case of *Bacillus* sp.ZBP4 [23] which according to the authors was due to nutritional stress.

5.4.4. Optimization of culture conditions using response surface methodology

The variables pH, carbon source (fructose) and nitrogen source (ammonium citrate) were chosen as independent variables based on the single factor experiments performed on the previous section. A three factor three level experiment was performed based on the values of the three variables as shown in the table 5.3. Total

17 sets of experiments were performed at 37 °C growth temperature, 18 h of incubation time and inoculum volume of 5% (v/v). Antimicrobial activities were tested against *Bacillus cereus* MTCC 430 and expressed as zones of inhibition.

Table 5.3. Box- Behnken Design arrangement for the independent variables and the response

Run	pH	Carbon source (g/l)	Nitrogen source (g/l)	Actual response (Zone of inhibition in mm)	Predicted response (Zone of inhibition in mm)
1	7	30	20	16.5 ± 0.70	16.81
2	7	40	30	15.5 ± 0.70	16.25
3	7	30	40	17.5 ± 0.70	17.88
4	7	20	30	13.5 ± 2.12	13.13
5	8	20	20	15.5 ± 0.70	14.75
6	8	30	30	14.5 ± 0.70	14.44
7	8	40	40	13 ± 1.41	13.06
8	8	30	30	12.5 ± 0.70	12.19
9	8	40	20	17 ± 1.41	17.69
10	8	30	30	22.5 ± 0.70	22.00
11	8	20	40	22.5 ± 0.70	22.00
12	8	30	30	20.5 ± 0.70	20.94
13	8	30	30	24.5 ± 0.70	23.81
14	9	40	30	22 ± 1.41	22.00
15	9	20	30	21.5 ± 0.70	22.00
16	9	30	40	21.5 ± 0.70	21.06
17	9	30	20	21.5 ± 0.70	22.00

The values of the independent variables and the actual response were analyzed using multiple regression analysis in Design Expert. The quadratic equation for the response was found to be as follows:

$$\text{Zone of inhibition (mm)} = 22 - 0.81A + 1.56B + 1.5C - 6.6A^2 + 0.125B^2$$

$$- 0.25C^2 + 0.13AB - 0.375AC - 0.125BC \quad (2)$$

Where A, B and C are the independent variables pH, carbon source and nitrogen source respectively. From table 5.4 it was found that the linear affects of A, B and C and quadratic effects of A and C significantly influenced antimicrobial activity in

terms of zone of inhibition. Higher F value and lower *P* value also indicates that the model fits the experimental data very efficiently [24]. Similarly, lack of fit value of 3.92 also denotes that it is not significant relative to pure error.

Table 5.4. ANOVA for Response Surface Quadratic Model

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**
Model	239.4449	9	26.60498	47.29775	< 0.0001*
A	5.28125	1	5.28125	9.388889	0.0182*
B	19.53125	1	19.53125	34.72222	0.0006*
C	18	1	18	32	0.0008*
A^2	184.8026	1	184.8026	328.538	< 0.0001*
B^2	0.065789	1	0.065789	0.116959	0.7424
C^2	6.578947	1	6.578947	11.69591	0.0111*
AB	0	1	0	0	1.0000
AC	0.5625	1	0.5625	1	0.3506
BC	0.0625	1	0.0625	0.111111	0.7486
Residual	3.9375	7	0.5625		
Lack of Fit	2.9375	3	0.979167	3.916667	0.1102
Pure Error	1	4	0.25		
Cor Total	243.3824	16			

For numerical optimization desired goal for the responses were set as maximum and importance was set as 5. The desirability ramp (Fig. 5.4D) shows that pH of 7.92, 40 g/l of carbon source and 35.61 g/l of nitrogen source resulted in maximum 24.10 mm of zone of inhibition with a desirability of 0.967.

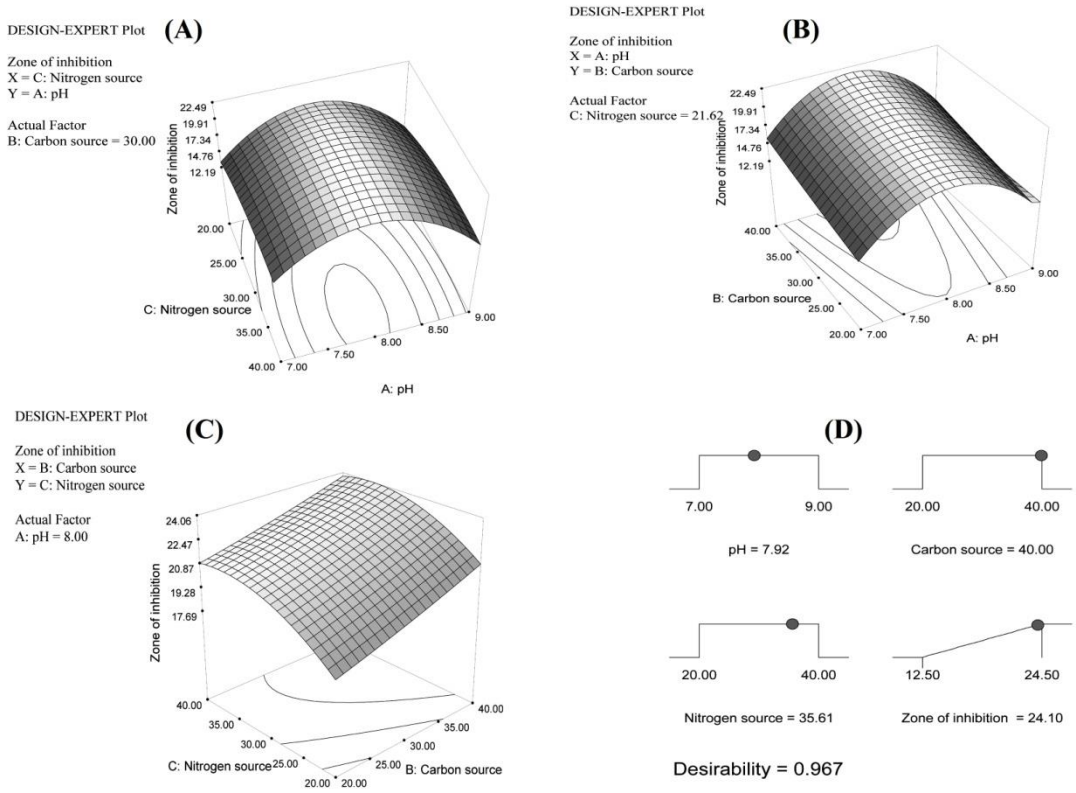

Fig 5.4. Optimization of different parameters antimicrobial activity; (A), (B) and (C) are the Design- Expert plots depicting effect of different variables in activity, (D) Multiresponse optimization showing desirability ramps for numerical optimization of parameters for antimicrobial activity

5.4.5. Validation of optimal culture conditions

For the validation of the optimized culture conditions experiments were performed keeping the initial pH 7.92, 40 g/l of carbon source and 35.61 g/l of nitrogen source. It was found that maximum bacteriocin activity was observed as zone of inhibtion of 23.5 ± 0.70 mm (Fig. 5.5.) which was very close to the predicted value of 24.1 mm. The effect of optimized and unoptimized media is depicted in the Fig. 5.5.

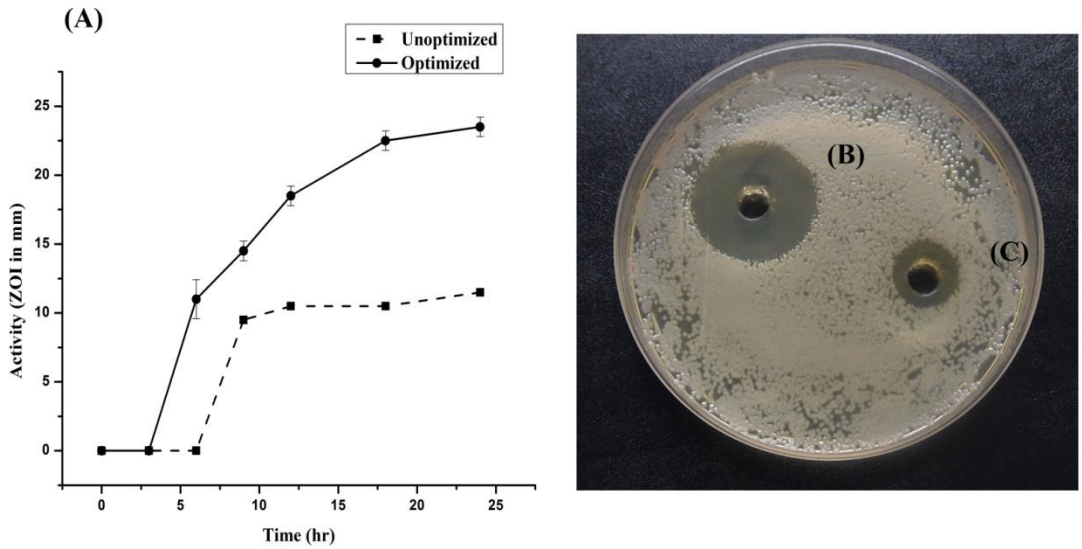

Fig. 5.5. (A) Bacteriocin activity at different time intervals and zones of inhibition against *Bacillus cereus* MTCC 430 observed in (B) optimized and (C) unoptimized media.

5.4.6. Cell lysis

The inhibitory activity of BLIS on the growth of *Bacillus cereus* MTCC 430 is shown in the Fig. 5.6. The addition of BLIS containing supernatant to lag and mid-logarithmic phase of bacterial culture resulted in diminished growth compared to the control. Cell lysis is confirmed by the decrease in $O.D._{600}$ which suggests that the mode of action is bacteriocidal. Similar bacteriocidal actions are reported in case of *Staphylococcus aureus and Enterococcus mundtii* [16, 25].

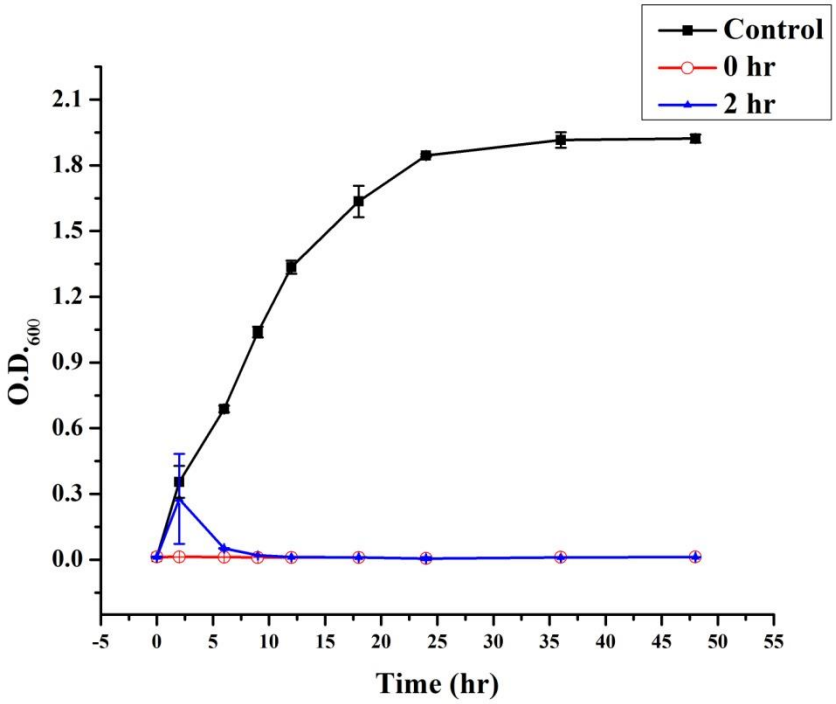

Fig. 5.6. The inhibitory activity of BLIS on the growth of *B. cereus* MTCC 430.

5.4.7. Partial purification of BLIS

The fractionation step after dialysis of the ammonium sulphate precipitate resulted in two fractions: concentrate and permeate. Both the fractions were tested for antimicrobial activity and it was found that the concentrate retained antimicrobial activity but the permeate did not. This suggested that the size of the antimicrobial substance is >10KDa. From the tricine- SDS PAGE and in gel bacteriocin activity it was found that the bacteriocin had an apparent size of 20 KDa (Fig. 5.7). Different members of the genus *Bacillus* are known to produce a diverse array of heat stable antimicrobial peptides active against food spoilage bacteria [26, 27, 28] without showing any antagonistic activities against lactic acid bacteria, which could suggest a potential application in food industry. The bacteriocins having intermediate size (10-30 KDa) have not been yet classified into any group due to limited information on their protein or DNA sequence. They can be represented as bacteriocin like substances (BLIS) [3].

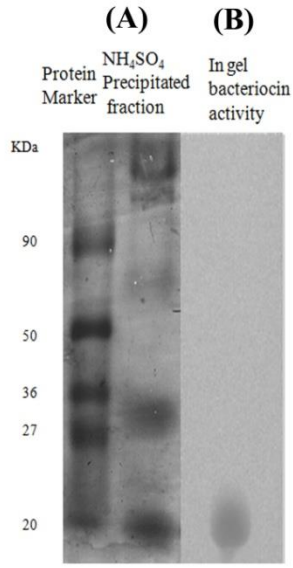

Fig. 5.7. (A) Native PAGE of bacteriocin and (B) in gel activity against *Bacillus cereus* MTCC 430

5.4.8. Effect of enzymes, pH, and temperature on BLIS activity

The sensitivity of bacteriocin- like substance was tested in presence of enzyme, pH and temperature can be observed from the Fig. 5.8. When treated with catalase, there was little or no change of activity as compared to the control. But treatment with proteolytic enzyme resulted in significant decrease in activity, maximum decrease was observed in samples treated with trypsin and proteinase K signifying proteinaceus nature of the antimicrobial substance. During food processing, factors such as temperature or pH might affect the bacteriocin activity [29]. At pH 2, 3, 9 and 10 significant decrease in activity was found and at near neutral pH values activity was observed to be comparable. Bacteriocin activity did not change significantly till 80 °C, but above this the activity kept on decreasing and there was no activity was found at 121 °C.

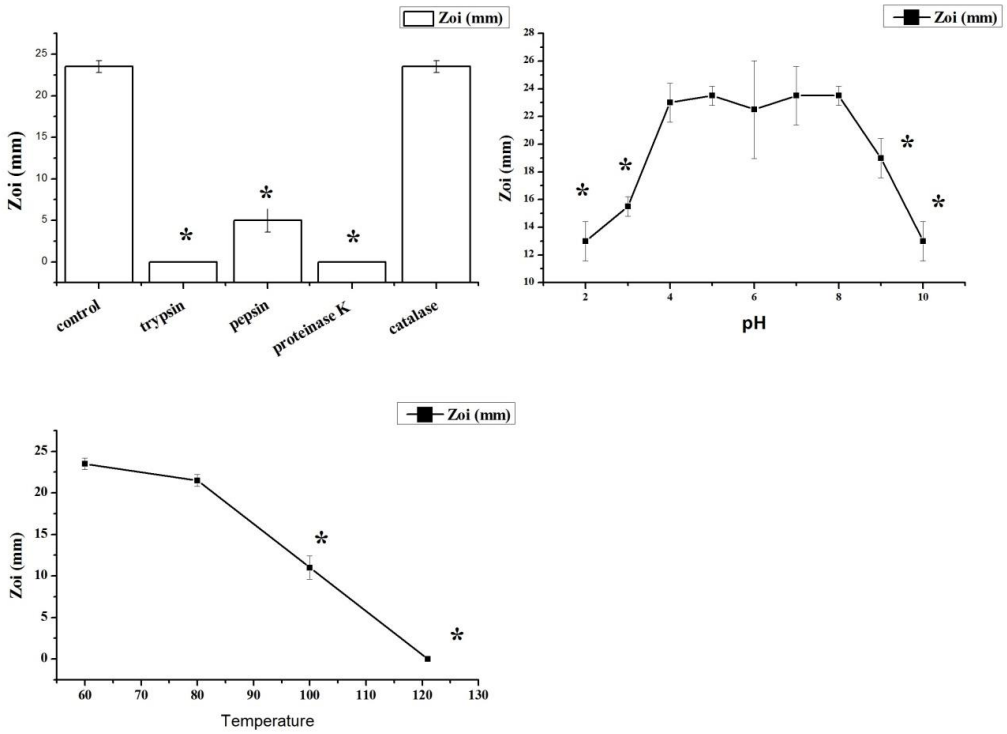

Fig. 5.8. Effect of enzymes, pH and temperature on bacteriocin action. *indicates significant difference (P <0.05).

5.4.9. Safety evaluations

Absence of hemolysis is an important attribute of probiotic strains [30]. As shown in the Fig. 5.9 the strain *Bacillus* sp. NK7 did not show any change in the blood agar plate (ϒ- hemolysis), whereas, *Bacillus cereus* MTCC 430 showed zone of inhibition (β- hemolysis). Antibiotic susceptibility is another very important property that any probiotic should possess and the strain *Bacillus* sp. NK7 showed susceptibility to most of the antibiotics except penicillin G as shown in the table 5.5. There are reports of penicillin G resistance of probiotics belonging to the genus *Bacillus* [31, 20].

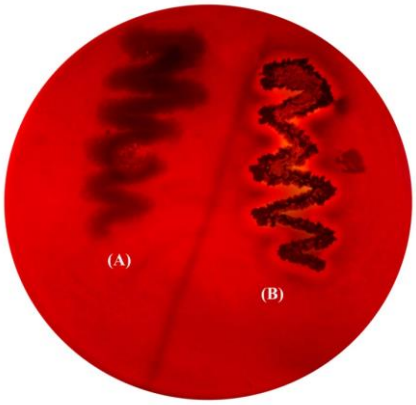

Fig. 5.9. Pattern of hemolysis on blood agar; (A) *Bacillus* sp. NK7 and (B) *Bacillus cereus* MTCC 430

Table 5.5. Antibiotic susceptibility test

Protein synthesis inhibitor	Zone of inhibition
Gentamycin	+++
Erythromycin	++
Chloramphenicol	++
Fusidic acid	++
Clindamycin	++
Tetracycline	+++
Cell wall synthesis inhibitor	
Vancomycin	++
oxacillin	+
Cephalothin	++
Penicillin G	–
Amoxyclav	++
Cotrimazol	+++
Methicillin	+++
DNA synthesis inhibitor	
Levofloxacin	++
Ciprofloxacin	++

Zone of inhibition are represented as mean ± S.D., + indicate 10–20mm, ++ indicate 20–30mm, +++ indicate >30mm and − shows no zone of inhibition

5.5. Conclusions:

In this study, BLIS production was optimized using response surface methodology and the effects of carbon source, nitrogen source and initial pH were found to be crucial for its production. The BLIS showed antagonistic properties against different gram positive bacteria, some of which were food- borne pathogens. Nonetheless, it did not show any antimicrobial activities against *Lactobacillus* and *Pediococcus*

strains suggesting possible food- grade application of the BLIS. Moreover, the putative probiotic properties and safety aspects also reveals that the strain *Bacillus* sp. NK7 has the potential to find its application as an alternative source of natural biopreservative.

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