Extraction and Purification of Bacteriocin from *Lactobacillus brevis CB-2* and *Lactobacillus zymae WHL-7* for their antimicrobial activity

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ABSTRACT

Antibiotic resistance and Food spoilage are the global issues and has detrimental impact on human health. In present study we isolated bacteriocinogenic *Lactobacillus brevis CB –2* and *Lactobacillus zymae WHL – 7* from cabbage and whey respectively and identified by phylogenetic method (16s rRNA gene sequencing). Fermentative production of bacteriocin from both isolates was carried out in optimized and modified MRS and M17medium separately. Purification of bacteriocin was done through SP- Sepharose, Sephadex – G and RP-HPLC with C18 column. Molecular weight of purified bacteriocin from CB - 2 and WHL - 7 was >40 and >15KD respectively. Effect of physical and chemical factors on purified bacteriocin revealed that, bacteriocins from CB - 2 and WHL - 7 was stable and active when heated with 80^{o}C for 15 min. and 80^{0}C for 30 min. and at pH 5 to 8 respectively. However, antibacterial activity of both samples completely lost at 80^{0}C for 45 and 60 min and at 2, 3, and 10. Activity of both bacteriocin loses when treated with proteolytic enzymes; however activity of WHL-7 was also loses with amylase hence it may be of glycoprotein in nature. Antimicrobial spectra of bacteriocin revealed their broad spectrum nature.

KEY WORDS: Food Preservation, Bacteriocin, Antimicrobial activity, *Lactobacillus sp*.

INTRODUCTION

Since ancient times, developed civilization of humans has been continuously engaged in development of new antimicrobial agents to combat infections. One of the great example of use of antimicrobials in the pre-antibiotic era is the use of potent anti-malarial drug, qinghaosu (Artemisinin), extracted from Artemisia plants, used by Chinese herbalists for thousands of years as a remedy for many illnesses (Chi and Su, 2009). The triumph of antibiotics to fight against microbial infections is one of the greatest accomplishments of Fleming's for Penicillin and this realm was auxiliary extended by Waksman for

Streptomycin from soil isolate *Streptomyces griseus*. Consequent discovery and production of a wide variety of antibiotics on a massive scale has revolutionized the human civilization. Now days the role of antibiotics has expanded from treating serious infections to preventing infections in surgical patients, cancer treatment, and impaired immune response and promoting growth in livestock. In agriculture, plant diseases and food spoilage are the major causes of considerable looses in fruits, vegetables, food and feed; results in significant reduction in their quality and quantity. In addition to economic losses, the toxin produced by moulds showed very destructive effect on health (Filtenborg *et al*., 1996). Nowadays, growers still rely profoundly on chemical pesticide and broad spectrum antibiotics to prevent or control these diseases. As due to the increased share of minimally processed fruits and vegetables in Ready to Use (RTU) form, the consumers have become more aware about the use of synthetic food additives, chemical food preservatives, antibiotics and pesticide residues in food. With the emergent consumers' rejection of chemical additives, there is growing demand for alternative antimicrobial agent and treatments. As far as live stock is concern, antibiotics have been used for therapeutic and prophylactic treatments to control a variety of bacterial infections. Different types of antibiotics have also been fed at sub-therapeutic levels to cattle, poultry, swine and aquaculture (fishes) to increase productivity and feed efficiency (Mc Dermott *et al*., 2002).

After the widespread use, the overuse and sometimes misuse of antimicrobials in human, plant and veterinary medicines, resulted the increasing threat of antibiotic resistance in microorganisms. Due to the upsetting antibiotic resistance, re-motivated research efforts are needed to find new and alternative arsenal to this problem. Numerous antibacterial agents are now being considered such as bacteriophage, Probiotic bacteria, Antimicrobial peptide and bacteriocins (Gillor, O., *et al*., 2005). According to Tagg (1991) bacteriocins are defined as "extracellularly released bacterial cationic membrane active peptides or protein molecules that are able to kill certain other closely related bacteria by a mechanism against which the producer cell exhibit a specific immunity". Class IV l antibiotics are complex peptides containing carbohydrate or lipid moieties which are important for activity (Begum Mothia, 2012). Presently more than 2000 different AMPs have been identified and several have entered into clinical trials (Jenssen *et al*., 2006). Conventional antibiotics generally target metabolic enzymes resulting into development of selective resistance, whereas AMPs act by generation of membrane pores, thus making it inherently difficult to develop resistance (Sang & Blecha, 2008). Thus, to enhance the bacteriocin arsenal against these undesirable microorganisms (food spoilage and pathogens) it is important, not only to advance the study (mode of antimicrobial action and their biosynthetic mechanisms) of known bacteriocins, but also to continue the search for more novel bacteriocins with promising properties. Therefore the present study was undertaken to study Bacteriocin from *Lactobacillus brevis CB-2* and *Lactobacillus zymae WHL-7* for their antimicrobial activity.

MATERIALS AND METHODS

Bacterial strains and cultural conditions

Bacteriocin producing Lactic acid bacteria (LAB) were isolated from whey and spoiled cabbage collected from local dairy plant and local market of Barshi (MS), India respectively and cultured in microaerophilic condition on MRS agar at 37^0C. After incubation, white colored, catalase and peroxidase negative, well isolated colonies were selected, purified and preserved in MRS agar (0.7%) overlaid with glycerol at -20^0C. Test microorganisms were procured from United States Department of Agriculture, USA (Table 1).

Table 1: Bacterial strains used for detection of antibacterial activity

Sr. No.	Test microorganism	Cultivation medium	Incubation temperature (°C)
1	*Bacillus cereus B*-3711	TSB / NA	37
2	*Bacillus coagulans NRS*- 609	TSB / NA	37
3	*Alkaligenes faecalis NRRL B* 170	TSB / NA	37
4	*Corynebacterium variabili NRRL B* 4201	TSB	28
5	*Enterobactor aerogenes B* 14144	TSB / NA	37
6	*Escherichia coli B* 14581	TSB / NA	37
7	*Klebsiella pneumoniae B* 41958	TSB / NA	37
8	*Kurthia gibsonii B* 41085	TSB / NA	28
9	*Proteus vulgaris B* 123	TSB / NA	37
10	*Pseudomonas aeruginosa B* 800	TSB / NA	37
11	*Staphylococcus epidermidis B* 2616	TSB / NA	37
12	*Enterococcus faecalis B* 537	TSB / NA	37

Screening of bacteriocin producing lactic acid bacteria

Broad spectrum antimicrobial activity showing LAB were screened from isolated colonies by agar overlay method (Hardy, 1982) against *Bacillus coagulans NRS*-609, *Escherichia coli B* 14581, *Pseudomonas aeruginosa B* 800 and *Klebsiella pneumoniae B* 41958.

Identification of promising bacteriocin producing strain

The promising bacteriocin producing strains were identified by morphological, biochemical and molecular methods (Heilig *et al.*, 2002). The phylogenetic identification of LAB isolates was carried out using universal Lac16S-forward primer (5' AATGAGAGTTTGATCCTGGCT 3') and Lac16S-reverse primer (5' GAGGTGATCCAGCCGCAGGTT 3'). The PCR was performed at 94°C for 2 min., 35 cycles (at 94°C for 30s, 52°C for 30s and 72°C for 30s), and 72°C for 5 min. The 600-1000 bp PCR product (data not shown) was sequenced by automatic gene sequencer (Hitachi) and phylogenetic tree was constructed by using Neighbor Joining Method (NJ) by Kimura 2 parameter with 1000 replicas in MEGA 4.0 and sequences were submitted to NCBI for accession numbers.

Screening of suitable fermentation medium for bacteriocin production

MRS broth, Bacteriocin Screening Medium (BSM) and M17 broth media were used to screen the suitable fermentation media (Jamriangrit, 2004) at 37^0C for 48 hrs. During fermentation, aliquots of samples were

removed for growth curve analysis, Protein estimation by lowery method (Lowry *et al.*, 1951) and antimicrobial activity by agar well diffusion method (Shillinger *et al.*, 1991). The medium showing maximum protein concentration and antibacterial activity was selected and used for further optimization studies.

Optimization of screened media and batch fermentation of bacteriocin

The screened medium was optimized for nutritional and environmental conditions such as optimization of carbon and nitrogen source, growth factor, pH and temperature. Optimization was done by changing one parameter and keeping other parameters constant. Bacteriocin production was carried out by inoculating 5ml inoculum (5×10^5 cfu/ml) of promising isolate in 100ml optimized medium and incubated microaerobically at 35^0C for 24 hrs.

Extraction and purification of bacteriocin

Fermented broth was centrifuged (4^0C, 8000 rpm, 15 min.) and pH of supernatant adjusted to 7.0. Cell free supernatant was precipitated by Ammonium sulfate at 70% saturation (4^0C), centrifuged (4^0C, 12000 rpm, 20 min.), and dialyzed in 50mM Sodium Phosphate buffer (SPB) (pH 6.8) for 24 hrs. Dialysate was collected, labeled as Crude bacteriocin preparation (CBP) and used for solvent extraction. The bacteriocin was extracted from CBP by treating 25 volumes of a mixture of Chloroform-Methanol (2:1 v/v) for 1 hrs at 4^0C. Fine grained white precipitate was collected, vacuum-dried and dissolved in 3ml of 50mM SPB (pH 6.8), and labeled as Partially Purified Bacteriocin (PPB). The PPB was applied to SP-Sepharose column (10 x 25mm) and eluted (flow rate of 2ml/min) with linear gradient of 0.2-2M NaCl in 20mM sodium phosphate buffer (pH 6.8), active fractions were loaded onto Sephadex G 50 (10 x 25mm) and eluted (flow rate of 2ml/min) with 50 mM phosphate buffer (pH 7.0). For final purification, active fractions from Sephadex G were applied to Agilent TC C_{18} (4.6 X 250 mm) Reverse Phase High Performance Liquid Chromatography column integrated in a high performance liquid chromatography system (Agilent 1100 series). Elution was carried out with linear gradient generated by 0.1% (v/v) Tri fluoro acetic acid (TFA) and 20-80% acetonitrile containing 0.1% TFA for 55 min. The Molecular weight of active fractions collected from RP-HPLC was determined by SDS-PAGE with standard peptide marker and gel was developed by Silver nitrate staining.

Effect of physical and chemical factors on purified bacteriocin

Purified bacteriocin (100µl) added into 100µl of enzyme buffer solution and incubated at 37^0C temperature for 2 hrs. The following enzymes (1mg/ml) were dissolved in appropriate buffer as follows: Trypsin, 0.04 M/l Tris-HCL and 0.1M/l $CaCl_2$ (pH 6.2), Pepsin, 0.2 M Citrate buffer (pH 6.0), α - amylase, 0.03 M/l sodium acetate buffer (pH 6.0), Lipase, 0.05 M/l Phosphate buffer (pH 6.8), Alkaline phosphatase, 0.2 M/l Phosphate buffer (pH 8.6) and Catalase, 0.2M/l Phosphate buffer (pH 6.8) (Dunder, 2006). To analyze thermal stability, 500µl of purified bacteriocin sample was heated separately at 40^0C

to 80^{0}C temperatures for 15, 30, 45, and 60 min. The effect of pH on their activity was determined by adjusting pH of samples from 2 to 10, incubated for 6 hrs and assayed by agar well diffusion method.

Antimicrobial susceptibility of purified bacteriocin

The antimicrobial spectrum of the purified bacteriocin was checked against test microorganisms (Table 1) by agar well diffusion method.

RESULTS AND DISCUSSION

Screening and identification of promising bacteriocin producing LAB

Bacteriocin producing strains *viz.* CB-2 and WHL-7 were isolated from Spoiled cabbage and Whey respectively by spot on lawn method (fig.1). Morphological and biochemical characteristics (Data not shown) revealed both strains belong to Lactic acid bacteria. Moreover, 16s rRNA partial sequencing and phylogenetic analysis, the isolates were identified as WHL7 – *Lactobacillus zymae* (Fig. 2, gene bank accession no. KJ 607885) and CB2 – *Lactobacillus brevis* (Fig. 3, gene bank accession no. KJ 531445). Numerous studies also reported the isolation of bacteriocinogenic lactic acid bacteria; Savadogo *et al*., (2004) isolated 08 strains from Burkina faso fermented milk; Deshmukh & Thorat, (2013) also isolated from Whey; in present study the percentage of getting bacteriocinogenic LAB was 56.66; it is much higher than Diep *et al*., (2002) get only 0.6% isolates

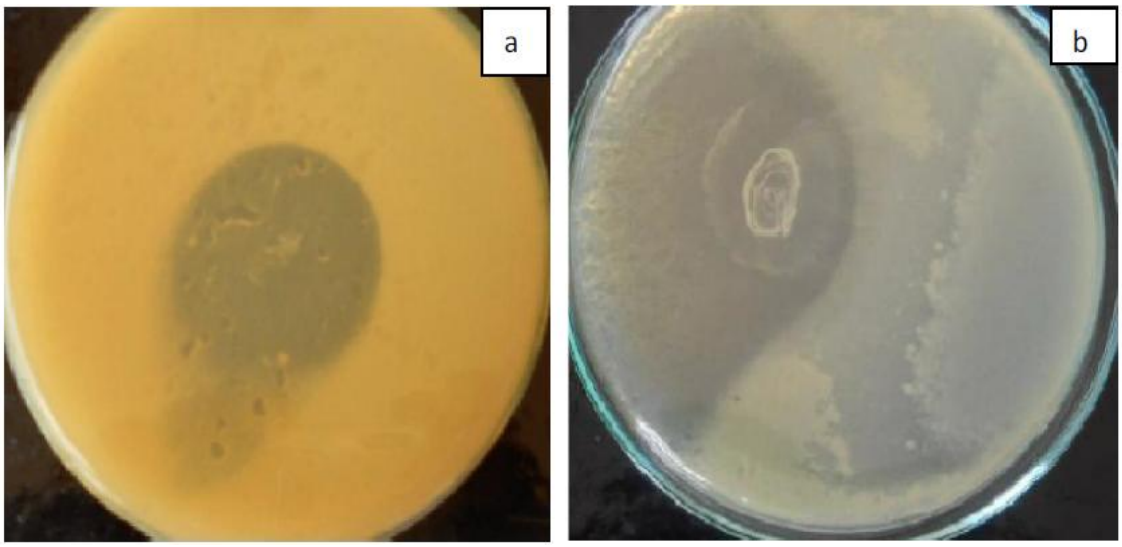


Fig 1: Antibacterial activity of (a) *Lactobacillus brevis CB-2* against *Bacillus coagulans B 609* and (b) *Lactobacillus zymae WHL-7* against *Pseudomonas aeruginosa* by agar overlay method.

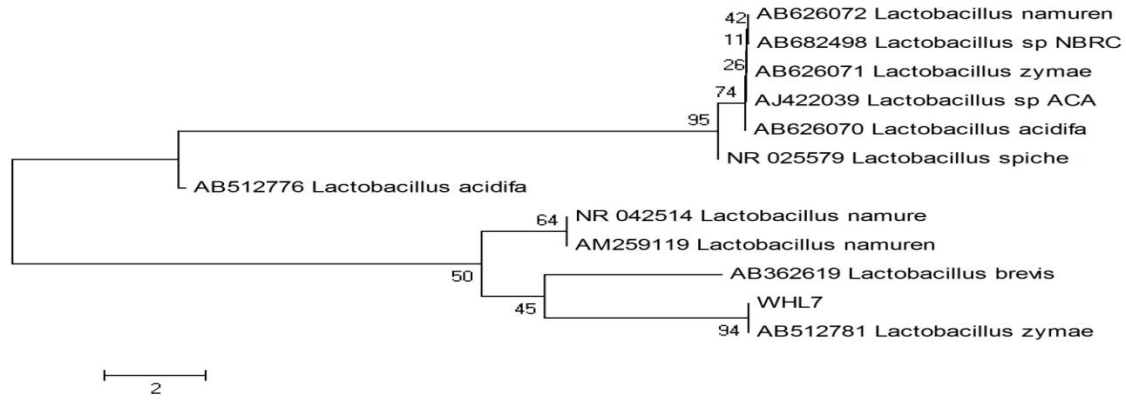


Fig 2: Phylogenetic tree of WHL7

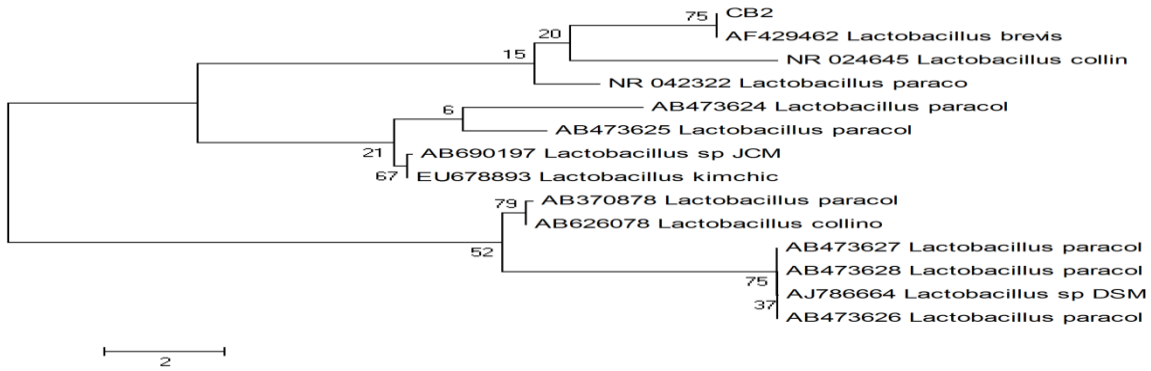


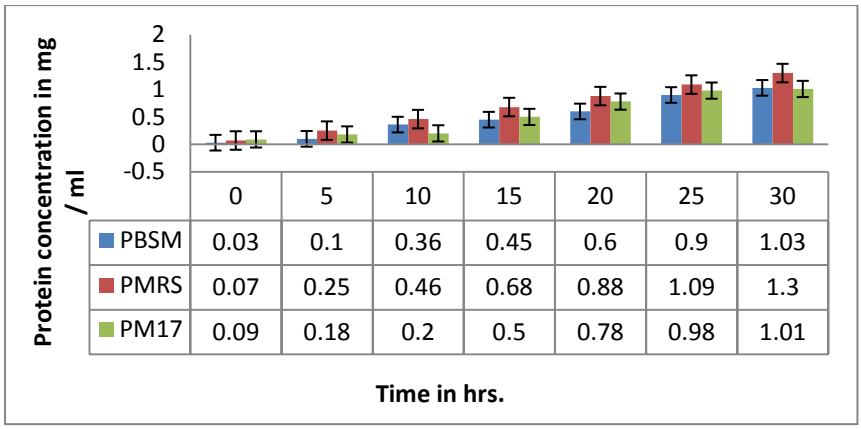
Fig 3: Phylogenetic tree of CB2

Screening of suitable fermentation medium for bacteriocin production

Ganzle *et al*., (1999) reported that, bacteriocin production was influenced by medium composition, incubation temperature, pH and microorganisms growth phase. Fig. 4 and Fig. 5 showed isolates CB2 and WHL7 showed maximum protein concentration i.e. 1.3 and 1.40 mg/ml and zone of growth inhibition i.e. 12 and 16 mm in MRS and M 17 medium respectively (Table 2). These results supported to Sansit, (2004); Oh *et al.*, (2000); Coeuret *et al.*, (2003), they achieved maximum protein concentration and antibacterial activity in BSM and M17 media,MRS medium respectively. The production and amount of bacteriocin released into medium is dependant on growth, physiological activity and biomass of producing strains. Almost all bacteriocins from lactic acid bacteria demonstrate primary metabolite kinetics (Zamfir *et al.*, 2000) since production occurs during mid – exponential phase and increases to reach maximal level at the end of exponential phase (Cheigh *et al.*, 2002).

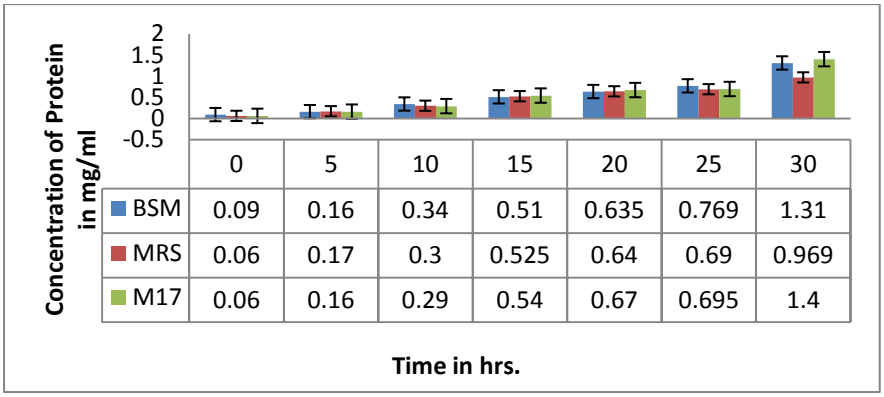
Table 2: Zone diameter of growth inhibition of *Bacillus coagulans NRS 609 by* promising LAB isolates in various media.

Sr. No.	Isolates	Zone diameter of growth inhibition of *Bacillus coagulans NRS 609* (in mm)			Standard Deviation	Coefficient of Variance
		BSM	MRS	M17		
1	CB 2	7	12	8	2.160	24.02
2	WHL 7	9	13	16	2.867	22.637



	0	5	10	15	20	25	30
PBSM	0.03	0.1	0.36	0.45	0.6	0.9	1.03
PMRS	0.07	0.25	0.46	0.68	0.88	1.09	1.3
PM17	0.09	0.18	0.2	0.5	0.78	0.98	1.01

Fig 4: Protein concentration of isolate CB2 in various media



	0	5	10	15	20	25	30
BSM	0.09	0.16	0.34	0.51	0.635	0.769	1.31
MRS	0.06	0.17	0.3	0.525	0.64	0.69	0.969
M17	0.06	0.16	0.29	0.54	0.67	0.695	1.4

Fig 5: Protein concentration (b) of isolate WHL7 in various media

(Notations: PBSM – Protein concentration in BSM medium, PMRS – Protein concentration in MRS medium and P M17 – Protein concentration in M 17 medium)

Optimization of screened media

Table 3: Set of parameters for bacteriocin production by WHL7 and CB 2

Sr. No.	Parameters	Zone diameter of growth inhibition of *Bacillus coagulans NRS 609* in mm	Parameters	Zone diameter of growth inhibition of *Bacillus coagulans NRS 609* in mm
		WHL7		**CB 2**
1	Basal M17	**16**	Basal MRS	**12**
2	Basal M17 (Except Glucose) + 1% Carbon source	**23** (Fructose)	Basal MRS (Except Glucose) + 1% Carbon source	**20** (Lactose)
3	Basal M17 (Except Peptone) + 1% Nitrogen source	**19** (BTA)	Basal MRS (Except Peptone) + 1% Nitrogen source	**21** (Tryptone)
4	Basal M17 + Optimized Yeast Extract Concentration	**25** (0.8g/100ml)	Basal MRS + Optimized Yeast Extract Concentration	**20** (08 g / 100ml)
5	Incubation Temperature(ºC)	**27** 35	Incubation Temperature (ºC)	**20** 35
6	Initial pH	**23** 7	Initial pH	**26** 7.0
7	Partially optimized M17	30	Partially optimized MRS	24

Todorov *et al.*, (2005) achieved elevated antimicrobial activity in medium containing Tryptone (20g/L) and, combination tryptone and yeast extract (1:0.6), at pH of 6.5-7.5 and 30^{o}C-40^{0}C incubation temperature. In addition they observed decreasing antimicrobial activity below and above 6.5-7.5 pH. Malini and Savitha, (2012) observed a maximum bacteriocin production at 37^{0}C incubation temperature, pH 7.0. Rate of bacteriocin production increases with increasing concentration of yeast extract up to certain extent (Ansen *et al.*, 2002). Decreased bacteriocin activity after 48 hrs. due to bacteriocin degradation by the proteases and declined pH (Torri *et al.*, 1994).

Batch fermentation, extraction and purification of bacteriocin

Batch fermentation of bacteriocin was carried out for both isolates separately in modified and optimized medium (Table 3). In Chloroform-Methanol extraction, antibacterial activity of interface was greater than solution collected from lower and upper layer and non extracted culture supernatant fluid. This may indicate that sufficient mixing of culture supernatant fluid with chloroform : methanol, concentrates bacteriocin aggregates at the interface (Burianek *et al.*, 2000). Bacteriocins are amphiphilic peptides with high affinity towards lipid membrane. The interface between chloroform and aqueous media creates ideal environment for the concentration of amphiphilic substances such as bacteriocins (Burdick and Jackson 1980).

The antibacterial activity of PPB against *Bacillus coagulans NRS 609* showed increased zone of growth inhibition as compared to zone observed in cell free supernatant (Sridevi *et al.*, 2008; Atta *et al.*, 2009). The "Selectivity" of RP – HPLC seperations can be substantially modified by the use of different ion-pairing reagents (Guo *et al.*, 1987) among them commonly used was Trifloroacetic acid (TFA) and acetonitrile. Fig. 6 and Fig. 7, implicates the peak showing graphs of reverse phase high performance liquid chromatography (RP-HPLC) of bacteriocins, in which fractions collected at 11.00 and 6.36 min of CB2 and WHL7 showed zone of growth inhibition respectively.

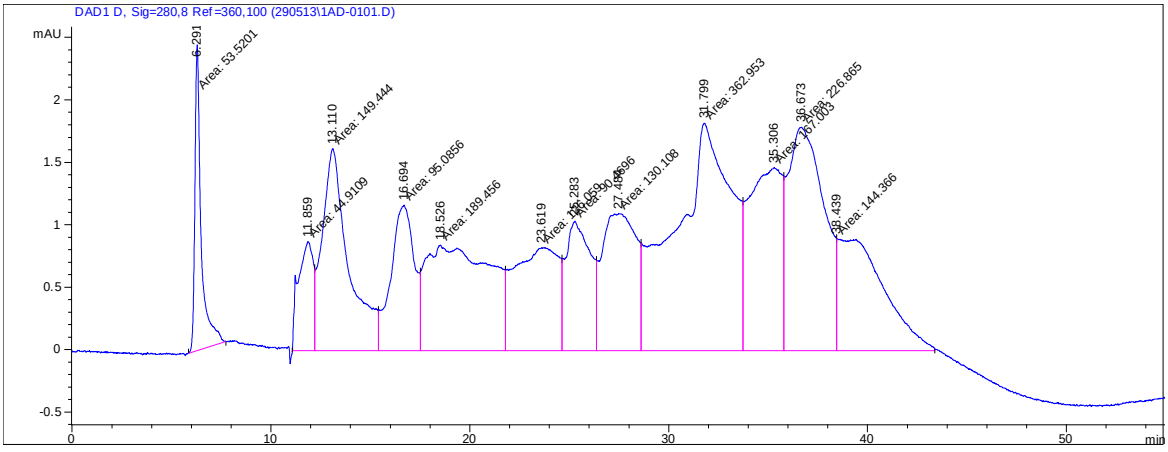


Fig 6: RP-HPLC purification of bacteriocin obtained from CB2 isolate

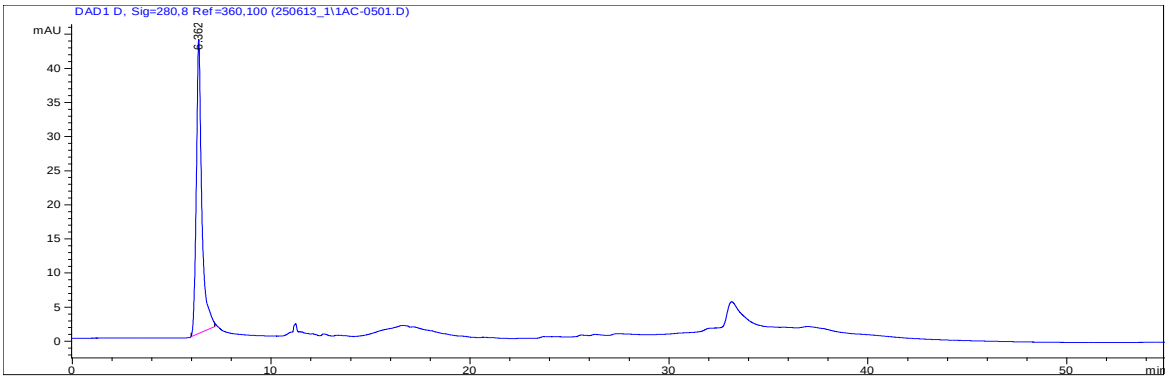


Figure 7: RP-HPLC purification of bacteriocin obtained from WHL7 isolate

Table 4: Zone diameter of growth inhibition of *Bacillus coagulans NRS 609* against bacteriocin from promising LAB isolates at subsequent purification step.

Sr. No.	Isolates	Zone diameter of growth inhibition against *B. coagulans* in (mm)				
		Cell free supernatant	(40%)Ammonium salt, Dialysis and solvent extraction	Chromatographic methods		
				Cation exchange	Gel filtration	RP – HPLC
1	CB 2	10	13	17	20	26
2	WHL 7	9	13	18	20	27

It is apparent from Table 4, both these bacteriocin samples showed increasing zone of growth inhibition as further purification occurs. These results support to the observations recorded by Ravishankar *et al.*, (2012) bacteriocin sample from *Lactobacillus plantarum* isolated from cow milk showed antimicrobial activity of 27.3, 155.3 , 1023.1 AU/ml in Culture filtrate, Ammonium sulfate precipitation and Sephadex G-100 gel filteraion chromatography respectively. Cheikhyoussef *et al.*, (2010) investigated Bifidin I from *Bifidobacterium infantis* BCRC 14602, and reported an increase in bacteriocin activity from 2.6 X 10^2 AU/mg for neutralised cell free supernatant to 3.7 X 10^5 AU/mg for the purified bacteriocin. Simova *et al.*, (2009) achieved a 10^5 fold increase in bacteriocin activity after a single peak was assessed upon C_{18} reverse phase liquid chromatography purification. Similarly, El – Shouny *et al.*, (2012) also observed an increased antimicrobial activity of bacteriocin from *Lactobacillus plantarum* SR 18 after gel filteration using Sephadex G 100.

Antimicrobial susceptibility of purified bacteriocin

Bacteriocin from both isolates showed growth inhibition against test bacteria. However, the isolate WHL7 inhibit the growth of all bacterial genera employed in present study, consequently it can be concluded, the bacteriocin preparation fom both isolates having broad spectrum antibacterial activity (Table 6). Sifour *et al.*, (2012) reported 35 lactic acid bacterial strains showed a broad spectrum antibacterial activity against *Escherichia coli ATCC* 29522, *Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Salmonella sp. Staphylococcus aureus ATCC* 29523, *Pseudomonas aeruginosa,*

Bacillus subtilis and *Listeria monocytogenes*. Yang *et al.*, (2012) observed growth of *Listeria innocua, Escherichia coli, Bacillus cereus, Pseudomonas fluorescens, Erwinia carotovora, Leuconostoc mesenteroides subsp. mesenteroides, Penecillium expansum, Botrytis cinerea* and *Monilinia frucitcola* were inhibited by bacteriocin from lactic acid bacteria isolated from cheese and yoghurt. Ravishankar *et al*., (2012) bacteriocin from *Lactobacillus plantarum* inhibited the growth of *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes and Enterococcus faecalis.* In addition they suggested that bacteriocins having broad spectrum of antimicrobial activity may be explored for development of new biopreservative.

Table 6: Antimicrobial susceptibility of purified bacteriocin against test microorganisms

Sr. No.	Test microorganism	Antimicrobial activity of purified bacteiocin	
		CB 2	WHL 7
1	*Bacillus cereus B*-3711	+	+
2	*Bacillus coagulans NRS*- 609	+	+
3	*Alkaligenes faecalis NRRL B* 170	+	+
4	*Corynebacterium variabili NRRL B* 4201	-	+
5	*Enterobactor aerogenes B* 14144	+	+
6	*Escherichia coli B* 14581	+	+
7	*Klebsiella pneumoniae B* 41958	+	+
8	*Kurthia gibsonii B* 41085	-	+
9	*Proteus vulgaris B* 123	+	+
10	*Pseudomonas aeruginosa B* 800	+	+
11	*Staphylococcus epidermidis B* 2616	+	+
12	*Enterococcus faecalis B* 537	+	+

(+ = presence of antimicrobial activity, - = absence of antimicrobial activity)

All the above results, showed conflict in the definition of bacteriocin; as bacteriocins are showing antimicrobial activity against closely related strains, but numerous reports recorded broad spectrum antimicrobial activity of bacteriocins. Hence, it is prime important to modify the definition of bacteriocin. Although the recent progress in food biotechnology, due to modern technologies and safety concepts (e.g. HACCP), the problem of food safety and security remains to be solved. Protective cultures and associated antagonistic substances should be considered an additional factor. However, nisin is the only bacteriocin which has been accepted by the WHO as a preservative in the food industry (Vandenbergh, 1993). Hence it is prior important to screen new biopreservative from Generally Recognised As Safe (GRAS) bacteria.

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