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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN FROM MARINE *Lactobacillus pentosus* B25

Bharti Ghude Wadekar
Assistant Professor, Microbiology ID,
ZSCT's Thakur Shyamnarayan Degree College, Kandivali East, Mumbai.
Email ID: bhartiwadekar@gmail.com

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ABSTRACT:
The bacteriocin B25 produced by Marine *Lactobacillus pentosus* B25. Purification was carried by ammonium sulphate precipitation, gel filtration chromatography. Gel filtration chromatography provides 3.04 fold increase in specific activity (553.84 AU/mg). The total production found 27 ml that contain 7.8 mg/ml bacteriocin concentration. This bacteriocin B25 was found to be stable up to 110^{0}C at 20 min, so it is heat stable protein and active over a wide pH range of 4.0-10.0. It was proteinases nature, because their activity inhibited by proteinase K enzyme. It remained active in Triton X-100, Tween-80, Tween 20 and SDS but lost its activity when treated by urea and EDTA. Molecular weight of bacteriocin estimated by Glycine SDS-PAGE and found to be <3.5kDa.
This study describes the production, purification and characterization of bacteriocin B25.

Key words: - Bacteriocin, *Lactobacillus pentosus* B25, gel filtration Chromatography, SDS-PAGE

INTRODUCTION :

Lactobacillus pentosus is important in many food fermentations either as a component of natural microflora or used as a starter culture. A number of *Lactobacillus* strains produce bacteriocins, which have been isolated and partially characterized (Maqueda *et al.*, 2008). Three major methods for the purification of bacteriocins by LAB to homogeneity can be distinguished. First, purification can be done by a conventional method that is based on a rather laborious series of subsequent steps of ammonium sulphate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography (Mortvedt *et al.*, 1991). Second, a simple three-step protocol has been developed (Callewaert *et al.*, 1999), including ammonium sulphate precipitation, chloroform/methanol extraction/precipitation and reversed-phase high-pressure liquid chromatography, the sole chromatographic steps involved. Third, bacteriocins can be isolated through a unique unit operation, i.e. expanded bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titre through pH adjustment of the crude fermentation medium (Callewaert and De Vuyst, 1999). Following the latter two methods, which are more rapid than the first conventional method and yet successful, complete purification of highly hydrophobic bacteriocins using reverse-phase HPLC is usually difficult, although the technique was successfully used to purify the amphophatic bacteriocins: nisin, lacticin S, lactacin 481, curvacin A, and sakacin P (Carolisssen-Mackey *at al.*, 1997).

In present study, we produced and purification of bacteriocin B25 has been carried out by ammonium sulphate precipitation, gel filtration chromatography using Sephadex G25 and

reverse phase HPLC. Molecular weight determined by SDS-PAGE. Characterization studies also performed.

MATERIALS AND METHODS

1. Mass production of Bacteriocin

Mass production of the bacteriocin was done by surface and fed-batch fermentation. In surface fermentation, mMRS broth 500 ml was prepared in one litter conical flask and autoclaved at 110^{0}C. The 2% inoculum of overnight culture was added in the sterile medium and incubated at 30^{0}C for 18 h without aeration and agitation. The medium was centrifuged at 10,000 rpm for 15 min. to remove bacterial cells. The supernatant was heated up to 80^{0}C for 15 min (Todorov and Dicks, 2004). This supernatant was filtered through 0.22 um cellulose acetate (Millipore) membrane filter to get cell free supernatant, which was purified and employed for further purification and characterization.

2. Purification

2.a Ammonium sulphate purification:

Fractionation with different concentration of ammonium sulphate viz., 40, 50, 60, 70, 80, 90 saturation has been carried out to precipitate bacteriocin. The salt was added slowly to the supernatant with continuous stirring with the help of magnetic stirrer and kept overnight under stirring at 4^{0}C. This solution was centrifuged at 10,000 rpm for 10 min. at 4^{0}C in a cooling centrifuge (Remi CM-12). Pellet and supernatant were collected and pellet dissolved in 50mM phosphate buffer and desalted by dialysis (dialysis membrane 70, Sigma) against 20mM phosphate buffer for 5 h with three changes of buffer and the dialysate was stored at 4°C till further used. Anti-microbial activity was checked for all samples and the one with maximum zone of inhibition was used as Fraction I for further purification. (Liliana *et al.*, 2008, Sapatnekar *et al.*, 2010).

2.b Gel filtration Chromatography:

The protein sample showing bacteriocin activity in ammonium sulphate precipitation Fraction I was purified by Sephadex G-25 (Sigma) gel filtration chromatography (Radha *et al.*, 2015). The column was equilibrated with buffer A 50mM Phosphate Buffer at a flow rate of 1 ml/min, and the bacteriocin was eluted using a buffer A in 1 ml fractions.

2.c Spectral analysis:

The protein concentration was monitored by measuring absorbance at 280 nm. Fraction of 2 ml aliquots were collected and assayed for antibacterial activity, the active fractions were pooled and used as Fraction II.

2.d FTIR Analysis:

Based on earlier investigations, the selected bacterial extract which have maximum antibacterial activity was analysed by Fourier Transform Infra-Red Spectroscopy (FTIR) (NEXUS-672 model) described by Kemp, (1991).The spectrum was taken in the mid IR Region of 1000-3500 cm^{-1}. The spectrum was recorded using ATR (Attenuated Total Reflectance) technique. The sample was directly placed in the sodium crystal and the spectrum was recorded in the transmittance mode. This work performed at Chemistry Department of Institute of Forensic Science, Aurangabad.

2.e Bacteriocin Activity Assay

For every experiment two sets were prepared. Finally concentration was calculated by using the following formula:

$$AU/ml = \frac{\text{Zone of Inhibition} \times 1000}{\text{Volume added in well}} \times \text{dilution factor}$$

2.f Protein Estimation:

The protein concentration was quantified by Lowry *et al.*, (1951). Bovine serum albumin was

used as a standard. (Concentration of std. Protein- 10 mg/ml).

3. Characterization of the semi purified antimicrobial peptide by Conventional method

The crude bacteriocins of mutant isolate AS5 of *Lactobacillus pentosus* B25 with strong inhibition zone were characterized with respect to stability to temperature, pH, enzymes, solvents, surfactants, metal ions, UV-radiation and NaCl concentration.

The conventional characterization was carried out by Ivanova *et al.*, (2000); Sharma and Gautam, (2009).

3.a Effect of temperature:

In order to test the effect of temperature (heat resistance), 10 ml of partially purified bacteriocin (PPB) preparation was exposed for 15 minutes to various (1) temperature: 30, 50, 70, 90, 100 (^{0}C) in a water bath for 20 min. and (2) autoclaved at 110 and 121^{0}C pressure (15 psi) for 10 min., 20 min., and 30 min. respectively. Residual bacteriocin activity was detected against selected pathogenic bacteria at each of these temperatures by using agar-well diffusion assay (Ogunbanwo *et al.*, 2003) with slight modification. Supernatant at 37^{0}C was kept as treatment control along with media and organism controls (Armour *et al.*, 2005).

3.b Effect of pH:

According to the method described by Karaoglu *et al.*, (2003) sensitivity of partially purified bacteriocin preparation to different pH values was tested by adjusting the pH of the bacteriocin to pH 2, 4, 5, 6, 7, 8, 9 and 10 with hydrochloric acid (1N HCl) and sodium hydroxide (1N NaOH), incubated for 2 h at room temperature. After 2 h of incubation at room temperature, residual activity of each of the samples was determined against the selected pathogenic bacteria under study by agar-well diffusion assay. Control was kept.

3.c Effect of enzymes:

Effect of enzymes was tested on the antagonistic activity of partially purified bacteriocin preparation by treatment with 1 mg/ml: trypsin, proteinase K, pepsin, papain, lipase, and α-amylase (Sigma Aldrich, USA). It was then incubated at room temperature for 2 hours and residual activity of bacteriocin was assayed and un-treated peptide samples were taken as respective controls (Armour *et al.*, 2005).

4. Molecular Characterization of Purified Bacteriocin B25

4.a Molecular weight determination by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE);

Purified bacteriocin was analysed by SDS PAGE with 20 % and 4 % concentration of acrylamide in separating and stacking gel respectively. Active fractions were pooled and loaded into the well along with a broad range marker protein (3.5 – 48 kDa, GeNei). The gel was run at constant current 25mA until the tracking dye (bromophenol blue) had migrated to the end. After running, gel was stained with Coomassie blue stain and destained by 20% v/v isopropanol, 10% v/v acetic acid and distilled water and kept at overnight and visualized the protein band (Malini and Savita, 2012). The molecular weight of bacteriocin B25 was determined using standard molecular weight marker.

4.b Reverse Phase HPLC:

The fraction from gel filtration chromatography was dissolved in HPLC Graded Methanol and water (60:40) and were further purified on analytical C-18 reverse phase Column (250 x 4.6 mm) using HPLC system. The column was equilibrated with methanol and fraction were eluted with a 100% linear gradient of buffer

(Methanol 60: Water 40) as an isocratic elution mode with UV detection (λ=280nm). The flow rate was maintained at 0.6 mL/min. and fraction were monitored by Photo Diode Array (PDA) detector. The peaks were obtained. (Kawai *et al.*, 2001). This work was carried at Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.

RESULT AND DISCUSSION

1. Production of Bacteriocin

Fed Batch Fermentation was carried out in flask level (500 ml) using 2% inoculum size and 2% NaCl with pH 7. The medium was centrifuged at 10,000 rpm for 15 min to remove bacterial cells. The supernatant heated and filtered through 0.22 um cellulose acetate (Millipore) membrane filter to get cell free supernatant, which was purified and employed for characterization.

2. Bacteriocin Purification

The supernatant of an overnight *Lactobacillus pentosus* B25 culture was clarified by centrifugation and supernatant heated at 80^{0}C (Todorov and Dicks, 2004) and precipitated with ammonium sulphate 60% w/v, final concentration. Fraction IV-60% had shown the specific activity 213.33 AU/mg of bacteriocin. The specific activity of dialysis extract was found to be 301.88 AU/mg. The high level of recovery by gel filtration chromatography was observed. Bacteriocin has good affinity with Sephadex G-25 and eluted with 50mM phosphate buffer at pH 7.2 and the elution was monitored by UV range 280 nm. Gel filtration chromatography provides 3.04 fold increase in specific activity (553.84 AU/mg). The total production found 27 ml that contain 7.8 mg/ml concentration of bacteriocin (Table 1). Figure 1 and 2 has represented the spectral analysis and purification profile of bacteriocin B25.

The statistical analysis of the result data obtained during the course of study revealed that the calculated values of F due to bacteria as well as due to samples are greater than their respective F values at 95 % probability level ($p < 0.05$) Hence, there is significant difference in the antagonistic activity of *Lactobacillus pentosus* B25.

Similar steps were carried out by Mackay *et al.*, (1997) and carried an ideal protocol for bacteriocin production should be one that is applicable to large scale purification, leading to bacteriocin yields higher than 50% and purity around 90.

3. Characterization of Semi Purified Bacteriocin

3.a Characterization by conventional method:

For the characterization and evaluation of the effect of physic-chemical parameter, on the antagonistic activity of semi purified bacteriocin sample. The effect of temperature, pH, surfactant, organic solvent, metal ions, enzyme, UV treatment and NaCl concentration on the partially purified bacteriocin B25 were investigated (Table 2). The bacteriocin activity was maintained up to 30 min at 110^{0}C (2720 AU/ml) and pH 2 to 10 and its increases at pH 7 (3360 AU/mL). The bacteriocin was completely digested by Proteinase K, pepsin and trypsin but maintained in lysozyme and alpha-amylase.

Anderson, (1986) also reported loss of activity after heat treatment at 121°C for 15 min. The phenomenon of heat stability of lactic acid bacteria bacteriocin have been reported earlier in Plantaricin C19 (Audisio *et al.*, 1999), a bacteriocin produced by *L. brevis* OGI (Ogunbanwo *et al.*, 2003). In Our findings, we are also in agreement with the above, by observing the activity of bacteriocin in *Lactobacillus pentosus* B25 after heat treatment at 110^{0}C for 15min. Therefore, it could be grouped under heat stable low molecular weight bacteriocin.

Maximum activity was noted at pH 2-8. Similar results were observed by Ivanova *et al.*, (2000); Ogunshe *et al.*, (2007).

In our investigation, we observed that the antibacterial activity by culture free supernatant was completely lost when exposed to proteinase K, pepsin, trypsin, (1mg/ml) revealing its proteinaceous nature and therefore identified as bacteriocin. Similar result obtained in the work of Todorov and Dicks (2004) had shown their bacteriocins were proteinaceous nature.

Molecular Characterization of Bacteriocin

4.a FTIR Analysis:

In FTIR analysis, the extract of *Lactobacillus pentosus* B25 strain revealed that the spectral range of obtained functional group ranged was between 1000 – 3500 cm^{-3}.

From the figure 3, observed that the peak signal recorded at 3262.81 cm^{-1} and 2944.1 cm^{-1} may be due to N-H and C-H group respectively. The small and sharp peak observed at 1617.92 cm^{-1}may be due to NH2 group. Again small peak observed at 1090.41 cm^{-1} represents the C-O group. All group represented the presence of nitro and hydroxyl grouping which are responsible for their antibacterial activity.

Similar result obtained by Shetukkarashi *et al.*, (2012) reported that the extract of marine bacterium *Bacillus* sp. MB19 subjected for FTIR analysis and obtained hydroxyl group.

4.b SDS-PAGE analysis:

Further the bacteriocin purity was estimated by Glycine SDS-PAGE which and the molecular weight was found to be <3.5kDa (Fig. 4).

4.c HPLC Analysis:

After gel filtration chromatography, the active fraction was applied in the rp-C18 SPE column to remove salts from active fractions, and bounded peptide was eluted with Methanol. HPLC analysis of this active fraction showed a single peak at retention time 15.22 min indicating the homogeneity of protein. A peak at 15 - 21 min could be identified as a potent bacteriocin-derived peak (Fig.5).

CONCLUSION:

The majority of studies on the teleosts' appetite-controlling mechanisms are conducted on domesticated fish that have been bred in captivity for many generations (e.g., salmon, carp, and cod). In comparison to wild fish exposed to less favourable conditions, it is highly likely that these fish, who are accustomed to the most favourable habitat (e.g., no predators, constant light exposure and temperatures) and feeding (e.g., satiation, lack of struggle), may have changed their feeding behaviour and systems controlling appetite. Finding the similarities between wild and captive populations could reveal crucial details about how domestication affects feeding behaviour. Therefore, research on fish feeding patterns and fish sampling in their natural habitat would be tremendously beneficial (Ronnestad et al, 2017).

Teleosts and mammals share many of the same endocrine systems that regulate central control of food intake, showing a common thread of conservation throughout vertebrate evolution. Current studies on several fish hormones, such as insulin, endocannabinoids, and members of the glucagon family of peptides, are insufficient to make accurate comparisons. Only a few number of groups, such as the salmoniformes, perciformes, pleuronectiformes, and cypriniformes, have undergone extensive research. Many fish species exhibit indeterminate growth, which means that they keep expanding during the course of their whole lives. Growth of mammals and other model animals, such as zebrafish (Danio rerio), which reach a maximum length size as adults, is in contrast to this. Therefore, even though controlling one's hunger and food intake is frequently seen as a behavioural aspect of maintaining an energy balance, the basic notion

of energy homeostasis needs to be applied with great caution in fish (Ronnestad et al, 2017). The effects on food intake of various neuroendocrine systems investigated in depth in fish, such as the NPY, melanocortin system, or CCK, were comparable to those in humans. In conclusion, we are unable to precisely identify evolutionary changes in all of their facets due to the still limited knowledge about central control of food intake in fish compared to mammals. The material that is now available mostly refers to neuroendocrine signalling. As a result, it is impossible to identify any obvious evolutionary patterns in teleost fish with relation to the regulation of food intake. The majority of studies and all existing data on nutrient signalling point to homeostatic control of food intake. Research on response to various cues like stress, environmental factors, circadian rhythms, nutrients in fish is though in a preliminary state, it is comparable to mammals and imparts a definitive direction in fish research and has immense potential.

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Table 1: Purification profile of Bacteriocin B25

Sr. No.	Fraction	Zone Inhibi. (mm)	AU/ml	mg/ml	Specific activity (AU/mg)	Purification fold	Quantity (ml)
1.	Extract I (PH 7)	17	2720	15	181.33	1	500
2.	Extract II (heat treatment at 80^{0}C for 10 min.)	16	2560	12	213.33	1.17	500
3.	Ammonium Sulphate ppt. 60%	18	2880	11.8	244.06	1.34	125
4.	Dialysis	20	3200	10.6	160	0.88	65
5.	Gel filtration Chromatography						
A.	Fraction I (min.2)	25	4000	5.2	796.23	4.39	-
B.	Fraction II(min.6)	27	4320	7.8	553.84	3.04	-
6.	Fraction II (min. 6)	27	4320	7.8	553.84	3.04	27

Fig. 1: Spectral analysis of purified fraction at λ 280

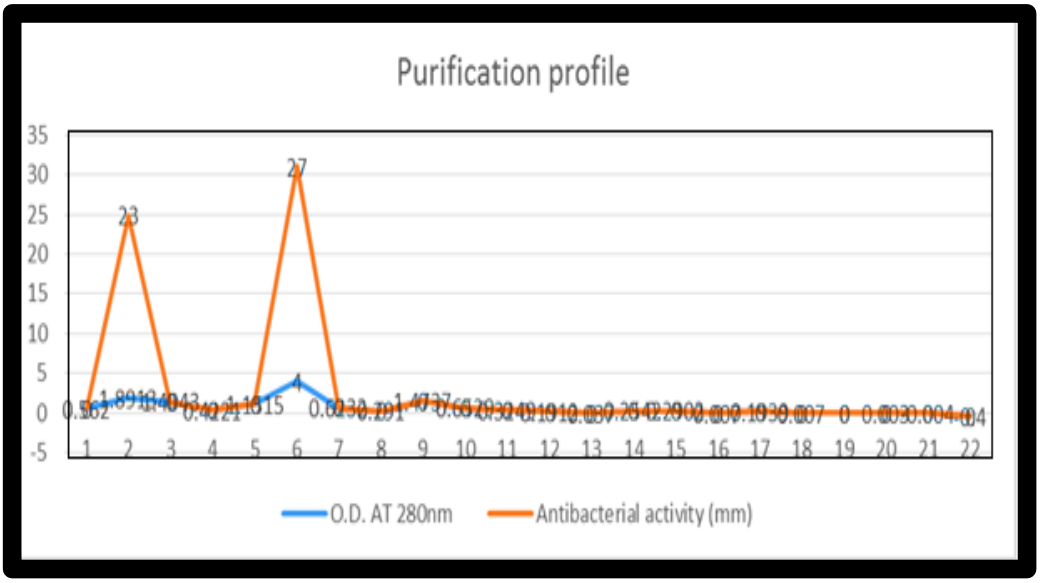

Fig. 2 purification Profile of Bacteriocin B25

Table 2: Characterization of semi-purified bacteriocin B25

Parameter	**AU/ml**	**Parameter**	**AU/ml**
Temperature(^{0}C)		**pH**	
Control	3280	2	2480
30 (20min)	3280	3	2720
50	3200	4	3200
70	3120	5	3200
90	2960	6	3280
100	3040	7	3360
110	2800	8	2560
110 (10 min.)	2720	9	2320
(20 min.)	2880	10	1760
(30 min.)	2720		
121	-		
Surfactant (0.5%)		**Enzyme**	
SDS	2720	Pepsin	-
Tween 80	3120	Alpha-amylase	2720
Tween20	2800	Pro.K	-
EDTA	-	Tripsin	-
Triton X-100	2960	Lysozyme	2960
Urea	-		

Table 3: Chemical group predicted by FTIR analysis

Sr. No.	Value for stretching Frequency	Chemical group predicted
1	326281	N-H
2	2944.1	C-H
3	1617.92	NH2
4	1090.41	C-O

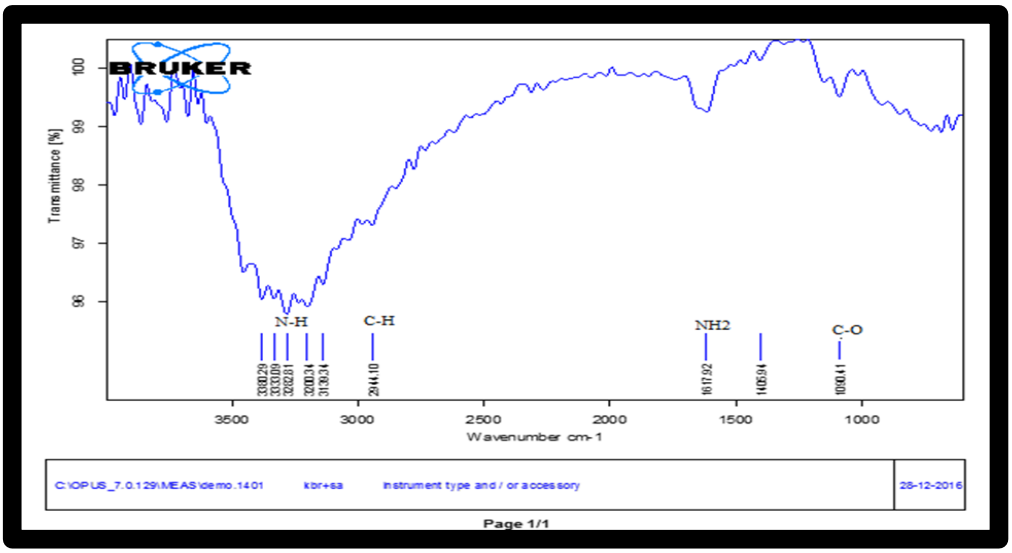

Fig. 3: FTIR analysis

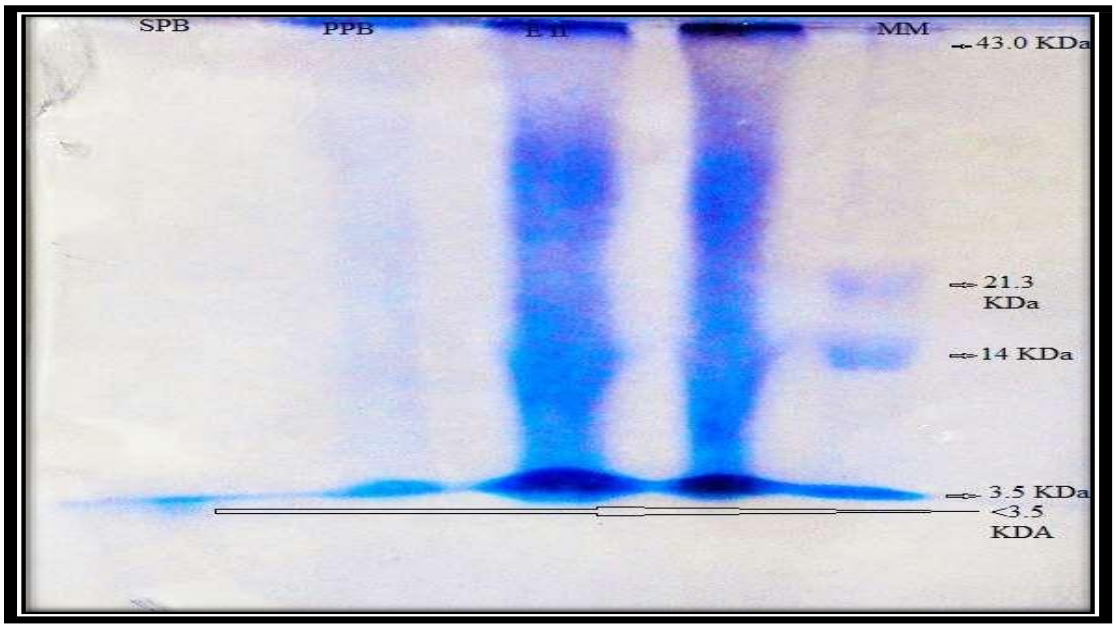

Fig. 4: SDS-PAGE Analysis
Legends: SPB: Semi Purified bacteriocin PPB: Partially Purified Bacteriocin
E II: Extract II of 60% Ammonium sulphate E I: Extract I of Supernatant
MM: Molecular Marker

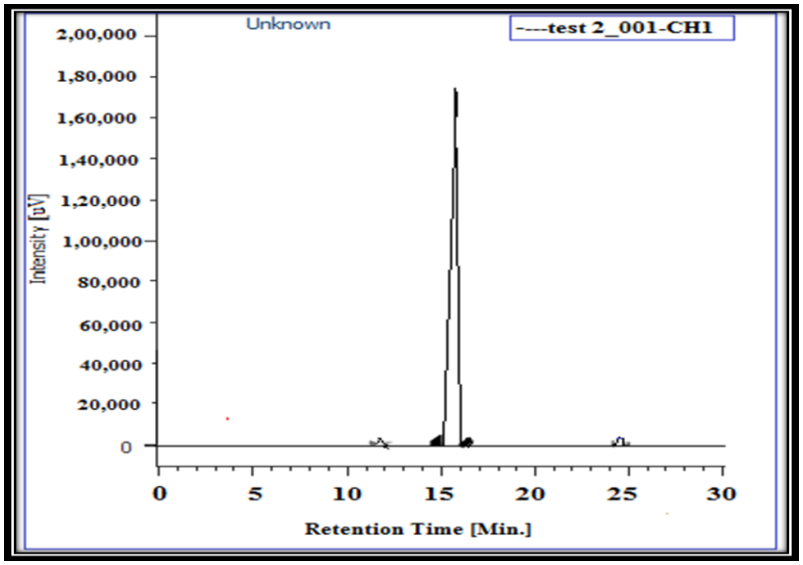

Fig. 5: HPLC Chromatogram