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Purification and Physicochemical Characterization of Anti-*Gardnerella vaginalis* Bacteriocin HV6b Produced by *Lactobacillus fermentum* Isolate from Human Vaginal Ecosystem

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ABSTRACT

Anti-*Gardnerella vaginalis, Lactobacillus fermentum* bacteriocin HV6b producing strain was isolated from vaginal swabs of healthy, fecund females. It was identified, purified and further characterized physicochemically. The relative molecular mass of bacteriocin was found to be less than 7 kDa. Maximum bacteriocin production was observed at 37°C, pH 6.5 after 16-18 h of incubation Proteinase K and pepsin strongly inhibited bacteriocin production. The bacteriocin has purified by absorption desorption method and characterized by reverse phase liquid chromatography. It exhibited high thermal stability and showed inhibitory activity over wide range of pH. The study revealed the possibility of using bacteriocin for bio-medical use and the *L. fermentum* strain as probiotic.

Key words: Bacteriocin, temperature, enzyme, stability, chromatography

INTRODUCTION

Many scientist in the field of biology and chemistry have given significant contribution to science and technology by utilizing the natural resources (Chauhan and Kaith, 2012; Aan *et al*., 2011; Abd El-Hady, 2011; Abd-El-Hady and Abd El-Baky, 2011; Abdi *et al*., 2010; Raja and Thilagavathi, 2011; Issaoui *et al*., 2011; Das *et al*., 2011; Rocco, 2011; Adedayo, 2012). Bacteriocins are biologically active peptides produced by several bacterial species against related species (Tagg *et al*., 1976). Bacteriocins have been classified into Class I, Class II, Class III, IV and Class V (Klaenhammer *et al*., 1993; Maqueda *et al*., 2004; Kawai *et al*., 2004).

Bacterial Vaginosis (BV) is an inflammation most common infection in the reproductive age. Bacterial vaginosis is an extremely prevalent vaginal condition and the cause of vaginitis among both pregnant and non-pregnant women. BV is a polymicrobial, superficial vaginal infection that occurs in the vagina and includes several strains of germs that cause bacterial vaginosis. It occurs when the natural balance of bacteria in the vagina is disturbed. Typically vagina contains a balance of healthy bacteria, known as lactobacilli, and dangerous bacteria, known as anaerobes. Lactobacillus microflora, the intrinsic stability of the resident micro flora is paramount to women's health. The lactobacilli species inhabit the vaginal tract and plays an important role in the maintenance of health and prevention of infection (Amsel *et al*., 1983; Ronnqvist *et al*., 2007; Tomas *et al*., 2003).

G. vaginalis recent studies have suggested that BV is a significant risk factor for upper genital tract infections in pregnant women that can result in adverse outcomes of pregnancy, Abnormal Vaginal Flora (AVF) before 14 gestational weeks is a risk factor for Preterm Birth (PTB) including preterm delivery bacterial colonization of the genital tract, subsequent preterm delivery, late miscarriage, low birth weight of infants, premature rupture of membranes, premature labor and impaired fetal development. BV may increase susceptibility to infection by human immune deficiency virus. However, with the exception of evidence for a relationship between *G. vaginalis* and *Prevotella bivia*, very little is known about the interactions between the microorganisms associated with BV. *G. vaginalis* is the predominant microorganism associated with Bacterial Vaginosis (BV) (Amsel *et al*., 1983). A survey of BV has been conducted among women in Delhi, India according to which a high percentage of woman was found to have BV. Increased rate of BV 43.5% was observed in 2008 (Madhivanan *et al*., 2009). Initially *G. vaginalis* was sensitive to antibiotics but now recurrent infections might occur due to the survival of metronidazole or clindamycin resistant bacteria in the vagina (Nagaraja, 2008). An overall 68% resistance of *G. vaginalis to* metronidazole at very high rate in the population was observed. Recurrence rates of up to 30% within three months after treatment have been reported in literature (Hay, 2000). Bacteriocin provides an alternative and attractive proposition for control of BV. The study reports the production, purification and physicochemical characterization of a new novel protein of *Lactobacillus fermentum* isolated from healthy human vagina and its anti *Gardnerella vaginalis* activity has been discussed that remained unexplored.

MATERIALS AND METHODS

Isolation and identification: One hundred vaginal swabs of healthy ladies of reproductive age group were collected from gynecologist (GMCH, Sector-32, Chandigarh) in the year 2009. They were aseptically sterilized in saline (NaCl) solution and transferred in MRS broth (pH 6.5) at 37°C for 18-20 h. Supernatants of overnight grown cultures were isolated and analyzed for detection of bacteriocin activity. The culture was purified by repeated streaking and purity was checked by gram staining.

Preparation of culture supernatant: The bacteriocin producing lactic acid bacteria were grown in MRS broth at 37°C for 18-20 h. The lactobacilli culture was centrifuged at 13,000 rpm for 10 min and boiled for 20 min then the supernatant was collected.

Bacteriocin assay: Bacteriocin activity was detected by agar well diffusion method. Twenty microliter culture supernatant was transferred to the wells in Casman media plates supplemented by selective media and 5% defibrinated blood. The plates kept for 2 h at 4°C and then incubated at 37°C for overnight and examined for the presence of inhibition zones around the wells and the antimicrobial activity expressed in millimeter (mm). *Gardnerella vaginalis* ATCC14018 was procured from American Type Culture Collection for demonstrating the anti- *Gardnerella* activity. Culture was revived and maintained in the Casman medium supplemented by *Gardnerella* supplement and 5% w/v defibrinated human blood. Bacteriocin activity assay was performed using spot-on-lawn method (Pucci *et al*., 1988), agar well diffusion method (Toba *et al*., 1991).

Production by adsorption and desorption: Bacteriocin was obtained and purified by pH dependent adsorption and desorption method on to producer cells. The protocol relies on the property of several bacteriocins to adsorb to the producer cells at neutral pH and their release

after being treated with a low pH. Culture of *Lactobacillus fermentum* inoculated initially in MRS broth was grown to late log phase (18 h, incubated at 37°C). The culture broth was then heated in boiling bath and allowed to cool. The pH was adjusted to 6.5 and kept for overnight stirring. Cells were harvested by centrifugation for 20 min at 9000 rpm and culture pellet were washed twice in 5 mM sodium phosphate buffer (pH 6.5) and re-suspend the pellet in 10 mL of 100 mM NaCl solution that had adjusted pH 1.5 with 5% (v/v) phosphoric acid. The suspension was stirred overnight and cells were harvested by centrifugation at 9000 rpm for 30 min. They should be stored at -20°C. Activity of bacteriocin was confirmed by well-diffusion assay showing inhibition of 22 mm against the *G. vaginalis* ATCC14018 (Yang *et al*., 1992; Ronnqvist *et al*., 2007; Tomas *et al*., 2003).

Purification reverse-phase (RP) HPLC: This analytical technique has been shown to be extremely valuable for the analysis of these antimicrobial peptides, since bacteriocins are generally resistant to different organic solvents used as mobile phases and the high pressures employed trough the chromatographic technique. The bacteriocin extract is loaded onto the HPLC column. The mobile phase consisted of acetonitrile and HPLC-grade water containing 0.1% Trifluoroacetic Acid (TFA). The sample was loaded on the C-18 column and separated by a linear biphasic gradient of 20 to 80% acetonitrile over 30 min at a flow rate of 0.5 mL min^{-1}.

Protein identification: peptide mass finger printing to identify protein: Proteins were identified by mass spectrometry, the most powerful techniques in protein chemistry, giving a 100 fold increase in sensitivity and 10 fold increase in speed. Accurate measuring of the mass-to-charge ratio of the resulting peptides gives enough information to identify the proteins in the sample. This can easily be done using the Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) procedure. This technique offers a fingerprint unique for the particular protein or protein mixtures. The experimental obtained mass-to-charge values can be matched against theoretical obtained mass data from already identified protein sequences and a score depending on the correlation can be given. This way of identifying proteins is called peptide mass searching or fingerprint MALDI-TOF MS and Liquid chromatography-Mass spectrometry.

MALDI-TOF analysis of pure protein: MALDI-TOF mass spectrometry was performed on Brukers Daltonics. Mass spectra were obtained in the positive ion mode, with an accelerating voltage of 25 kV. One microliter of the trypsin digested protein was mixed with 1 μL of matrix (10 mg of sinapinic acid and 0.1% 4-hydroxy-α-cyanocinnamic acid) in 1 mL of distilled water and 0.3 μL of this mixture was applied to the Teflon-coated plate. BSA was used for calibration.

Liquid chromatography-mass spectrometry: The chromatographic system consisted of a Agilent MS-6460 triple quadra pole, equipped with an auto sampler. Analysis was done using an Agilent eclipse C-18 5 μm column 150×4.6 mm internal diameter with a flow of 0.5 mL min^{-1}. The mobile phase was 0.1% formic acid 70% in purified water and 30% ACN. The injection volume 5 μL. The system was coupled online to HPLC-MS-MS-triple quadruple. Mass spectrometer equipped with a pneumatically assisted electrospray ionization source (Barber *et al*., 1981).

Anti-*Gardnerella* activity: The overnight grown *Gardnerella vaginalis* culture (cells-$1\times10^{5-7}$) 1 mL culture was transferred to six different Effendorfs with different concentrations of bacteriocin. Spreading was done on Casman media plates and kept overnight at 37°C for colony counting.

RESULTS AND DISCUSSION

Isolation and purification: *Lactobacillus fermentum* was isolated from healthy vaginal ecosystem of 11 bacteriocin producing lab strains. Only 8 isolates namely HV6A, HV6B, HV54A, HV59A, HV59C, HV59D, HV69, HV75 were found to inhibit *G. vaginalis*. But, only HV6b was showing maximum inhibition against *G. vaginalis* that was selected for further study and identified as *Lactobacillus fermentum* and its culture supernatant showed a vast antimicrobial range.

Production by adsorption and desorption: The crude and purified bacteriocin was purified and then subjected to bio-assay; the results obtained are given in Table 1. The result clearly shows that the purified bacteriocin has significantly increased activity and a high recovery was observed (Kawai *et al*., 1997; Miteva *et al*., 1998; Boris *et al*., 2001). The crude protein was showing 6.5×10^5 activity units per mL and pure protein was showing 4.4×10^6 activity units per mL. The specific activity have been increased 20.84 folds while the total recovery of pure protein is approximately 32%.

Evaluation of physicochemical characterization and stability of bacteriocin

Effect of temperature on bacteriocin assay: Temperature stability was investigated by heating 50 μL of each culture supernatant at 40, 60, 80, 100°C and 121°C for 30 min. The samples were then assayed for antimicrobial activity using the well diffusion method (Fig. 1). It is active even at 90°C for 20 min. The results clearly shows that it has its maximum activity at 37°C and up to 90°C it indicates that the temperature does not affect the activity of bacteriocin. It remains stable even at 90 for 20 min.

Effect of pH on bacteriocin assay: To determine the effect of pH, purified bacteriocin was adjusted at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and incubated for 2 h at 37°C. Each pH treated bacteriocin was assayed by well diffusion method (Table 2, Fig. 1). The result obtained clearly show the higher growth at pH 6-6.5 and 9.5 due to maximum production of bacteriocin at 6.5, 9.5.

Effect of time on bacteriocin assay: To determine the effect of time of incubation, the bacteriocin production was observed by taking supernatant after 4, 8, 12,16, 20, 22, 26 to 48 h. Each supernatant was assayed by well diffusion method. It indicates maximum production of bacteriocin in between 16-20 h that has optimized our method of bacteriocin production (Table 3, Fig. 1).

Effect of various enzymes and surfactants: Effect of enzymes on activity have been observed by adding different enzymes (proteolytic enzymes, proteinase k or α-chymotrypsin) to bacteriocin, the results clearly indicate that the bacteriocin activity was lost after enzyme treatment.

Table 1: Purification data after absorption-desorption method

Sample	Volume (mL)	Activity (AU mL^{-1})	Total activity	Protein (mg mL^{-1})	Specific activity ($\times10^4$)	Purification (folds)	Recovery (%)
Crude bacteriocin	1000	6.5×10^5	6.5×10^5	17.00	3.80	1.00	100.00
Purified bacteriocin	10	4.4×10^6	4.4×10^7	5.30	83.00	20.84	31.17

Purification folds represents increase in specific activity, Recovery % is remaining protein concentration

Table 2: Inhibition zone at different pH

pH	Inhibition zone (mm)
2.0	11.0
3.0	11.5
4.0	-
5.0	-
5.5	11.0
6.0	12.0
6.5	13.0
7.0	11.0
8.0	12.0
9.5	15.0
10.0	12.0

-: No inhibition

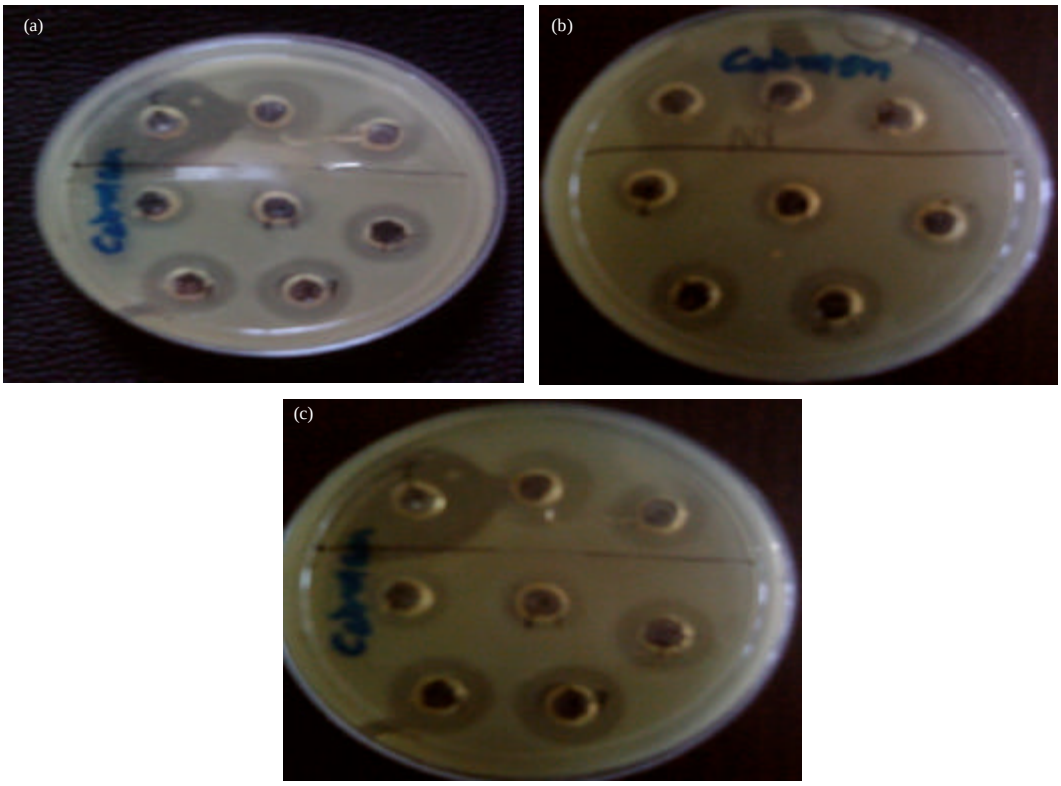


Fig. 1(a-c): Anti microbial activity depicting effect of zones at different (a) Dilutions, (b) pH and (c) Temperature

Stability of bacteriocin during storage: It retains its activity for 3 to 5 months at refrigerator (0-4°C) and for one year at deep freezer (-20°C) in liquid and lyophilized form. It showed its stability even after prolonged storage. To determine the stability of bacteriocin, it was stored in an incubator (37°C), the refrigerator (4°C) and freezer (-20°C). At different time interval (every 30 days) the bacteriocin were taken from the stored bacteriocin for detection of antimicrobial

Table 3: Inhibition zone at different time intervals

Time (h)	Inhibition zone (mm)
4	-
8	2.0
12	12.0
16	22.0
20	18.0
24	16.5
28	15.0
32	15.5
36	16.0
40	13.8
44	12.0
48	11.0

-: No inhibition

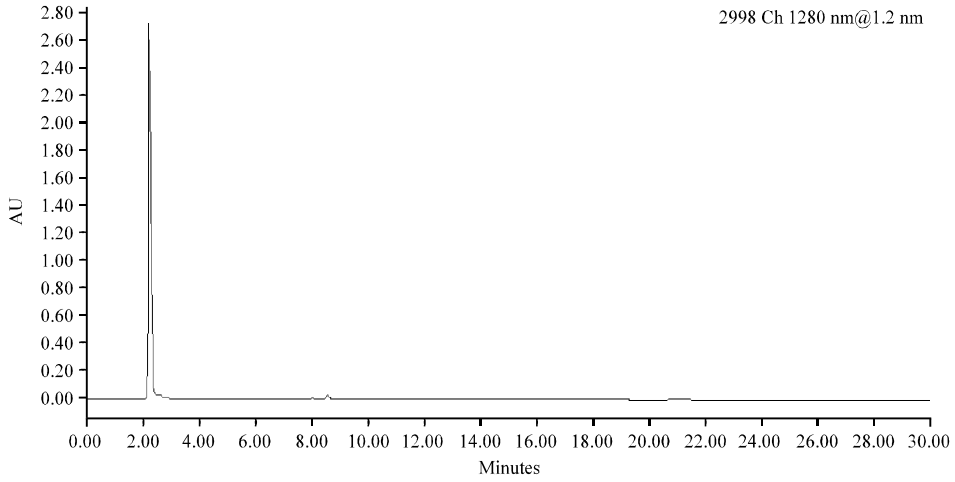
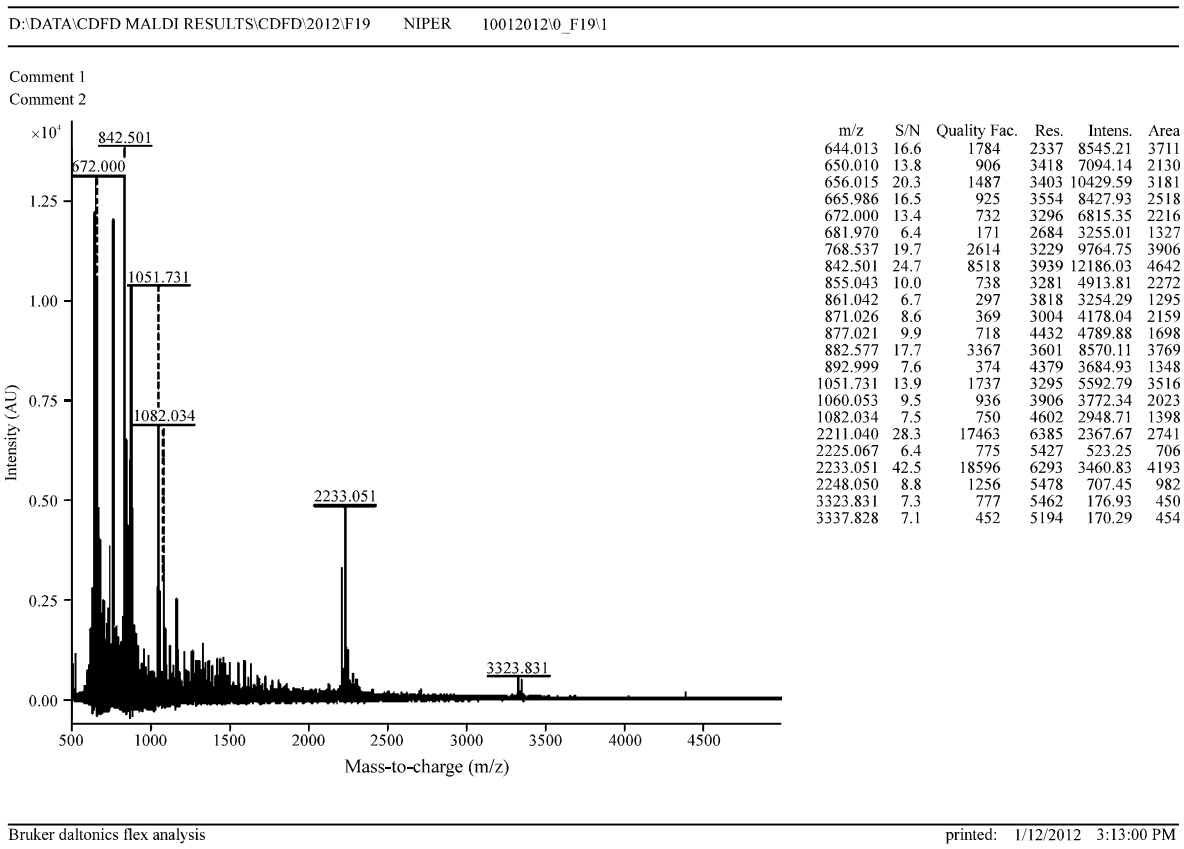


Fig. 2: HPLC pattern of the bacteriocin

activity using well diffusion assay and degradation pattern were observed on RP-HPLC. A single peak of bacteriocin remained constantly same throughout (Fig. 2). The reverse phase HPLC of the bacteriocin gave a single peak at 2 minute time interval of 2.6 intensity and it gave the same results after preservation till 8 months there after it started forming the degradation product. Reverse phase HPLC has confirmed its stability. It was performed at regular intervals of time at absorbance of 280 nm. There was a decrease in absorbance at 280 with time and the single peak starts splitting after a prolonged storage which indicates degradation after 1 year of storage at -20°C. The X axis refers to retention time and Y axis refers to Absorbance Unit Full Scale (AUFS). The absorbance at 280 nm is 2.7632 AU, and retention time is 2.214 min.

MALDI-TOF: After trypsin digestion the bacteriocin was fragmented that give the fragmentation pattern (after deleting the peaks of contamination and trypsinzation: 842.51, 1045.56, 2211.10, 2225.12. Protein identification was performed by sending trypsin digested peptide masses to bacterial databases of National Centre for Biotechnology Information (NCBI) and Swiss port using



m/z	S/N	Quality Fac.	Res.	Intens.	Area
644.013	16.6	1784	2337	8545.21	3711
650.010	13.8	906	3418	7094.14	2130
656.015	20.3	1487	3403	10429.59	3181
665.986	16.5	925	3554	8427.93	2518
672.000	13.4	732	3296	6815.35	2216
681.970	6.4	171	2684	3255.01	1327
768.537	19.7	2614	3229	9764.75	3906
842.501	24.7	8518	3939	12186.03	4642
855.043	10.0	738	3281	4913.81	2272
861.042	6.7	297	3818	3254.29	1295
871.026	8.6	369	3004	4178.04	2159
877.021	9.9	718	4432	4789.88	1698
882.577	17.7	3367	3601	8570.11	3769
892.999	7.6	374	4379	3684.93	1348
1051.731	13.9	1737	3295	5592.79	3516
1060.053	9.5	936	3906	3772.34	2023
1082.034	7.5	750	4602	2948.71	1398
2211.040	28.3	17463	6385	2367.67	2741
2225.067	6.4	775	5427	523.25	706
2233.051	42.5	18596	6293	3460.83	4193
2248.050	8.8	1256	5478	707.45	982
3323.831	7.3	777	5462	176.93	450
3337.828	7.1	452	5194	170.29	454

Fig. 3: The MALDI TOFF of the bacteriocin

the MASCOT (Matrix Science) Peptide Mass Finger printing programme. The mono-isotopic masses were used and the mass tolerance was set to 0.5 kDa. Almost no match to the protein data was available in various protein search engines that indicates it may be an unidentified noble protein. The following peak list in the m/z ratio was available: 644.013, 650.010, 656.015, 665.986, 672.000, 681.970, 768.537, 842.501, 855.043, 871.026, 877.021, 882.577, 892.99, 1051.731, 1060.053, 1082.034, 2211.040, 2225.067, 2233.051, 2248.050, 3323.831, 3337.828 (Fig. 3) (Yang *et al*., 1992).

LCMS: The resulting MS/MS spectra of trypsin digested protein (Fig. 4) were submitted to MASCOT for searching. The Y-axis is peak intensity and the X-axis refers to m/z. Searching was against gram positive bacteria NCBI and Swiss port databases and was performed but the but their ion score of individual peptides failed to reach a significant score level that indicates that the data of this particular protein has yet not been notices or explored (Wu *et al*., 2009).

Evaluation of the anti-*Gardnerella vaginalis* activity: The bacteriocin was evaluated for anti-*Gardnerella vaginalis* activity. The results obtained (Table 4) were significant as seen in Fig. 1, a/b/c and table shows that number of colonies have been decreased drastically with increase in the concentration of protein and 500 μg of bacteriocin completely inhibit the growth of-*Gardnerella vaginalis* which has given us a excellent treatment of bacterial vaginosis. 100 μg of protein sample kills 50% of bacteria, while 500 μg of protein completely kills *G. vaginalis*.

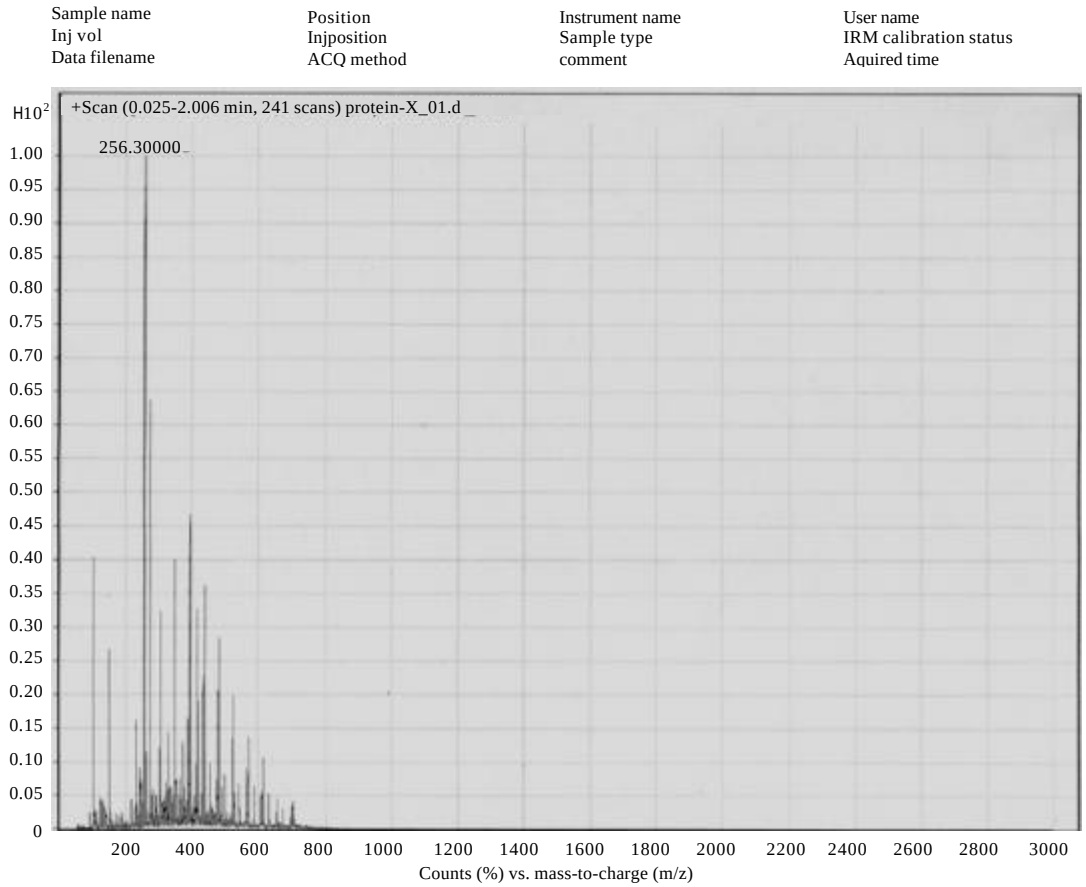


Fig. 4: LCMS mass fragmentation patter of the bacteriocin

Table 4: Effect of bacteriocin on viability of *G. vaginalis* cell

Bacteriocin (µg)	Culture (mL)	No. of colonies
0	1.0	210
100	0.9	136
200	0.8	89
300	0.7	45
400	0.6	9
500	0.5	0

CONCLUSION

Based on the presented results, it may be concluded that the antimicrobial entity procured from the culture supernatant of *L. fermentum* is a peptide with a diverse spectrum of antimicrobial activity. It shows stability in acidic, neutral and basic pH range (up to pH 11), it is sensitive to proteolytic cleavage. Heat stability at various temperatures allows bacteriocin HV6b competitive advantage to use *L. fermentum* as probiotic preparations and bacteriocin for the treatment of bacterial vaginosis and other infections. Maldi TOF- TOF peptide mass fingerprinting and LCMS-MS mascot ion search results indicates that it is a novel protein. It was effective against *Gardnerella vaginalis*.

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