

Partial identification of a peptide from *Lacticaseibacillus casei* using MALDI TOF and its cytotoxic activity against MDA-MB-231 cell line

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ABSTRACT

The current study focused to extract and purify the peptide of interest efficaciously from *Lacticaseibacillus casei*. The peptide of interest was partially identified and checked for anti-cancer activity using MDA-MB-231 triple negative breast cancer cell line. To begin with, it was purified via ammonium sulfate precipitation, ultrafiltration, and gel filtration chromatography. It was partially identified using mass matrix assisted laser desorption/ionization-mass spectrometry (MS-MALDI) and checked for cytotoxic activity using (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. As an outcome, the active part collected after ultra-filtration, 880 µg/ml of protein content with 15 mm zone diameter, was loaded on sephadexG50 column. The purified protein content was found to be 118 µg/ml with 15 mm of zone of inhibition against *Listeria monocytogenes*. A peak was obtained at a retention time of 9.371 minutes on subjected to high performance liquid chromatography. The result of MS-MALDI revealed it as an unnamed peptide with 75% query sequence similarity with the hypothetical protein of *Lactobacillus reuteri*. The cytotoxic study revealed its potential as an anti-cancer peptide, with the IC₅₀ value of 108 µg/ml for MDA-MB-231 cancer cell line. In conclusion, this is the first report of this unknown peptide which showed both antibacterial and cytotoxic activity. It may act as a promising agent and can be further explored in future.

1. INTRODUCTION

Cancer is still one of the deadliest diseases with high mortality rate [1]. Cancer treatment, such as radiation therapy, chemotherapy or medicine come with pain and prolonged suffering. In case of traditional therapeutics, it has showed an immense failure due to the resistance by the cancer cells. Hence, an intent need has arisen to find a cure or an alternative novel metabolite which will selectively target the cancer cells as stated by Raguz and Yague [2].

One of the trending metabolites like bacteriocin has come to light. Vast ongoing research are being carried forward to discover its role against cancer cell lines. Lactic acid bacteria produce bacteriocins and those are positively charged peptides. It has anti-microbial activity and are, ribosomally synthesized, narrow or broad-spectrum, antimicrobial peptides [3]. Bacteriocins are broadly

classified into three classes: class I post translationally modified, class II unmodified, and class III <10 kDa in size as described by Arnison *et al.* [4] and Cotter *et al.* [5].

It acts against the closely related species and act against the negatively charged cancerous cells other than normal cells as stated by Kaur and Kaur [6]. Cancer cell surface possess negatively charged ubiquitous components in a significant amount. Components like gangliosides, anionic phosphatidyl serine, O-glycosylated mucins, and heparin sulphates, whereas in the outer surface of non-cancer cells, components like neutral phospholipids including sphingomyelins and phosphatidyl choline are present. In case of the inner surface amino-phospholipids can be found as described by Riedl *et al.* [7].

For the first time, as observed by Farkas-Himsley and Cheung [8] bacteriocin proved to have toxic effect on eukaryotic cells. In addition, the effect on mammalian cells has also been reviewed earlier by Cornut *et al.* [9]. As per the report of Singh *et al.* [10] strain GI-9 belong to the genus *Brevibacillus* sp. produced bacteriocin named laterosporulin which showed broad spectrum

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antibacterial activity. Based on its biochemical and genomic characterization, it was discovered as a novel bacteriocin.

In the study, as carried by Baidara *et al.* [11] the anti-cancer activity of LS10 a defensin like peptide obtained from *Brevibacillus* sp. strain SKDU10 was examined against varied cancer cell lines, such as MCF-7, HEK293T, HT1080, HeLa, and H1299. It was revealed, the bacteriocin LS10 induced apoptosis at 2.5 μ M concentration. The study has also confirmed both the necrotic and apoptotic death of the cancer cells at higher and lower concentration respectively.

Another report suggested the lethal effect of bacteriocin, which showed a visible decrease of the sensitive indicator strain, *Mycobacterium diernhoferi* ATCC 19340 by the bacteriocin. In this study, Saito *et al.* [12] demonstrated the inhibition of the growth of HeLa-S3 cells by the bacteriocin preparation.

The identification of the unknown active peptide is indeed necessary to carry out the research commercially in future. Hence, its identification is one of the important parameters to learn in depth about the unknown peptide. Similar finding like, De Giani *et al.* [13] reported a study in identifying a bacteriocin-like compound from *Lactobacillus plantarum*, which showed antimicrobial activity and cytotoxic effects on normal and cancerogenic human intestinal cells.

The study was continued with the major cancer cell line such as triple negative breast cancer MDA MB-231 for the initial screening of cytotoxic activity.

In previous studies, *Lacticaseibacillus casei* MH137744 was isolated from fermented Cumin by authors. Effect of pH, temperature and enzymes were checked. Peptide extracted from *L. casei* was proved to be of proteinaceous in nature. The bacteriocin activity was found to be 800 AU/ml.

The current research dealt with the purification of the peptide and checks its activity against major food borne pathogens as preliminary screening, due to its pore forming characteristics in the bacterial cell wall. This study was conducted to check the cytotoxic role against triple negative breast cancer MDA-MB-231 and partial identification of the unknown peptide.

2. MATERIALS AND METHODS

2.1. Sample Preparation

The isolate *L. casei* MH137744 was retrieved from 20% (v/v) of glycerol stock and sub cultured in triplicate to carry out the further assays. It was grown in 200 ml of De Man, Rogosa and Sharpe medium for 48 hours at 30°C. The culture was centrifuged (13,000 rpm, 20 minutes, 4°C) and the cell free supernatant (CFS) was collected and purified via three-step purification step, ammonium sulphate precipitation, ultrafiltration and gel filtration chromatography using sephadexG50. After each purification step, the protein concentration was measured using Lowry's method at 660 nm using bovine serum albumin as a standard and the antibacterial activity was checked by agar well diffusion assay (AWDA) using *Listeria monocytogenes* as an indicator organism.

2.2. Purification

2.2.1. Ammonium sulfate precipitation

The supernatant was subjected to ammonium sulfate precipitation. Throughout the experiment the supernatant was kept in ice, and the powdered ammonium sulfate was added slowly with continuous stirring, for 1 hour. The ammonium sulfate was added, consecutively for 3 days, starting from 0% to 30%, 30% to 60%, 60% to 80%, 80% to 100%. Each day the precipitate and supernatant were collected and checked for antibacterial activity using AWDA.

The supernatant was desalted using sephadexG15 before being loaded to the fine ultra-filtration membrane. The active fractions were loaded onto the column bed (15 \times 2.5 cm) packed with Sephadex G10 for desalting using 50 mM of sodium acetate buffer as an eluent (pH 5.5). The sample were collected, at a flow rate of 0.5 ml/minute and subjected to ultra-filtration technique.

2.2.2. Ultra-filtration technique

The ultra-filtration technique was carried out using 10 and 3 kDa ampicon centrifugal unit (Sigma-Aldrich) consecutively and centrifuged (4,000 \times g, 30 minutes, 4°C). The permeate and retentate were both collected and checked for protein content using Lowry's method and antibacterial activity by AWDA.

2.2.3. Gel filtration chromatography using sephadexG50

The active fractions were loaded onto the column bed (15 \times 2.5 cm) packed with Sephadex G50 for gel filtration chromatography using 50 mM of sodium acetate buffer as an eluent (pH 5.5). The 20 fractions were collected, at a flow rate of 0.5 ml/minute and checked at 220/280 nm in UV visible spectrophotometer (Remi) for the presence of peptide.

2.3. Partial Identification of the Peptide

2.3.1. UV-visible spectrophotometer

Initially, the crude CFS containing the peptide of interest was analyzed using UV Visible spectrophotometer. The spectrum obtained given an overview idea of the peptides of interest present in the crude supernatant. All amino acids absorb at 200–220 nm due to the C=O group. But, high density of spectrum at 220 nm, suggest the presence of peptide bond (amide) which has given absorbance. It can also be inferred as, the aromatic amino acid (Tyr, Phe, and Trp), absorbed UV with two different peaks. The peak at ~270–280 nm is due to the conjugation system of the aromatic ring and the absorbance peak at lower wavelengths is attributed to carboxylic acid moiety of the amino acid. However, the presence of disulfide bridges (also a result of oxidation) may have showed a weak extinction factor at 280 nm.

2.3.2. Fourier transform infra-red (FTIR) analysis

Surface functional groups of purified peptides were unraveled by Perkin-Elmer infrared spectrophotometer (India). Purified peptide was lyophilized and mixed with potassium bromide (spectroscopic grade). Pellet was prepared with the size of about

10–13 mm diameter and 1 mm thickness. Sample was scanned in transmission mode with a resolution of 4 cm^{-1} at $4,000\text{--}500\text{ cm}^{-1}$ range and functional groups were compared with the literature.

2.3.3. Reverse phase–high performance liquid chromatography (HPLC)

A sample volume of $20\ \mu\text{l}$ of purified and partially purified were injected simultaneously in HPLC (Detector—UV VIS 2487; Column length—250 mm—C18; Make: Water; Model No: 1525 Pump; elution temperature, flow rate $300\ \mu\text{l}/\text{minute}$; wavelength—220/280 nm) peptide concentration of $100\ \mu\text{g}/\text{ml}$ dissolved in 50 mM of sodium acetate buffer (pH 5.5). The HPLC profile was recorded out using 1:1 acetonitrile:water constituted with trifluoroacetic acid 0.05%. The purified peptide of interest was lyophilized and stored at -80°C for further assay.

2.3.4. MS MALDI analysis

The peptide of interest after trypsinization was directly subjected for the analysis by MS MALDI (BRUKER Ultra flex III MALDI TOF TOF). The sequence was obtained from mascot search and the sequence homology was performed using basic local alignment search tool (BLAST).

2.4. Cytotoxic Activity of the Peptide Using MTT Assay

The purified bacterial peptide was checked for cytotoxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for (MTT) assay [14] against major cancer cell line, triple negative breast cancer MDA-MB-231 an epithelial human breast cancer cell line.

The cell line (Sigma-Aldrich) was maintained in Dulbecco's modified eagle medium with 10% fetal bovine serum at 37°C and 5% CO_2 . Cells were trypsinized and 5 ml of complete media was added to the trypsinized cells. The cells were centrifuged at 500 rpm in the swinging bucket rotor ($\sim 400 \times g$) for 5 minutes.

The media was removed and later resuspended with complete media. The $100\ \mu\text{l}$ of cells were added into each well and left for incubation for overnight.

On the second day, the cells were treated with 50, 100, 250, and $500\ \mu\text{g}/\text{ml}$ of peptide of interest (in triplicate), respectively, and the percentage viability was calculated as described by Sharma *et al.*

[15]. The final volume was maintained at $100\ \mu\text{l}$ per well. On the third day, $20\ \mu\text{l}$ of $5\ \text{mg}/\text{ml}$ MTT was added to each well. The setup was incubated for 4 hours at 37°C without any disturbance. The MTT solvent of $150\ \mu\text{l}$ was added and the cells were agitated on orbital shaker for 15 minutes. The absorbance was checked at 570 nm with a reference filter of 620 nm [16].

3. RESULTS AND DISCUSSION

In continuation of the previous study, a large number of LAB were isolated from fermented *Cuminum cyminum* or commonly known as cumin, which were also found to be an active hub of bacteriocin like substance.

As studied earlier, by the authors among 110 isolates one potent strain VITCM05 was selected based on their broad-spectrum antibacterial activity against major food borne pathogens. Using BLAST, it showed 93% maximum identity and *E*-value equal to 0.00 for all closely related taxa. The isolate was identified as *L. casei*, MH137744 and the CFS extracted was of proteinaceous in nature. *Lacticaseibacillus casei* has been transferred to a new genus *Lacticaseibacillus* (the *L. casei* group). This group comprises 26 species and two subspecies as reported by Zheng *et al.* [17].

The 200 ml of culture *L. casei*, 16×10^7 cells/ml was centrifuged and the extracted CFS was consist of extracellular peptide. The total protein content was found to be $3.4\ \text{mg}/\text{ml}$ with 25 mm zone of inhibition against *L. monocytogenes* MTCC5260.

As peptides are small in nature, it generally secretes out of the cell membrane, and thus can be found in extracellular environment as stated by Daw and Falkiner [18].

3.1. Purification

The purification was carried out via ammonium sulfate precipitation, ultrafiltration, and gel filtration technique. Simultaneously in each step, the activity was checked using AWDA, which showed a clear zone of inhibition for the active portions. In step 1 and 2 of purification, the yield was found to be 100%, due to negligible loss of peptide. Simultaneously, the specific activity increased with the decrease of yield as can be observed in Table 1.

The CFS was subjected for ammonium sulfate precipitation till the protein of interest gets precipitated out. Interestingly, even at 100% of saturation, the protein of interest did not precipitate out

Table 1. Purification table.

Sample	Vol (ml)	$\text{mg}^{-1}\ \text{ml}$	Total protein (mg)	Activity units (AU/ml)	Total activity (AU)	Specific activity (AU/mg)	Purification (fold)	Yield (%)
Crude	200	3.4	720	800	160,000	222.222	1	100
Ammonium Sulphate ppt.	100	1.94	194	1,600	160,000	824.742	0.371	100
Desalted	10	1.4	14	1,600	16,000	1,142.857	5.142	10
Ultrafiltration	10	0.880	4.4	3,200	32,000	7,272.727	32.727	20
Gel filtration	2	0.118	0.236	3,200	6,400	27,118	122.03	4

The purification table shows the specific activity of the purified peptide was increased to 27,118.64 when compared to the crude specific activity of 222.22, the final step showed the purified peptide has total Activity (AU) 6,400 with 4% of yield and 122.03 purification fold.

as a pellet and remained in the supernatant. The pellet showed no zone of inhibition against the indicator organism but the remaining supernatant showed 20 mm of zone of inhibition. The protein content was found to be 1.94 mg/ml. In this case, it can also be stated that, most of the proteins has already been excluded as a pellet so very few and only low molecular weight proteins were left out in the supernatant. Hence, the yield was found to be 100% even after performing ammonium sulfate precipitation, as can be seen in Table 1.

The supernatant was desalted using Sephadex G15, protein content was found to be 1.4 mg/ml with 15 mm zone of inhibition. On being subjected to ultrafiltration ($4,000 \times g$, 30 minutes, 4°C) using 10 and 3 kDa ampicon centrifugal unit (Sigma-Aldrich) consecutively. Both the permeate and retentate were pulled out and checked for its content and antibacterial activity. The permeate obtained from 10 kDa showed activity of 15 mm with a content of approximate $980 \mu\text{g/ml}$ and retentate showed no activity, which indicates the peptide to be of less than 10 kDa. Further the active permeate fraction were ultrafiltered with 3 kDa membrane. There were no permeate obtained and the retentate showed a 15 mm of zone of inhibition with $880 \mu\text{g/ml}$ of protein content. From the observation it is evident that the protein of interest range between 4 and 9 kDa.

The active fraction eluted from the gel filtration chromatography showed a 15 mm of zone of inhibition with a protein content of $118 \mu\text{g/ml}$.

The CFS showed a specific activity of 222.222 with a 160,000 AU and as the purification step was carried out, gradually specific activity increased to 7,272.727 with 20% yield and 32,000 AU. In the final step of purification, the total peptide content of 0.23 mg showed 6,400 AU with a vast increase in specific activity of 27,118 with maximum of 4% yield. As reported by Sabo *et al.* [19], the AU 3,200 with yield of 2% is quite less when compared with this present work.

3.2. Partial Identification of the Peptide

3.2.1. UV-visible spectrophotometer

In this study, the crude CFS was checked spectrophotometrically using UV visible spectrophotometer. The reading was captured at a range of 220–300 nm. The sharp absorption at 220 nm, showed a possibility for the presence of peptide, as shown in Figure 1.

3.2.2. FTIR analysis

In the analysis of FTIR, the IR absorbance was checked in the range $500\text{--}4,000 \text{ cm}^{-1}$ of wavelength. The FTIR spectra peaks at $1,542$, $1,630$, and $3,270 \text{ cm}^{-1}$ showed the existence of peptide bonds in the sample as can be observed in Figure 2.

The wavelength $1,542.06 \text{ cm}^{-1}$ reveals an amide II (generally a NH deformation) which can be found in the range of $1,540\text{--}1,550 \text{ cm}^{-1}$. It is also used to determine the secondary structure of the protein, which also reveals it to be α helix in nature. The major functional group, (amide I) group range from $1,625\text{--}1,640 \text{ cm}^{-1}$,

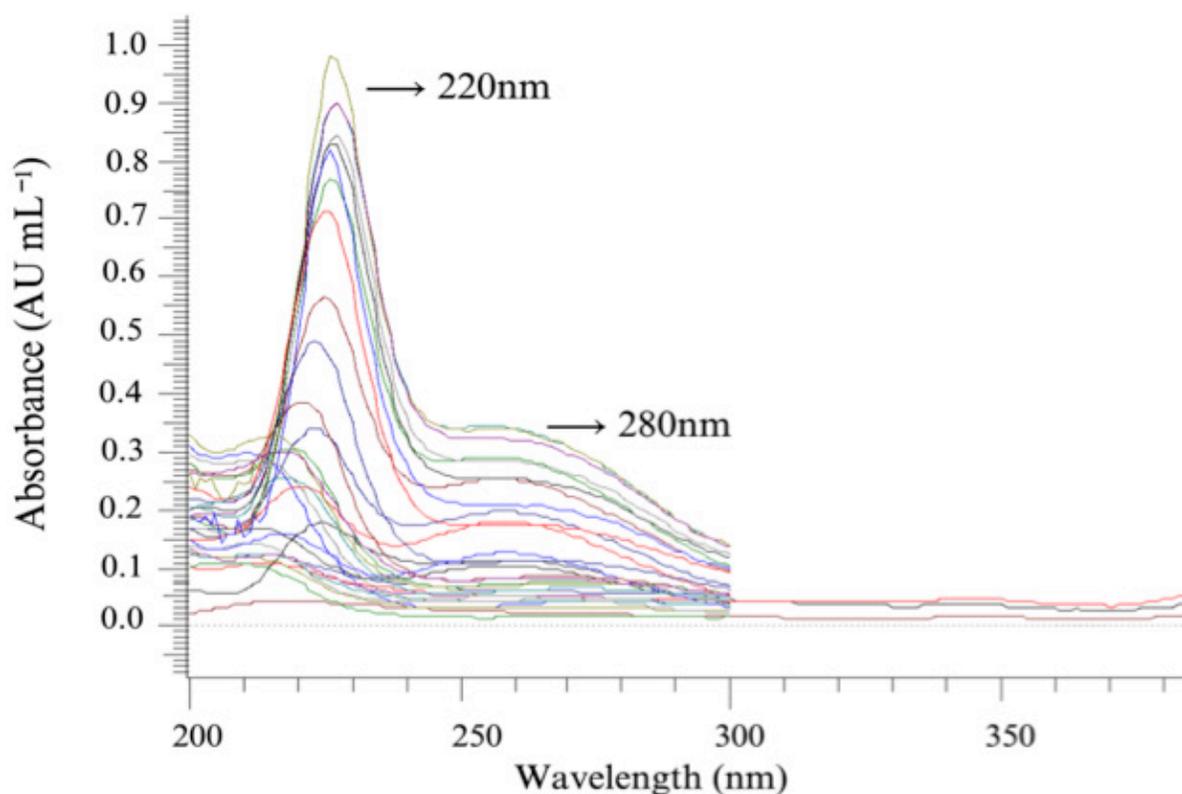


Figure 1. On the X-axis, “nm” is plotted, whereas on the Y axis, the absorbance unit is shown (AU ml^{-1}). The figure depicts the crude supernatant were a mixture of peptide or protein, as it showed absorbance at 220 and 280 nm simultaneously. The UV visible spectrophotometer reading captured at 220–300 nm. Sharp absorption at 220, 280 nm indicates the total concentration of the crude extract.

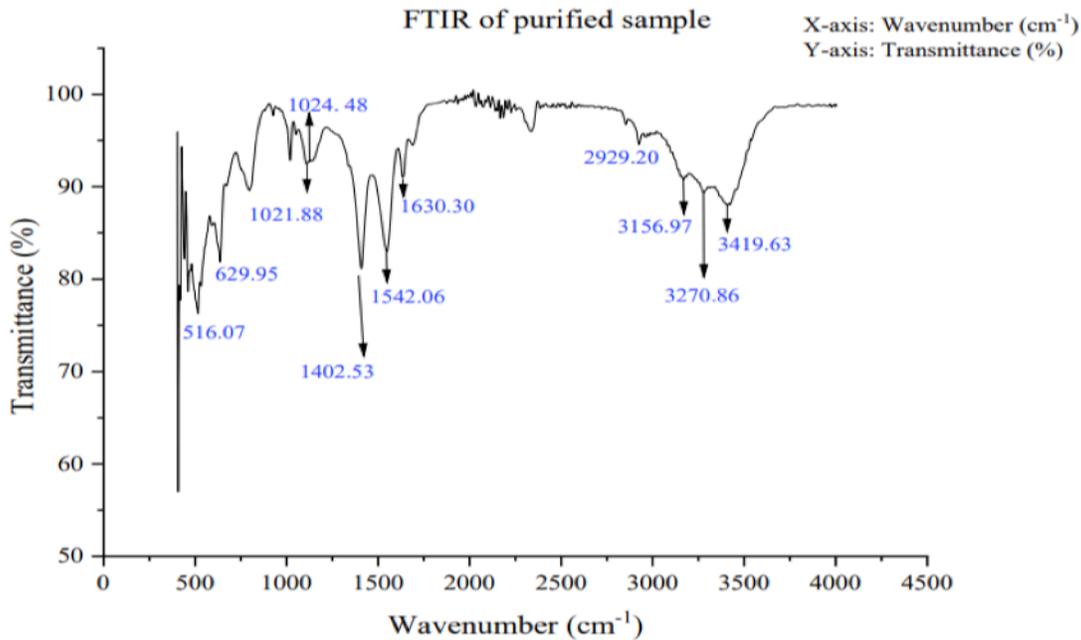


Figure 2. The FTIR spectra of showed peaks at 1,542, 1,630, and 3,270 cm^{-1} shows the the existence of peptide bonds in the sample. Wavelength versus transmittance% is plotted, and the respective peak depicts the functional bonds.

which is specifically used to determine structure. The peak at 1,630.30 cm^{-1} reveals the presence of primary amino group (amide I) C=O stretch [20]. We can also conclude the presence of a β sheet, as a secondary protein structure. The peak at 3,156 cm^{-1} confirmed aromatic C–H stretch as reported by Coates [21].

Generally, in this range of 3,300–3,500 cm^{-1} , the wavelength shows a NH stretch. Greater than 3,400 cm^{-1} , at higher wavelength, an asymmetric stretch of (R-NH2) wavelength generally can be found. The presence of a peak at 3,419.63 cm^{-1} depicts a presence of amide stretch as well. Further few peaks can be seen in the fingerprint region, below 1,500 cm^{-1} . The 1,402.53 cm^{-1} wavelength reveals OH bend.

3.2.3. Reverse phase–HPLC

Figure 3a shows the peak / presence of peptide; purified and Figure 3b partially purified sample checked at 220/280 nm in HPLC. A peak was obtained at 9.371 minutes, was pulled out and checked for concentration and antibacterial activity.

The partially purified sample showed a number of peaks, at different retention time, such as at 1.697, 7.983, 8.462, 8.803, 9.072th minute, which represented, number of peptides present in the sample. In comparison, a sharp single peak was obtained at a retention time of 9.371 minute, which was further pulled off for later study. Similar study was also carried out by Zhang *et al.* [22] where instead of ultrafiltration membrane, they have used dialysis membrane flowed by AKTA (GE Healthcare, Uppsala, Sweden), equipped with SP Sepharose Fast Flow column, for purification.

In this study, dialysis method has been omitted due to its inability to retain the low molecular weight metabolites.

3.2.4. Mass matrix assisted laser desorption/ionization-mass spectrometry (MS-MALDI) analysis

The active fraction was further subjected to MS MALDI. Using the search parameters, the peptide of interest was identified as using MS MALDI analysis using the peaks obtained: 780.389, 879.466, 951.507, 1,105.580, 1,252.607, 1,310.0466, 1,412.697, 1,542.731, and 1,719.854.

Search Parameters used as given below:
Type of search: Peptide Mass Fingerprint, Enzyme: Trypsin, Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M), Mass values: Monoisotopic, Protein Mass: Unrestricted
Peptide Mass Tolerance: ± 100 ppm
Peptide Charge State: 1+; Max Missed Cleavages: 2
Number of queries: 14; Selected for scoring: 13

In Figure 4 MS MALDI analysis showed a major peak in 1,252.607 m/z . Corresponding to that m/z value peptide sequence coverage were checked by MASCOT search as given. Remaining peak search revealed no result. Moreover, the Mascot result revealed, the peptide sequence similarity of 75% with the hypothetical protein of *Lactobacillus reuteri*.

Figure 5(a) and (b) confirms that the peptide showed no exact similarity with the already discovered protein, peptide or even with any type of bacteriocin as shown in Figure 6 using BLAST analysis. The peptide showed 75% sequence similarity with the hypothetical protein of *L. reuteri* ($p < 0.5$). The partial protein sequence, were aligned using BLAST tools. Hence, it is the first

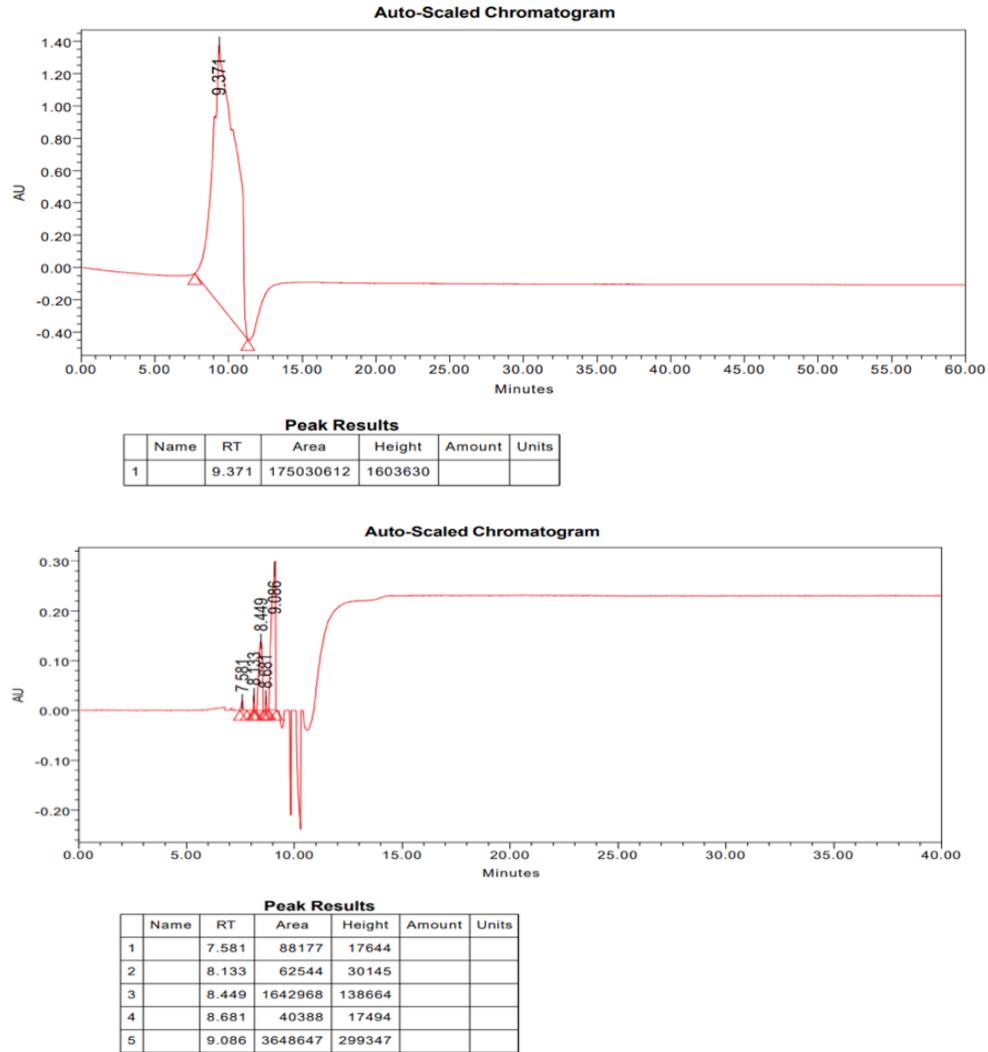


Figure 3. (a) The purified (b) partially purified sample checked at 220/280 nm in HPLC.

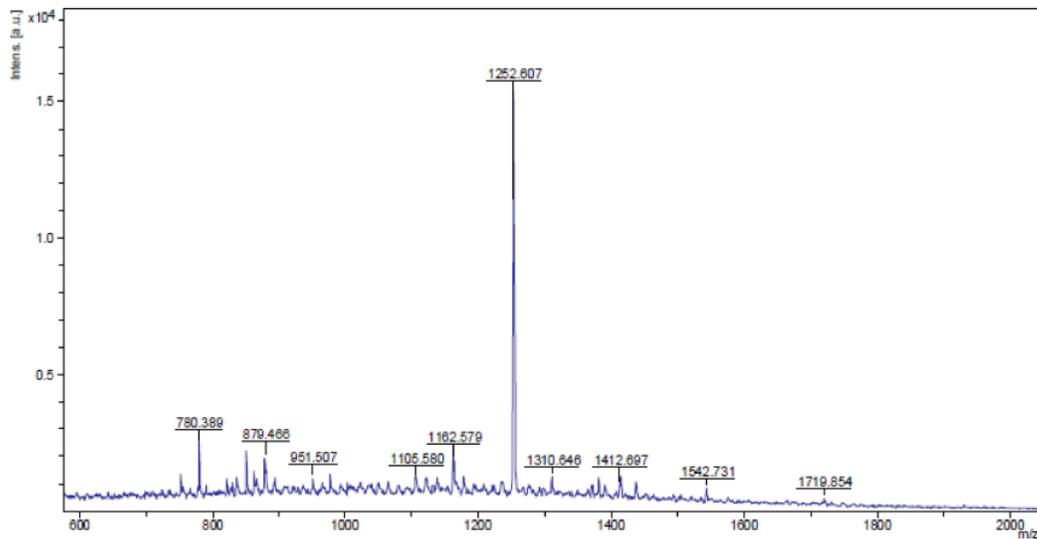


Figure 4. MS MALDI analysis showed a major peak in 1,252.607 m/z .

MASCOT Search Results

Protein View: CUR41808.1

hypothetical protein LRLP16767_LRLP167_00157 [Lactobacillus reuteri]

Database: NCBIprot
 Score: 72
 Expect: 1.3
 Monoisotopic mass (M₀): 5792
 Calculated pI: 4.93
 Taxonomy: Lactobacillus reuteri

Sequence similarity is available as an [NCBI BLAST search of CUR41808.1 against nr](#).

Search parameters

MS data file: peaklist.xml
 Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
 Fixed modifications: Carbamidomethyl (C)
 Variable modifications: Oxidation (M)
 Mass values searched: 14
 Mass values matched: 5

Protein sequence coverage: 75%

Matched peptides shown in **bold red**.

1 **NHLMTKLELPL DRETRRIIRD NFQIKIQDIN KINSQMKKQ** DIYSNGGGH

Unformatted sequence string: **49 residues** (for pasting into other applications).

Sort by residue number increasing mass decreasing mass

Show matched peptides only predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calcd)	ppm	M	Peptide
2 - 12	1310.6461	1309.6388	1309.7241	-65.1	1	NHLMTKLELPLDR .B
7 - 16	1252.6072	1251.5999	1251.7047	-83.8	2	K.LPLDRDRETRR .I
16 - 24	1162.5795	1161.5722	1161.6142	-36.2	1	R.RIINDNTPQK .I
25 - 38	1719.8544	1718.8471	1718.8872	-23.4	2	K.IQDINKINSQMKK .Q + Oxidation (M)
32 - 38	879.4659	878.4586	878.4531	6.24	1	K.INSQMKK .Q + Oxidation (M)

No match to: 780.3899, 851.4281, 851.4438, 951.5074, 978.5131, 1105.5800, 1258.5710, 1412.6970, 1542.7308

Figure 5. a, b: Mascot result showed the best match with the protein sequence of hypothetical protein of *L. reuteri*. As per the mascot score histogram, protein score $\geq 75\%$ are significant. Sequence similarity is available as an NCBI BLAST search of CUR41808.1 against nr. ELETKLPLP DRETRRIIRD NFQIKIQDIN KIESQMKK.

Query Length 37

Descriptions

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
hypothetical protein LRLP16767_LRLP167_00157 [Lactobacillus reuteri]	73.9	73.9	100%	1e-17	100.00%	CUR41808.1
hypothetical protein [Lactobacillus vaginalis]	49.3	49.3	100%	8e-08	56.76%	WP_003717883.1
hypothetical protein [Lactobacillus reuteri]	42.0	42.0	89%	6e-05	60.61%	WP_153700650.1
hypothetical protein [Lactobacillus reuteri]	38.5	38.5	100%	0.003	54.05%	WP_152742994.1
hypothetical protein BBP11_02510 [Lactobacillus reuteri]	38.1	76.2	81%	0.006	63.33%	OCW61559.1
hypothetical protein BHL90_02055 [Lactobacillus reuteri]	38.1	38.1	81%	0.006	63.33%	OTA48249.1
hypothetical protein Lreu23DRAFT_3171 [Lactobacillus reuteri 100-23]	37.7	37.7	81%	0.007	63.33%	EDX41660.1
hypothetical protein [Lactobacillus reuteri]	37.7	37.7	100%	0.009	54.05%	WP_170090569.1
serine hydrolase [Bacillus sp. VT-16-64]	30.4	30.4	81%	8.1	50.00%	WP_077114121.1

Graphic Summary

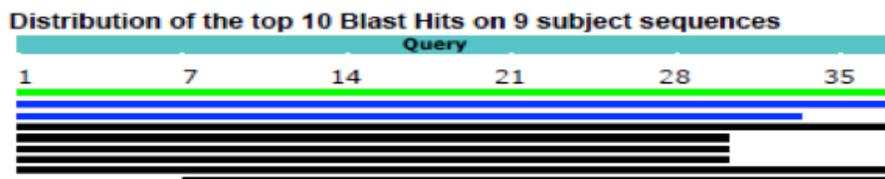


Figure 6. The BLAST analysis revealed, there are no match with the closely related nine peptides. The peptide showed no similarity with the already discovered bacteriocin as well.

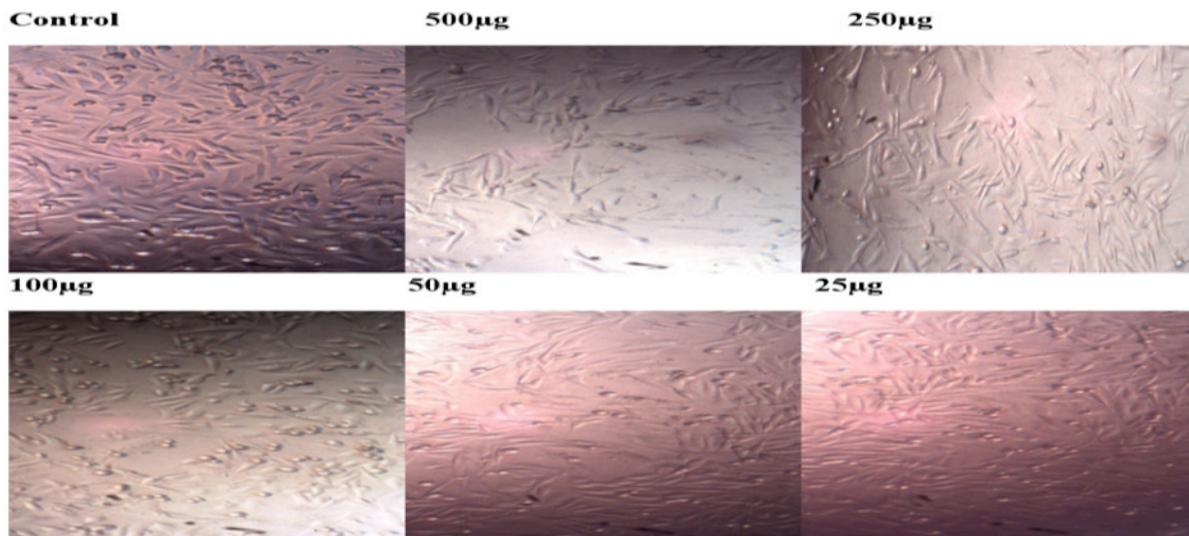
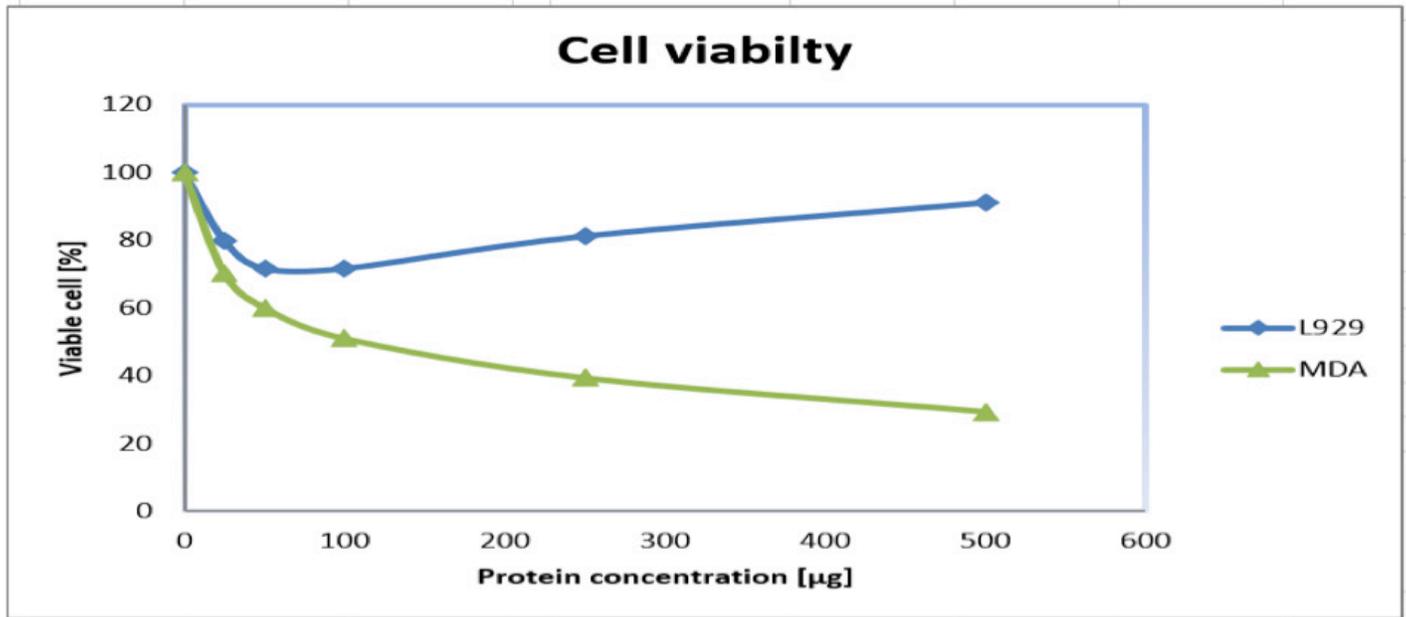


Figure 7. (a) The cell viability assay shows, with the increase in concentration of the peptide, the cells were killed when compared to the normal cell line L929, which was not affected by the peptide. (b) The MTT assay showed the cytotoxic activity of the peptide of interest. It depicts, in dose dependent manner, the cells were observed to get reduced in number. The cells were observed at 40× magnification.

report of this kind of peptide that was extracted and successfully purified from *L. casei*.

3.3. Cytotoxic Activity of the Peptide Using MTT Assay

The cell viability (%) were plotted against peptide concentration (µg/ml) as shown in Figure 7(a). Blue line indicates Normal cell line L929 and green line indicates triple negative breast cancer cell line MDA-MB-231. In Figure 7(b) the MTT assay revealed a visible death of the cancer cells with the increase in sample concentration. The cells have been treated for 24 hours with

the different concentration of sample, before measuring the cell viability at 570 nm.

The IC_{50} value for MDA MB-231 cell line as reported by Pilco-Ferreto and Calaf [23], in the article “Influence of doxorubicin on apoptosis and oxidative stress in breast cancer cell lines”. When treated with Doxorubicin, the IC_{50} value was found to be 543.52 µg/ml. So, with this back reference we have carried out the assay, with the cells treated with varied concentration of the peptide (50–500 µg/ml) along with a negative control (without any incorporation of the peptide of interest). We have found out the IC_{50} value for the

peptide of interest is 108 µg/ml against MDA-MB-231 cell line, which showed five-fold better performance than the commercial drug available in the market.

This is the first study to report about this unnamed peptide, checked for its cytotoxic activity against triple negative breast cancer MDA-MB-231 by MTT assay. The IC₅₀ value was showed to be 108 µg/ml.

4. CONCLUSION

The peptide extracted using the simplest three-step purification process from the potent strain *L. casei* MH137744. The purified peptide of interest was found to be of 1.3 mg ml⁻¹ of concentration. On partial identification, it did not show any match with any peptides in the databases, neither in the bacteriocin database as well. The unnamed protein product was checked for anti-cancer activity against major cancer cell line triple negative breast cancer MDA-MB-231 and the IC₅₀ value was found to be 108 µg/ml. The work will be further carried out solely with adenocarcinoma cell line A549 as another major cancer cell line and the structure and name of the unknown peptide shall be identified, in the future study.

5. ACKNOWLEDGMENTS

The authors are thankful to the VIT management for their continued support to carry out this research.

6. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. FUNDING

This work was financially supported by Vellore Institute of Technology.

8. CONSENT TO PARTICIPATE

The authors give consent to participate.

9. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

10. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

11. AVAILABILITY OF DATA AND MATERIAL (DATA TRANSPARENCY)

Data are available on request to the corresponding author.

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