CHAPTER 2

HETEROLOGOUS EXPRESSION OF PLANTARICIN GENES, AND PURIFICATION OF ACTIVE PLANTARICIN PEPTIDES

While bacteriocins have been recommended as important food biopreservatives, their therapeutic values have also been under serious considerations. All these require purification of the active agents and their proper characterization. Many bacteriocins have been purified by standard methods of three, -four -or -more steps, involving affinity chromatography, gel filtration, and RP-HPLC/ FPLC, but peptide recovery is generally low. In order to overcome these shortcomings, heterologous expression system for bacteriocin production offer several advantages over native systems. Relieving the constraints of inherent regulation in the native strain, facilitating the controlled gene expression and achieving increased production levels, are the main objectives of this line of study. In recent years, several such bacteriocins have been isolated and purified through heterologous expression with enhanced yield.

Studies on Plantaricins:
Previous studies in the lab, on *Lb. plantarum* strain LR/14 identified a 2-peptide bacteriocin with a broad inhibition spectrum. However, a major constraint has been the low yield of the purified peptides from the spent culture filtrate. A full pln operon has also been characterized from this strain and the structural genes for PlnE/F and PlnJ/K have been identified. As described in Chapter 1, these gene sequences could also be retrieved from soil metagenome. Except for *plnJ*, the other three were absolutely identical with the known sequences. Therefore, with the purpose of increasing the yield, heterologous expression of PlnE, -F, -J, and –K peptides in *E. coli* has been carried out during the present study.
As described in Chapter 1, PCR amplification from metagenomic cloned library DNA, using nisin, enterocin and plantaricin gene-specific primers led to the identification of the respective structural genes. Nisin and enterocin have been very well studied, so further work was focussed on plantaricins. Interest in plantaricins also emanated from the fact that previous work has shown broad inhibition spectrum of plantaricins from
strain LR/14. Thus, soil metagenome – derived plantaricin genes/proteins were studied further.

![Resolution of PCR product (a) and clone confirmation (b)](image)

Fig 2.1 (a): Resolution of PCR product obtained using *pln EFI* gene specific primers and cloned metagenomic DNA as template by agarose gel electrophoresis. Lane 1-1 kb ladder, lane 2- *Lb. plantarum* WCFS1 (control) genomic DNA, lane 3- DNA from soil sample. (b): Confirmation of clone by restriction digestion (*NotI*). lane 1- DNA size ladder (1 kb), lane 2- Positive clone carrying vector backbone (3 kb) and *plnEFI* insert (1.5 kb). (c): pGEM-T vector.

Total *plnEFI* gene stretch obtained through PCR amplification from metagenomic DNA was cloned in pGEM-T vector and maintained in *E. coli*. In order to confirm the results of PCR amplification, genomic DNA of *Lb. plantarum* WCFS1 was also used as control. Confirmation of clone was done by restriction digestion (*NotI*), which released the insert from vector backbone (Figure 2.1a-b) as well as by sequencing (Central Instrument Facility, University of Delhi, South Campus, USA).
Sequence of plnEFI region:

plnE
1  gtgctagtt ttgagaagtt acaatatcc aggtgcgcgct aaaaaagct tgccaaaata
61  tctggtggtt ttaatcgggg gcgttataac tttgtaaaaa gtgttgcaca tggcttgaat
121  gcaatttggtt cagtgcaggg cattcgtggtt attttgaaaa gtattcgtaa attttctttg
plnF
181  gggagatcaaa cattttttaa gaaaaattttaa gttttgcttg accgtaaatt aatatctatt
241  tcaggtggcgc ttttccatgc cttatagcccg cttggcggttc ggaataatta taaaagtgtct
301  gttgggccctg cggattgggt ctttagcgcgt gtccgaggatt cttccacgg attttttaag
361  ccatacattt taaggactat aagaaagcc tgcatttattg actgggcttg cagttgtcag
plnI
421  ctttttttagt ttatatgccc cggatattttta cttcaccatt aataaaagta ataattcagct
481  ctattttacta cctaatagtg ccttttttaa tgaaccccttt gacctgatatt atgggaatctt
541  aagacggacc ctttcaattc atattaactg aatcctattt ataatcgcg ataatcattc
601  tgaataggcg atatgttaag caacctatcc actggctacc cgtaatatct atgtcaatat
661  taagaagaaa tagtctgcct ttgagccctc caataaatatt tctactatttt tcttttcgaa
721  acccacgta tcatatatat atctctatt tcatctgtat gatttgagcg ataaactcagcag
781  aatagcgttt tcgggctagt atatcagaa cgcttttagc actaaccctt aaaaaatttg
841  ccacattgca ggccacaccg cgttcgagta tgtctgcttt gctttatgtt tggctgatgc
901  atttgcctaa ctttttatca cagcccgtat ggtcagtttg tgtcctcttt ctctatgtta
961  ttgggttagg tattttactt gcagcaatct attttaaac cggagaacctt tgtggcttca
1021 tttagtgctca ttgagata cgtttattta cttcctattc tcaaggtacct gaccccaactc

Contd..
The representative initiation and termination codons have been represented by colour box: green box: start codon, grey box: stop codon.

These results suggested that the metagenomic library not only contained isolated plantaricin genes but also the whole transcription unit that consisted of the two genes coding for two synergistically active peptides, PlnE and PlnF and their cognate immunity protein PlnI. The plantaricin peptides are synthesized as pre-peptides and are processed by removal of the signal peptide so as to form the mature active peptides.

Subsequently, gene-specific primers were designed on the basis of structural genes, pln E, -F, -J, and -K (without leader sequence). PCR amplicons of around 100-110 bp were obtained from metagenomic library (Figure 2.2a), which were gel eluted (Qiagen kit, Qiagen, Germany) and digested with BamHI and XhoI.
The resultant gene fragments were purified and ligated into expression vector, pET 32a (+) (Novagen, USA) digested with the same enzymes in order to be cloned in-frame with thioredoxin (TRX) and (His)$_6$ tag. Ligation mixtures were transformed into *E. coli* XL1-Blue competent cells (as described in Materials and Methods) and transformants were selected on LB agar containing ampicillin (50 µg/ ml) and tetracycline (50 µg/ ml) as selection marker. The recombinant plasmids were confirmed by restriction digestion by *Bam*HI and *Xho*I (Figure 2.2a and b). Confirmation of clones was also done by colony PCR (Figure 2.3a and b).


The cloned PCR fragments were once again sequenced at Central Instrument Facility, South Campus, University of Delhi, New Delhi, analysed and were submitted to NCBI database (accession no(s)- KC445629, KC445630, KC445631, KC445632). These sequences along with the derived protein sequences are represented below:
Sequence of *plnE* (102 nucleotide)

1  tttaatcggg gcggttataa cttttgcaa aaagtgttcgc agtttggtga tgcaatttgg
tttggcaaa agtgttcgac atgttgttga tgcaatttgg aatattttgaa

PlnE Protein:
F N R G Y N F G K R
Stop

Sequence of *plnF* (105 nucleotide)

1  gttttccatg cctatagcgc gcgtggtcgtt cggaataatt atatattgtgga tataaaaagtgc ttttgggct
ttgcttgcttg tgttgggcct gcattcgtgg tctagttgcag gcattcgtgg
tttttgaaa agtgttcgac atgttgttga tgcaatttgg aatattttgaa

PlnF Protein:
V F H A Y S A R G V R N N Y K S A V G P A D W V I S A V R G F I H
G Stop

Sequence of *plnJ* (78 nucleotide)

1  ggccgattggg aaaaattctg ctctagttta agaaaaagat tttatgatgg ccagagctgg
tcctagttta gcgtggtcgtt gcattcgtgg tctagttta atatattgtgga tataaaaagtgc ttttgggct
tttttgaaa agtgttcgac atgttgttga tgcaatttgg aatattttgaa

PlnJ Protein:

Sequence of *plnK* (99 nucleotide)

1  cgtcggagtc gtaaaaatg gataaaaatg gtaaaaatg gtaaaaatg gtaaaaatg gtaaaaatg gtaaaaatg
ggcgcttggg aaaaattctg atatattgtgga tataaaaagtgc ttttgggct
tttttgaaa agtgttcgac atgttgttga tgcaatttgg aatattttgaa

PlnK Peptide:

**Fig 2.4:** Multiple sequence alignment of (a): *plnJ* nucleotide sequence (b): PlnJ Protein sequence
The *pln E, -F, -K* gene regions coding for mature plantaricins showed 100% sequence similarity with similar genes of known strains of *Lb. plantarum* available in database, whereas *pln J* showed some differences in its nucleotide sequence (Figure 2.4a). Nucleotide sequence alignment of *plnJ* gene was done with two *Lb. plantarum* strains, such as WCFS1 (accession no - AL935263.2) and LR/14 (accession no - JQ436928.1), a strain characterised in our lab. Compared to *Lb. plantarum* WCFS1, a single nucleotide change in 10\(^{th}\) (transition of A\(\rightarrow\)G) and 52\(^{nd}\) position (transition of G\(\rightarrow\)A) was observed. However, in comparison with *Lb. plantarum* LR14, difference was found only at 10\(^{th}\) position (Figure 2.4a).

To see, whether these nucleotide changes gave rise to any amino acid change or not, these sequences were analysed by expasy tool. In *Lb. plantarum* WCFS1 PlnJ peptide, lysine and aspartic acid were present at 4\(^{th}\) and 18\(^{th}\) position, respectively. PCR amplicon of soil metagenomic *plnJ* gene when translated by expasy tool, showed substitution of amino acid at these places, such as *Lys 4 Asp* (K4 E) and *Asp 18 Lys* (E18 K). In *Lb. plantarum* LR14, lysine was found to be present in both 4\(^{th}\) and 18\(^{th}\) position (Figure 2.4b).

**Heterologous Expression of Plantaricins**

In order to characterize these peptides, it became mandatory to express them in a suitable host. Thus it was decided to express them in a suitable host. Thus, it was decided to express them in a heterologous host so as to get a higher yield. There has been a limited information on plantaricin peptides expressed this way.

**E. coli** as expression system:

Though both prokaryotic and eukaryotic host systems have been tried, *E. coli* is still the most well-studied expression system. The low cost, high yield, and short turnaround time along with its ability to grow in high cell density and a strong genetic background, make it a popular choice for large scale protein production. Gram positive expression system, such as that of *Bacillus subtilis* may have limited use as it produces and secretes high levels of extracellular proteases which may degrade the secreted foreign proteins. Moreover, plantaricin peptides are very small (~ 3.0 kDa) and do not express on their own in *E. coli*, as described earlier.
In this study, PlnE, F, J, and K mature peptides were, therefore, heterologously expressed as His\(_6\) tagged-thioredoxin fusion proteins in *E. coli* background. Plantaricin EF (PlnEF) and plantaricin JK (PlnJK) are both two-peptide bacteriocins that belong to the large group of small, heat-stable non-lantibiotics termed class IIb bacteriocins, as described earlier. PlnE, PlnF, PlnJ, and PlnK are cationic peptides that consist of 33, 34, 25, and 32 amino acids, respectively.

The *pln* E, F, J, and K structural genes (without leader sequence) cloned in pET 32a (+), as described above, were further transformed into *E. coli* BL21 (DE3), as described in Materials and Methods, for expression. These *E. coli* BL21 clones carrying pET 32a-*pln*(s) were maintained on LB agar containing ampicillin (50 µg/ml). The composition of all the buffers/solutions and electrophoresis conditions have been presented in Annexure I.

**Expression of *pln* E, F, J and K genes in *E. coli***

For protein expression, 10 ml each of overnight culture of *E. coli* BL21 (DE3) harbouring recombinant plasmids carrying different *pln* genes were inoculated into 1 litre LB broth containing ampicillin (50 µg/ml) and incubated at 37°C with agitation. Once the OD\(_{600}\) reached 0.4, cultures were induced with IPTG (1 mM) and further grown for additional 5 h at 16 and 25°C and 4 h at 30 and 37°C, as described in Materials and Methods.

After the induction conditions were optimized (induction for 5 h, at 25°C), cells were harvested (8000 g, 10 min, 4°C), and cell pellets were washed and suspended in 15 ml of lysis buffer containing 20 mM Tris, 50 mM NaCl, 10 mM imidazole (pH 8). The cells were disrupted by sonication (60% amplitude) for 10 min. Sonication was done at room temperature, but the samples were maintained on ice. Supernatant containing cell-free extract was collected by centrifugation (8000g, 10 min, 4°C) and was used as the soluble cytoplasmic fraction. Pellets were dissolved in buffer containing urea (as described in Materials and Methods) and treated as insoluble fraction. Both soluble and insoluble fractions were analysed by SDS-PAGE (as described in Materials and Methods). Such an analysis revealed that the fusion protein accumulated essentially as soluble material in the cytoplasmic fraction (Figure 2.5a-d) of *E. coli*. In each case,
Irrespective of all the temperatures and IPTG concentrations tried, proteins were found to be expressed in soluble fraction. Maximum amount of protein was obtained when induction was given at 25°C (5 h) and 30°C (4 h) with 1 mM IPTG concentration. Majority of the protein was found to be present in soluble fraction, whereas little amount of the protein could be recovered in the pellet fraction as well.
The apparent molecular mass of the expressed fusion proteins were ~21-22 kDa as confirmed by SDS-PAGE (Fig 2.5a-d). The yield of fused peptides as estimated by BCA kit (Sigma Aldrich, USA) was 8-12 mg/litre, in each case.

**Purification of recombinant proteins PlnE, - F, - J and - K by Ni-NTA affinity chromatography**

Since all the Pln peptides were expressed with a (His)$_6$ tag, it provided a tool to purify the peptides by affinity chromatography. The high affinity of the Ni-NTA resins for His-tagged proteins or peptides is due to both the specificity of the interaction between histidine residues and immobilized nickel ions and to the strength with which these ions are held to the NTA resin.

Fig 2.6: SDS-PAGE Analysis showing purification of fused protein in Tris-Cl buffer using different imidazole concentrations (100 to 500mM). (a) PlnE: Eluted protein in different imidazole concentration (lane 1, 2, 3, 4, 5), molecular mass marker, Bangalore Genei, India (lane 6), induced PlnE at 25°C (lane 7), flow through (lanes 8, 9). (b) PlnF: uninduced sample (lane 1), Flow through after passing the cell lysate through the column (lane 2), molecular mass marker, Bangalore Genei, India (lane 3), induced PlnF at 25°C (lane 4), Eluted protein in different imidazole concentration (lane 5-9). (c) PlnK: Uninduced at 25°C (lane 1), induced at 25°C, eluted protein in different imidazole concentration (lane 3, 4, 5, 6, 8), molecular mass marker, GeneDirex, USA (lane 7). (d) PlnJ: Eluted protein in different imidazole concentration (lane 1, 2, 4, 5), molecular mass marker GeneDirex, USA (lane 3), First flow through (lane 6), induced PlnJ at 25°C (lane 7).
This protein purification system is based on the remarkable selectivity of Ni-NTA resin for recombinant proteins carrying a small affinity tag consisting of 6 consecutive histidine residues, the (His)_6 tag. NTA has a tetradeutate chelating group that occupies four of six sites in the nickel coordination sphere.

In order to purify the fused TRX-(His)_6-Plantaricin E, - F, - J and - K proteins, the soluble cytoplasmic fractions was loaded onto Ni-NTA column (Qiagen, Germany). At first, 10 ml of lysis buffer containing 20 mM Tris and 50 mM NaCl was passed through the column. Then the column was washed by binding buffers containing 10, 20, and 50 mM imidazole, and then immobilized protein was eluted with a gradient of 100 mM to 500 mM imidazole. All the buffers were maintained at pH 8.0. The efficiency of purification procedure was checked by SDS-PAGE (Figure 2.6a-d).

Each of the eluted fraction was collected and total protein was quantified spectrophotometrically, as described in Materials and Methods. Heterologous expression in E. coli enabled a yield of about 8-12 mg of fused purified protein/litre by this purification procedure. Maximum amount of protein was found to be present in 200 mM and 300 mM imidazole eluted fractions.

In some of the initial attempts, recombinant cell lysis was carried out in sodium phosphate buffer (pH 8), containing 50 mM sodium phosphate and 300 mM NaCl and elution was done in the same buffer, additionally containing 100 mM to 500 mM imidazole. However, better yield and resolution were obtained in Tris-Cl buffer, therefore, this system was chosen for protein purification. The composition of all the buffers are mentioned in Annexure II.

**Recovery of Peptides:**

After purification, the eluted TRX-(His)_6-Plantaricin fusion peptides were dialysed using buffer containing 20 mM Tris and 50 mM NaCl (pH 8) for overnight at 4°C.

After dialysis the samples were resolved on SDS-PAGE (as described in Materials and Methods). As shown in Figure 2.7, all the fused peptides appeared in the size range of ~22kDa. From 100 ml culture, total proteins in the cell lysate of uninduced and induced sample was 25-30 mg/ml and 12-15 mg/ml, respectively.
Fig 2.7: SDS-PAGE showing fused Plantaricin peptides after dialysis. PlnE (lane 1), PlnF (lane 2), PlnJ (lane 3), PlnK (lane 4), protein molecular mass marker, Sigma, USA (lane 5).

**Enterokinase treatment**

The eluted, dialyzed protein samples were subjected to an overnight cleavage at 23°C with the help of enterokinase (NEB, USA) in the same buffer additionally containing 10 mM of CaCl₂ (as described in Materials and Methods). One microlitre of enzyme (stock 2µg/ml) was used to digest 1 mg protein.

Fig 2.8: Tricine SDS-PAGE showing digestion of fused Pln peptides by enterokinase treatment. (a): PlnE and PlnF: digested PlnE passed through Ni-NTA column (lane 1), digested PlnF passed through Ni-NTA Column (lane 2), molecular mass marker (lane 3), digested PlnE and PlnF before passing through column (lane 4 and lane 5). (b): PlnJ: digested protein (lane 1), molecular mass marker (lane 2), undigested protein (lane 3). (c): PlnK: molecular mass marker (lane 1), digested protein (lane 2 and 3).
Enterokinase treatment led to the release of plantaricin peptides from the TRX tag (Figure 2.8).

A significant yield of fused and cleaved peptides was obtained in the range of 8-12 mg/litre and 1-1.5 mg/litre of the culture, respectively.

The enterokinase-treated fractions were analysed by Tricine-SDS-PAGE as small peptides could be resolved better after silver staining (See, Materials and Methods). The enterokinase proteolytic products consisted of four peptides, in the range of ~3-3.5, 15, 17, and 20 kDa. The bands of 15 and 17 kDa identified the two TRX forms. The weak band of 21-22 kDa observed by Tricine-SDS-PAGE corresponded to the remaining uncleaved Trx-(His)$_6$-Plantaricin fusion proteins (Figure 2.8a-c). In order to purify the cleaved plantaricins, the enterokinase cleavage products were passed through Ni-NTA column (Qiagen, Germany) and flow through fractions were collected and resolved by Tricine-SDS-PAGE (Figure 2.8a-c). As it is clear from this figure, the four Pln peptides appeared in the size range of 3.0-3.5 kDa.

**Standardization of enterokinase digestion:**

In order to recover the fully active plantaricin peptides from TRX fusion tag, some parameters pertaining to enterokinase cleavage needed to be standardized. This standardization was carried out with Trx-(His)$_6$PlnE. Initially, 1 microlitre of enzyme (stock 2 µg/ml) was mixed with 1 mg of fused protein for 5 h and 10 h digestion at 23ºC. The products of this digestion were resolved on tricine-SDS-PAGE.

As shown in Figure 2.9a, Some amount of protein was left undigested which clearly indicated that longer treatment is required for complete digestion. So the digestion was carried out for overnight under the conditions described above.

In the next step, same amount of enzyme was used to digest different concentrations (0.8, 1.0, 1.2 and 1.5 mg/ml) of fusion protein, overnight at 23ºC. The results shown in Figure 2.9b indicated complete digestion in all the cases and thus this amount of enzyme was sufficient for digestion.
By Tricine SDS-PAGE, all the four peptides appeared at their expected molecular mass.

![Image](108x568 to 514x688)

(a)

(b)

Fig 2.9: Tricine SDS-PAGE analysis showing digestion of PnE fused peptide by enterokinase treatment. (a): enterokinase cleavage at different time intervals. Lane 1- molecular mass marker, Sigma, USA (3.5 kDa-26.6 kDa), lane 2- 10 h digestion, lane 3- 5 h digestion.

(b) enterokinase treatment using different protein concentrations: Lane 1, 2, 3, 4 - overnight enterokinase-digested protein at 1.5, 1.2, 1.0, and 0.8 mg/ml concentration of protein, lane 5 – molecular mass marker, Sigma, USA (2.5 to 16.9 kDa).

**Plantaricin quantification and assay.**

The concentration of purified plantaricins was determined by two methods: UV absorption at 280 nm and quantification by BCA method using Bicinchoninic Acid Kit for Protein Determination (BCA1-1KT, Sigma-Aldrich, USA). The yield of purified fused and cleaved peptides was found to be in the range of 10-12 mg/litre and 1.5-2 mg/litre, respectively. The purified plantaricins were stored at (-20°C) in Tris-Cl buffer, pH 8, and used within 1 month of purification. After 4 months, 50% loss of activity was observed.

**Mass spectrometry**

MALDI-TOF

Though the mass analysis from tricine-SDS-PAGE indicated that the peptides purified by this procedure are the desired plantaricins, it was important that it is confirmed by mass spectrometry.

The molecular mass of the purified plantaricins was determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS).
The samples in Tris-Cl buffer, pH 8.0, were analysed by a Service Provider Company (Sandor Proteomics, India) as described in Materials and Methods. The MALDI-TOF analysis generated the mass spectra that conformed to the one reported in literature. The masses obtained for PlnE, -F, -J, -K are 3.543 kDa, 3.702 kDa, 2.994 and 3.499 kDa, respectively (Figure 2.10a).

![MALDI-TOF MS chromatogram](image)

**Fig 2.10a**: MALDI-TOF MS chromatogram of the purified plantaricins, PlnE, PlnF, PlnJ, and PlnK.

ESI-MS

The molecular mass of the purified plantaricins was also confirmed by ESI-MS analysis by GenProtech, Delhi ((as described in Materials and Methods), which also generated the same molecular mass, indicating the expression and purification of the
correct plantaricins (Figure 2.10b). The masses obtained for PlnE, -F, -J, -K are 3.547 kDa, 3.703 kDa, 2.929 and 3.503 kDa, respectively.

Fig 2.10b: ESI-MS chromatogram of the purified plantaricins, PlnE, PlnF, PlnJ, and PlnK.
**Antimicrobial activity assay of Plantaricins**

Though mass analysis confirmed the purification of plantaricin peptides, they were further analyzed for their antimicrobial function, for which they have been described in literature.

Plantaricins belong to the class of two-peptide bacteriocins (class IIb). As the name suggests, they are novel in that they consist of two very different peptides and optimal activity requires the presence of both the peptides in about equi-molar ratio.

Furthermore, circular dichroism (CD) studies reported in literature on the three two-peptide bacteriocins (plantaricin E/F, plantaricin J/K, and lactococcin G) revealed that the two peptides interact and structure each other upon exposure to target membranes as part of their antimicrobial action. In this study, both qualitative and quantitative analyses were done taking all the four heterologously expressed and purified peptides individually as well as in combination, against a set of related and unrelated Gram-positive bacteria.

**Agar-well Diffusion assay**

For the detection of a potential inhibitory activity of peptides, the agar well diffusion assay was carried out as described in **Materials and Methods**. Six indicator strains chosen were *Listeria innocua* B33314, *Micrococcus luteus* MTCC 106, *Enterococcus casseliflavus* B3502, *Lactococcus lactis* ssp. *lactis* 1821, *Lactobacillus curvatus* B4562, and *Lactobacillus plantarum* B4496 (listed in **Materials and Methods**). All these strains were reported to be inhibited by the crude bacteriocin preparation of *Lb. plantarum* LR/14 and also synthesized PlnE, -F, -J, and -K. For this experiment, *Listeria* was grown in Brain Heart infusion (BHI) medium (Himedia, India) at 37\(^\circ\)C, *Micrococcus* in nutrient broth (NB) (Himedia, India) at 37\(^\circ\)C, and lactic acid bacteria were grown in MRS (Himedia, India) at 37\(^\circ\)C. The details of media composition are described in **Annexure I**.

As described in **Materials and Methods**, the indicator organisms seeded in 5 ml of soft agar were overlaid onto a solidified base agar. Cells were exposed to plantaricins by placing 100 µl of the cleaved peptides (10µg in 100µl water) in wells made on this plate. After incubation overnight at 37\(^\circ\)C, a faint zone of inhibition was observed, in
all the cases. The extent of inhibition was increased when these were tested in combination suggesting their independent as well as synergistic action (Figure 2.11a and b). In case of *Listeria* only, where the fused peptides showed antimicrobial activity, it provided a first level screening for the positive clones. The activity was also checked against *Pediococcus acidilactici* and *Leuconostoc mesenteroides*, but these organisms were found to be insensitive towards plantaricins.

![Fig 2.11: Agar well diffusion assay showing zone of inhibition against *Listeria innocua* by purified Plantaricins. (a): PlnE, PlnF and PlnE+PlnF, (b):PlnJ, PlnK and PlnJ+PlnK.](image)

**Quantitative analysis for antimicrobial activity: Percent inhibition of cell viability**

Plantaricins’ antimicrobial activity was quantified with a microtiter plate assay, (as described in Materials and Methods). Indicator strains derived from a fresh, overnight culture, were subcultured (initial OD<sub>600</sub> of 0.01) in 1 ml of their respective medium in 24-well microtitre plate with different concentrations of the peptides singly as well as in combination (PlnE-PlnF and PlnJ-PlnK) for 12-14 h for all the assays. The concentrations tested for inhibition, as reported in literature, were in the range of 0.1 ng/ml to 100 µg/ml. In the present study, however, none of the selected strains was inhibited at the concentration of 0.1 ng/ml. Therefore, the minimum concentration used of these peptides was 5 ng/ml and the maximum was set at 5 µg/ml. No significant inhibition was observed at 5 ng/ml but at 5 µg/ml concentration, complete loss of viability (100%) took place. The concentration range, therefore, was adjusted between 50 ng to 1.0 or 2.0 µg/ml and effect on cell viability was determined. The inhibitory effect on these strains was typically dose-dependent and
strain-specific (Table 1). The fusion peptides did not show activity against the indicator organisms (except *Listeria*) indicating that free N-terminus region is necessary for the activity of Pln peptides.

**Table 1:** Percent inhibition of cell viability by purified plantaricins against indicator organisms at different concentrations:

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<th>0.2</th>
<th>0.5</th>
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<td>50-55</td>
<td>60-70</td>
<td>80</td>
<td>≥90</td>
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<td>55-65</td>
<td>70-75</td>
<td>80-90</td>
<td>≥95</td>
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<tr>
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<td>50-55</td>
<td>65-75</td>
<td>80-85</td>
<td>≥90</td>
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<td>40-50</td>
<td>60-65</td>
<td>80-85</td>
<td>≥90</td>
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<td>75-80</td>
<td>&gt;90</td>
<td></td>
</tr>
<tr>
<td><em>En. casselilavus</em> B3502</td>
<td>25-30</td>
<td>35-45</td>
<td>60-65</td>
<td>75-80</td>
<td>80-85</td>
<td></td>
</tr>
<tr>
<td><em>En. lactis lactis</em> 1821</td>
<td>10-15</td>
<td>25-35</td>
<td>45-50</td>
<td>60-65</td>
<td>65-75</td>
<td></td>
</tr>
<tr>
<td><em>Lb. curvatus</em> B4562</td>
<td>5</td>
<td>10-15</td>
<td>25-30</td>
<td>55-60</td>
<td>65-75</td>
<td></td>
</tr>
<tr>
<td><em>Lb. plantarum</em> B4496</td>
<td>10-15</td>
<td>30-35</td>
<td>40-45</td>
<td>60-65</td>
<td>75-80</td>
<td></td>
</tr>
</tbody>
</table>

Contd…
Percent inhibition of viable cells at PlnK concentration (µg/ ml)

<table>
<thead>
<tr>
<th>Strains</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. luteus MTCC106</td>
<td>&lt;5</td>
<td>5-10</td>
<td>10-15</td>
<td>25-35</td>
<td>35-45</td>
<td>55-60</td>
</tr>
<tr>
<td>L. innocua B33314</td>
<td>15-25</td>
<td>30-35</td>
<td>50-55</td>
<td>60-65</td>
<td>70-75</td>
<td>&gt;80</td>
</tr>
<tr>
<td>En. Casseliflavus B3502</td>
<td>25-35</td>
<td>50-55</td>
<td>55-60</td>
<td>60-65</td>
<td>70-80</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Le. lactis lactis 1821</td>
<td>15-20</td>
<td>25-35</td>
<td>40-48</td>
<td>55-60</td>
<td>65-70</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Lb. curvatus B4562</td>
<td>&lt;5</td>
<td>10-15</td>
<td>25-35</td>
<td>40-45</td>
<td>50-55</td>
<td>60-65</td>
</tr>
<tr>
<td>Lb. plantarum B4496</td>
<td>10-20</td>
<td>20-30</td>
<td>40-45</td>
<td>55-60</td>
<td>65-70</td>
<td>60-75</td>
</tr>
</tbody>
</table>

Percent inhibition of viable cells at PlnE+PlnF concentration (µg/ ml)

<table>
<thead>
<tr>
<th>Strains</th>
<th>0.025+0.025</th>
<th>0.03+0.03</th>
<th>0.04+0.04</th>
<th>0.05+0.05</th>
<th>0.075+0.075</th>
<th>0.1+0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. luteus MTCC106</td>
<td>20-30</td>
<td>30-35</td>
<td>50</td>
<td>65-70</td>
<td>75-80</td>
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<tr>
<td>L. innocua B33314</td>
<td>40-45</td>
<td>50-55</td>
<td>65-75</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>En. casseliflavus B3502</td>
<td>25-30</td>
<td>30-40</td>
<td>50-55</td>
<td>65-70</td>
<td>85-90</td>
<td>100</td>
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<tr>
<td>Le. lactis lactis 1821</td>
<td>15-20</td>
<td>25-30</td>
<td>35-45</td>
<td>55-65</td>
<td>70-80</td>
<td>100</td>
</tr>
<tr>
<td>Lb. curvatus B4562</td>
<td>5-10</td>
<td>10-20</td>
<td>25-30</td>
<td>35-45</td>
<td>50-55</td>
<td>70-75</td>
</tr>
<tr>
<td>Lb. plantarum B4496</td>
<td>10-20</td>
<td>25-35</td>
<td>40-45</td>
<td>50-55</td>
<td>65-70</td>
<td>80</td>
</tr>
</tbody>
</table>

Percent inhibition of viable cells at PlnJ+PlnK concentration (µg/ ml)

<table>
<thead>
<tr>
<th>Strains</th>
<th>0.025+0.025</th>
<th>0.05+0.05</th>
<th>0.075+0.075</th>
<th>0.1+0.1</th>
<th>0.2+0.2</th>
<th>0.5+0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. luteus MTCC106</td>
<td>&lt;5</td>
<td>5-10</td>
<td>15-25</td>
<td>25-30</td>
<td>35-45</td>
<td>60-70</td>
</tr>
<tr>
<td>L. innocua B33314</td>
<td>25-35</td>
<td>50-60</td>
<td>&gt;90</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>En. casseliflavus B3502</td>
<td>50-55</td>
<td>70-75</td>
<td>&gt;90</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Le. lactis lactis 1821</td>
<td>35-40</td>
<td>50-55</td>
<td>55-65</td>
<td>90-100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lb. curvatus 4562</td>
<td>&lt;5</td>
<td>15-20</td>
<td>25-30</td>
<td>35-45</td>
<td>55-60</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Lb. plantarum B4496</td>
<td>5-10</td>
<td>15-20</td>
<td>30-40</td>
<td>50-55</td>
<td>80-90</td>
<td>100</td>
</tr>
</tbody>
</table>

**ND** – Not determined

The percent cell viability inhibition was calculated taking different concentration range (**Table 1**) and the one that gave 50% inhibition of viable cell count, in comparison to untreated control (100% cell viability) was taken as its IC<sub>50</sub> value (**Table 2**).
Dose-dependent, strain-specific effect of different peptides

*M. luteus* has been commonly used as indicator organism in our lab because of its wide range of sensitivity towards bacteriocins of a number of lactic acid bacteria. *Listeria* is a food-borne pathogen and commonly used as indicator organism for class IIa bacteriocins. Keeping this in mind, *M. luteus* and *L. innocua* were used as indicator organisms to check the activity of the Pln peptides. Since bacteriocins are known to inhibit similar or closely-related species, four Gram positive LAB strains, such as *En. casselilavus* 3502, *Lc. lactis lactis* 1821, *Lb. curvatus* B4562, *Lb. plantarum* B4496 were also included as indicator organisms. PlnE, PlnF, PlnJ and PlnK were found to be active against all of these and their IC$_{50}$ values could be determined against the strains tested (Table 1 and Table2).

### Table 2. Activities of purified plantaricins individually and in combination towards different indicator strains.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>IC$_{50}$ Concentration (µg/ml) of plantaricins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PlnE</td>
</tr>
<tr>
<td><em>M. luteus</em> MTCC 106</td>
<td>0.1</td>
</tr>
<tr>
<td><em>L. innocua</em> NRRL B33314</td>
<td>0.09</td>
</tr>
<tr>
<td><em>En. casselilavus</em> NRRL B3502</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Lc. lactis ssp. lactis</em> NRRL 1821</td>
<td>0.12</td>
</tr>
<tr>
<td><em>Lb. curvatus</em> NRRL B4562</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Lb. plantarum</em> NRRL B4496</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Among the four peptides, PlnE was found to be most effective which inhibited (~10%) *M. luteus* at a very low concentration of 0.01 µg/ml. However, other peptides have had no effect on *M. luteus* at this concentration. PlnK was found to be least active against *M. luteus*. Synergistic activity of PlnE-PlnF as well as PlnJ-PlnK was observed (2-3 folds increase) which was also found to be dose-dependent. Combination of PlnE and PlnF exerted a higher inhibition in comparison to PlnJ and
PlnK. The IC$_{50}$ values were observed to be 0.1, 0.2, 0.1, 0.75 µg/ml, respectively when PlnE, -F, -J, and -K were used individually, whereas in combination IC$_{50}$ of PlnE-PlnF and PlnJ-PlnK was 0.08 µg and 0.75 µg/ml, respectively.

*L. innocua* was found to be more sensitive than *M. luteus*. 50% growth inhibition was caused at much lower concentration like 0.09, 0.15, 0.08, 0.2 µg/ml of PlnE, PlnF, PlnJ, PlnK individually, and 0.06 and 0.07 µg/ml of PlnE-PlnF and PlnJ-PlnK combined.

A similar pattern of inhibition was observed in case of all the four Gram positive LAB strains. Synergistic activity was observed significantly in a dose-dependent manner. The results clearly demonstrated that PlnE and PlnF were more effective than PlnJ and PlnK. The IC$_{50}$ value of PlnE was calculated and it was found to be 0.1, 0.12, 0.25, 0.15 µg/ml for *En. casseliflavus, Lc. lactis lactis, Lb. curvatus*, and *Lb. plantarum*, respectively. PlnF showed lesser activity as it exerted 50% inhibition at comparatively higher concentrations of 0.15, 0.2, 0.4, 0.25 µg/ml, respectively. PlnE and PlnF together showed enhanced activity and their IC$_{50}$ values were 0.08, 0.09, 0.15, 0.1 µg/ml for all these four strains. In between J and K, J was found to be more active than K as it was showing 50% inhibition against *En. casseliflavus, Lc. lactis lactis, Lb. curvatus*, and *Lb. plantarum* at a concentration of 0.06, 0.15, 0.4, 0.25 µg/ml. In combination of PlnK and PlnJ, the IC$_{50}$ values were 0.05, 0.07, 0.05, 0.1 µg/ml, respectively whereas PlnK alone showed 50% inhibition at much higher concentrations, such as, 0.09, 0.2, 0.5, 0.3 µg/ml. These results were derived from viable cell count and for each concentration plating was done in triplicate. Two independent experiments were performed with virtually the same results.

The IC$_{50}$ value of Pln E, -F, -J, - K recombinant peptides was compared with synthetic peptides available in the lab (based on the data from *Lb. plantarum* C11) The results showed comparable activity, indicating that these are biologically active peptides. Any difference observed on the inhibitory concentrations as reported in literature can thus be explained on the basis of target strain differences. This study also demonstrated the feasibility of the heterologous expression strategy to produce these peptides.
The results clearly demonstrated that PlnE was most active amongst the four peptides and PlnK was having least activity. Though all the four peptides were active individually, synergistic activity of PlnE-PlnF as well as PlnJ-PlnK was also observed in case of all the strains (Table 2; Figure 2.12a and b).

![Graph](image)

**Fig 2.12:** Determination of IC$_{50}$ Concentration of (a): PlnE, PlnF, and PlnE+PlnF (b): Determination of IC$_{50}$ Concentration of PlnJ, PlnK, and PlnJ+PlnK

**Activity of Synthetic-Hybrid peptides:**

It has been reported that N-terminal domain of a bacteriocin contributes to the membrane binding and C-terminal part of the molecule is responsible for target specificity. However, as shown above and also in literature, the 2-peptides showed differential effectivity. It was therefore decided to swap the N- and C- terminii of PlnE and PlnF to generate two hybrid peptides. These peptides were synthesized based on
the amino acid sequence of PlnE and PlnF, and were labelled as $E_N+F_C$ (16 amino acids from PlnE+16 amino acids of PlnF) and $F_N+E_C$ (17 amino acids from PlnF+18 amino acids from PlnE).

Sequence of hybrid peptides:

$E_N+F_C$: F N R G G Y N F G K S V R H V V G P A D W V I S A V R G F I H G


(The arrow demarcates the two swapped parts of PlnE and PlnF peptides)

Antimicrobial activity of these peptides was assessed by overnight treatment of the same six indicator strains as described above.

At 0.5 $\mu$g/ml, *L. innocua* showed maximum growth inhibition in comparison to other strains. For $E_N+F_C$ peptide, the percentage inhibition was 50% and $F_N+E_C$ peptide it was slightly higher (~54%). Against *Listeria*, PlnE and PlnF peptides at this concentration showed ~82% and ~73% inhibition, respectively. All other strains such as *M. luteus*, *En. casseliflavus*, *Lc. lactis lactis*, *Lf. curvatus*, and *Lb. plantarum* also showed inhibition (~17 - 30%) at this higher concentration, but compared to individual PlnE and PlnF, the effect was low. PlnE gave ~70-85% inhibition against all these indicator strains, and PlnF around ~60 - 75% (*Fig 2.13a*).

PlnE was more effective than PlnF and $F_N+E_C$ showed higher activity in comparison to $E_N+F_C$. At 5 $\mu$g/ml concentration, $E_N+F_C$ and $F_N+E_C$ peptide showed around ~71% and ~79% inhibition against the most sensitive indicator strain, *L. innocua*. $E_N+F_C$ peptides inhibited *M. luteus* and the four lactic acid bacterial strain in a range of ~53 to 70%. Individual PlnE and PlnF showed higher activity against all the indicator organisms. ~95 to 99% inhibition in case of PlnE and ~83 to 98% inhibition in case of PlnF was observed against all these indicator organisms (*Figure 2.13b*).

The combined effect of these peptides was 10-20% lower than the combination of individual PlnE and PlnF in case of lactic acid bacteria. Interestingly, these peptide combinations were equally effective against non-lactic acid bacteria, *M. luteus* and *L. innocua* (*Figure 2.13c*). The effect of all the plantaricins were dose-dependent and slightly increased inhibitory activity was observed in case of hybrid $F_N+E_C$ as
compared to $E_N+F_C$. Inhibition was 15-30% lesser in hybrid peptides activity compared to the individual parental peptides signifying the requirement of their respective N and C termini, respectively, for full inhibition.

Fig 2.13: Percent inhibition of cell survival of different strains by hybrid peptides at (a): 0.5 µg/ml concentration, (b): 5 µg/ml concentration, (c): 0.5 µg/ml concentration of PnE+PnF and synthetic $E_N+F_C$ (1) and $F_N+E_C$ (2) peptides in combination
The activity of hybrid peptides were also checked against some other lactobacilli strains, such as, *Pediococcus acidilactici* and *Leuconostoc mesenteroides*, which are otherwise not sensitive to Pln E, -F, -J, and -K. A concentration range of 0.5 µg/ml to 10 µg/ml was tried for both individual and hybrid peptides. However, hybrid peptides also did not show any inhibition against these indicator organisms.

**Structural analysis of the peptides:**

ORF *plnE* has a GC content of 30%. PlnE is translated as pre-peptide with double glycine in the leader sequence which is cleaved off during export to produce active peptide with 33 amino acids. The mature PlnE peptide is a small peptide with molecular weight of ~3.5 kDa and a pI of 11.0. The peptide has 5 strongly basic, 1 strongly acidic and 12 hydrophobic amino acids. Blast results of the peptide showed 100% similarity with plantaricinE of *Lb. plantarum* (strains WCFS11, C11, NC8, and LR/14).

ORF *plnF* has a GC content of 50% and the putative mature peptide is 100% similar to that of PlnFs of different strains. PlnF is also translated as pre-peptide with double glycine in the leader sequence suggesting the site at which the peptide is cleaved off during export to produce active peptide with 34 amino acids. The mature PlnF peptide is small peptide with molecular weight of ~3.7 kDa and pI 10.3. The peptide has 4 strongly basic, 1 strongly acidic, and 15 hydrophobic amino acids.

ORF *plnJ* has a GC content of 40%. Its protein product differs in having amino acid lysine (K) instead of amino acid glutamic acid (E) and a glutamic acid in place of lysine at 4\(^{\text{th}}\) and 18\(^{\text{th}}\) position, respectively, in the peptide. A leader sequence carrying a G-G site could also be identified. This ORF which may, therefore, be translated as pre-peptide, should be cleaved off at double glycine site during export to produce an active peptide of 25 amino acids. The deduced molecular weight of mature peptide is ~2.9 kDa and has a pI of 11.6. It has 7 strongly basic, 1 strongly acidic, and 9 hydrophobic amino acids.

ORF *plnK* has a GC content of 50% The homology search using Blast software showed it to 100% similar to putative *plnK* sequence of *Lb. plantarum* strains like WCFS1, C11, ATCC 14917, ST-III, and LR/14. The PlnK peptide is made up of 32
amino acids with the molecular mass of 3.5 kDa, and pI of 10.6. The mature peptide consisted of 7 strongly basic, 2 strongly acidic and 10 hydrophobic amino acids. Structural analysis based on bioinformatic analysis, NMR spectroscopy and CD analysis has revealed important structural attributes.

Figure 2.14: Representation of three-dimentional structure of PlnE and PlnF
RCSB-PDB PROTEIN DATA BANK (http://rcsb.org/pdb)

Figure 2.15: Representation of three-dimentional structure of PlnJ and PlnK
RCSB-PDB PROTEIN DATA BANK (http://rcsb.org/pdb)

PlnE has an N-terminal alpha-helix (residues 10-21), and a C-terminal alpha-helix-like structure (residues 25-31). PlnF has a long central alpha-helix (residues 7-32) with a kink of 38+/−7 degrees at Pro20. There is some flexibility in the helix in the kink region. Both helices in PlnE are amphiphilic, while the helix in PlnF is polar in its N-terminal half and amphiphilic in its C-terminal half. The alpha-helical content obtained by NMR spectroscopy is in agreement with CD studies. PlnE has two GxxxG motifs which are putative helix-helix interaction motifs, one at residues 5 to 9
and one at residues 20 to 24, while PlnF has one such motif at residues 30 to 34 (Figure 2.16 a and b). The peptides are flexible in these GxxxG regions. It is suggested that the two peptides lie parallel in a staggered fashion relative to each other and interact through helix-helix interactions involving the GxxxG motifs (Figure 2.14). The difference in activity of hybrid peptides with that of individual PlnE and PlnF might have arisen because of the number and positional difference of alpha helix and beta pleated sheets of the peptides. Three dimensional structures of all the peptides were elucidated on the basis of homology search from the structural database using the programme (PDB). In addition to PlnE and PlnF described above, PlnJ, a 25 residue peptide, has an N-terminal amphiphilic α-helix between Try-3 and Tyr-15. The 32 residues long PlnK forms a central amphiphilic helix between Gly-9 and Leu-24 (Figure 2.15a and b).

**Structure of PlnE, PlnF, and hybrid peptides:**

The modified synthetic peptides were also analyzed and compared with the two constituent peptides, as shown in Figure 2.16. The structure of hybrid peptides was deduced with the help of PDB software (http://www.rcsb.org/pdb). The deduced two dimensional structure of the hybrid peptides showed a completely changed topology. The secondary structure of EN+FC consisted of 3 helices (Gly4 – Asn7, Gly9 – Val16 and Ala19 – Gly28) and 2 helix-helix interactions. FN+EC in comparison consisted of 3 helices (Tyr5 – Arg8, Arg11 – Ile18, Val21 – Ser30), 3 helix-helix interactions, 2 beta turns (Val2 – Ala4, Phe2 – Tyr5) and 3 gamma turns (Gly9 – Arg11, Ile18 – Ser20, Ser30 – Arg32) (Fig 2.16c).

While EN+FC peptide has 4 strongly basic, 1 acidic, and 10 hydrophobic amino acids, FN+EC peptide has 3 strongly basic, 1 acidic and 9 hydrophobic amino acids (Fig 2.16d). Information available from CD and NMR analysis of PlnE and PlnF as described above revealed that E peptide with its two helical regions separated by a flexible GxxxG motif (G20xxxG24), and an additional GxxxG motif (G5xxxG9), is involved in inter-peptide helix-helix interactions, with F-peptide having one such motif (G30xxxG34). In case of hybrid peptides, EN+FC is containing two GxxxG motifs
$G_{5\text{xxx}G_{9}}$ and $G_{28\text{xxx}G_{32}}$, whereas $F_{N}+E_{C}$ contains only one GxxxG motif ($G_{22\text{xxx}G_{26}}$).

Fig 2.16: Two dimensional structure of (a) PlnE, (b) PlnF, and two hybrid peptides (c) $E_{N}+F_{C}$ and (d) $F_{N}+E_{C}$.

**Construction of gene fusion:**
Another modification carried out was to bring about a head to tail fusion of $plnJ$ and $plnK$ genes and to study the antimicrobial effect of the derived peptide. Based on sequence similarity, both $plnJ$ and $plnK$ genes were amplified from *Lb. plantarum* LR/14, with the former as $NcoI(F)$ and $BamHI(R)$ fragment, and the latter contained
BamHI(F) and XhoI(R) sites in reverse primer (Fig 2.17). The plnK fragment was first cloned in pET 32a vector, which was digested with BamHI and XhoI restriction enzymes. The recombinant plasmid was linearised by NcoI and BamHI. The plnJ PCR product was digested with the same set of enzymes (NcoI and BamHI). Both the digested products were eluted and a construct was formed by ligation. The ligated pET 32a (+) carried both plnJ and plnK cloned in frame with thioredoxin and (His)$_6$ tag (as described in Materials and Methods).

![fig2.17.png]

**Fig 2.17: Construction of gene fusion to form hybrid peptide**

The construct was confirmed by PCR (Fig 2.18), as well as by sequencing (Biochem Life Sciences, New Delhi).

**Sequence of fused gene:** (restriction enzyme sites are underlined)

\[
\text{plnJ} \rightarrow \]

1.  
ccatggggcg cttggaaaaa tttctgtgctct agttaagaa aaggattttta tggatggcaaa 

\[
\text{plnK} \leftarrow \]

61.  
gctggcagag caatccgtcg tggatccgct cggagtcgta aaaatggaat tggatatgcct

121.  
atttgttatg agttgggacgc ggtggaacgg gccgtgcttg gtggttcagc tttgtgggta

181.  
actcgag
Expression of *plnJ-K*

Expression and purification was carried out in the same manner as described above and in Materials and Methods. IPTG induction (0.5 mM) was given for 5 h at 25°C and cells were processed to get different fractions. As shown in Fig 2.19, the desired protein was expressed in both soluble and pellet fraction to almost the same level. A large amount of protein could be eluted with 50 and 100 mM of imidazole concentration and only some in 200 and 300 mM of imidazole concentration. The apparent molecular mass of the purified fused peptide was ~ 25 kDa as confirmed by SDS-PAGE (Fig 2.20).
These samples of Pln J-K were subjected to an overnight cleavage at 23°C with the help of enterokinase as described above. The undigested and digested samples were resolved by Tricine-SDS-PAGE (Fig 2.21).

**Activity of PlnJ-K fused peptides:**

The activity of fused PlnJ-K was checked against *L. innocua*, *En. casseliflavus*, and *Lc. lactis lactis*. The IC₅₀ concentration was determined as described above and compared to that of PlnJ and PlnK peptides (Fig 2.22).
As is clear from the results, the activity of fused PlnJ-K was found to be higher than PlnK and almost similar to that of PlnJ. When both PlnJ and PlnK were added in combination, the IC\textsubscript{50} concentration is found to be lower, which indicates the higher activity of peptide in combination compared to that of fused PlnJ-K. The effect was strictly strain-dependent.

The activity of fused peptide was also checked against some other lactobacilli strains, which were resistant to PlnE, -F, -J, -and K, such as, \textit{Pediococcus acidilactici} and \textit{Leuconostoc mesenteroides}, but fused peptide did not show any inhibition against these indicator organisms.

As described under the strategy to produce J-K fused peptide, the TRX tag was present at N-terminus end of the peptide. After enterokinase treatment, the TRX-tag was removed and thus the N-terminus of PlnJ, which was blocked earlier, would have got free. Because of head-to tail fusion of \textit{pln J} and -K, the N terminus region of \textit{plnK} and C terminus of \textit{plnJ} were blocked. When the tag was removed, N-terminus end of PlnJ became free, and its C-terminus was blocked, where as the N-terminus of PlnK is blocked and C-terminus was free. The activity of fused peptide was found to be comparable as that of PlnJ and no additive effect of PlnK was observed. It indicates that free N-terminus is essential for the activity of plantaricins, whereas effect of C-terminus is not that much significant.
Large scale production of Plantaricins:
The work described so far has demonstrated that soil-metagenome derived plantaricins could be successfully expressed and purified from the heterologous host, *E. coli*. One of the long sought goals in recombinant protein production is to achieve high levels of the desired protein. Keeping this in mind, experiments were designed to enhance the level of plantaricin production by scaling up the production culture volume and taking it to the level of a bioreactor.

(i) Shake-flask Batch cultivation by increasing production volume
The *plnE*, *-F*, *-J*, *-K* genes cloned individually in pET32a vector and expressed in *E. coli* BL21 (DE3) were used for this study. Primary culture was raised from freshly transformed colony and grown in LB overnight at 37 ºC, 200 rpm, in the presence of 50 µg/ml of ampicillin. Subculturing was done at 37ºC, 200 rpm till OD₆₀₀ reached ~ 0.4. Cultures were then induced by addition of IPTG (0.5 and 1 mM), followed by growth for 4 h at 30, and 37ºC and 5 h at 16 and 25ºC. Initially 15 ml of culture was checked for protein expression. For this, cells were harvested by centrifugation at 10,000g for 10 min, and processed as described in Materials and Methods. Maximum amount of protein was found to be expressed at 25 and 30 ºC, at both the IPTG concentrations. Gradual scaling up was done raising 50 ml, 100 ml, 1 litre, and 2 litre cultures under shake-flask optimized growth conditions, and protein expression was monitored. When 100 ml batch culture was raised, the eluted fused protein was quantified to be in the range of 1.0 to 1.4 mg. Further scaling up could be done up to 1 and 2 litre of batch culture using same temperature (30ºC) and IPTG (1 mM) concentration. Total 10-12 mg and 20-25 mg of fused PInE, -F, -J, and –K peptides were obtained in the soluble fractions from 1 and 2 litre of culture, respectively. All the four fused proteins were produced at almost a similar yield.

Effect of Growth medium on Plantaricin Production
The synthesis of a protein at high levels is often linked with high biomass generation. The latter is influenced by growth conditions and one of the important parameters for growth is the culture medium. For *E. coli* different types of media are known, but the most popularly used medium is LB. As described above, all the expression studies
were carried out in LB. However, alternate complex medium, such as terrific broth (TB) has been recommended for high density culturing of *E. coli*.

Terrific Broth is an enriched medium (composition provided in **Annexure I**) recommended for the cultivation of recombinant strains of *E. coli*. This medium has been shown to help the recombinant strains of *E. coli* to maintain an extended growth phase. Increased amount of tryptone and yeast extract in the medium provide additional nutrients and growth factors required for enhanced growth of *E. coli* and glycerol as an additional carbon source also serves the same purpose. Potassium phosphates are present to buffer the medium during bacterial growth. The higher growth is expected to allow the bacteria to maintain a higher plasmid yield and should lead to higher protein production.

In the present study, growth and plantaricin production, taking PlnE as a representative peptide, was compared in LB and TB. Primary culture (initial OD$_{600}$ ~ 0.01) was raised in LB in presence of 50 µg/ml of ampicillin, overnight at 37°C, 200 rpm. Secondary culture was raised in 100 ml both in LB and TB at 37°C, 200 rpm, with an initial OD$_{600}$ of 0.01. After 2 h, the temperature was lowered to 22°C for 30 min and 0.5 mM of IPTG induction was given. Uninduced control cells in both LB and TB were grown parallely. The growth of all the samples was measured spectrophotometrically (OD$_{600}$) at an interval of 2 h. After a further growth of 5 h and 9 h, induced cells were harvested by centrifugation for 10 min at 10,000g and total proteins were isolated. An aliquot from both the induced samples were serially diluted and plated on LB agar plate, and number of colonies were compared in terms of CFU/ml. After 5 h induction, the number of colonies in TB was 4-5 times higher than LB, but after 9 h it was found to be similar. To check the stability of recombinant plasmids, some 100 colonies (from both LB and TB) were chosen randomly and were patched on LB agar containing ampicillin (50 µg/ml).

In terrific broth, no expression of protein was observed when cultures were grown above 25°C. With lowering of temperature (22°C), expression of PlnE was observed in TB-grown cultures, but to a lesser level in comparison to LB. After a brief acclimatization (30 min) to lower temperature, both the cultures were induced with IPTG (0.5 mM). The OD$_{600}$ of cultures at this stage of induction in LB and TB was
0.61 and 0.4, respectively. Post induction, the growth was monitored turbidometrically for 12 h.

As shown in Fig 2.23a, initially the growth under induction was higher in LB, but subsequently, the growth of the induced samples was found to be almost similar in two media. In comparison, however, uninduced TB-grown cells maintained higher growth profile compared to the cells grown in LB (Fig 2.23b). Some hundred colonies from these cultures when patched on LB+Amp, were able to survive in this medium, indicating the high stability of recombinant plasmids.

**Fig 2.23**: Growth of *E. coli* BL21 host strain (containing recombinant plasmid, pET32aplnE) in LB and TB under different condition: (a) induced (b) uninduced
Both the uninduced and induced cells grown in LB and TB were harvested by centrifugation, and processed for protein isolation as described in Materials and Methods. Induced soluble fractions were run on SDS-PAGE (Fig 2.24). The total protein in 5 h-induced samples was estimated to be 13 and 23 mg/ml for LB- and TB-grown cultures, respectively, whereas after 9 h induction, total proteins in both types of cells was ~25 mg/ml. After purification using affinity chromatography, the proportion of TRX(His)_6PlnE protein was quantified to be 1.2 and 0.76 mg/ml, respectively. Thus, TB-grown cells appear to have synthesized a lower level of plantaricin E, in comparison to that of LB-grown cells.

![SDS PAGE analysis showing expression of PlnE fusion protein in E. coli. Induced supernatant fraction after 5 and 9 h induction (a) in LB (lane 1 and 2), (b) in TB (lane 3 and 4), protein molecular mass marker (lane 5).](image)

The optimization of cell growth conditions and media thus seem to be target protein-dependent and there does not seem to be any empirical rule reported to date on this aspect.

(ii) Scaling-up in Fermenter:
In the present investigation, plantaricin production was further scaled up in a 30 L stirred bioreactor (Scigenics, Pvt. Ltd., Chennai, India) (Fig 2.25) using LB. Twelve litre LB medium (pH 7) prepared and sterilized in situ was inoculated with 1.2% inoculum from two representative recombinant strains expressing PlnE and PlnJ raised as overnight growth in LB at 30 °C. The impeller was maintained at 200 rpm, and compressed air was sparged into the medium at a constant rate of 0.5vvm. The fermentation parameters, such as temperature, pH, dissolved oxygen, and airflow were
continuously monitored. During the fermenter run, the foaming was controlled by using appropriate amount of silicon oil as anti-foaming agent. After 2 h of growth at 30 ºC, when OD₆₀₀ reached 0.4, IPTG induction (0.5 mM) was given to both the cultures. The samples (10 ml) were withdrawn periodically at 1, 2, and 3 h post-induction and OD₆₀₀ was checked. These samples were centrifuged at 10,000g, 10 min, and the cell pellet was processed for protein purification (as described earlier and in Materials and Methods).

Fig 2.25. Fermenter used for large scale production of plantaricins

Fig 2.26: SDS PAGE analysis showing expression of Trx-(His)₆-PlnE fusion protein in E. coli. (a) Induced supernatant fraction after 1, 2, and 3 h (lanes 1,2,3), protein molecular mass markers (lane 4). (b) induced pellet fraction after 1, 2, and 3 h (lane 1,2,3), protein molecular mass marker (lane 4).
Post IPTG induction at 1, 2 and 3 h interval, OD$_{600}$ of PlnE-expressing cultures was found to be 0.6, 0.75 and 0.9, respectively and in case of PlnJ expressing culture, the same was 0.65, 0.82, 1.09, respectively. The expression level of PlnE, in 1 h-induced sample was lesser compared to 2 and 3 h induced samples. Soluble fractions from two strains were run on SDS-PAGE to check the expression level (Fig 2.26a and b). No significant difference was observed between 2 and 3 h induction. Similarly, the induced samples from PlnJ expressing strain were also analysed by SDS-PAGE (Fig 2.27).

Fig 2.27: SDS PAGE analysis showing expression of PlnJ fusion protein in E. coli. Induced PlnJ pellet and supernatant fractions after 1 h (lane 1 and 2), induced pellet fraction (lane 3), protein molecular mass markers (lane 4), induced supernatant fraction (lane 5).

Fig 2.28a: SDS-PAGE analysis showing purification of fused PlnE protein in Tris-Cl buffer using different imidazole concentrations. Lane 1: flow through, eluted protein in different 10, 20, 50 mM imidazole concentration (lane 2, lane 3, lane 4, lane 5), 100, 200 mM imidazole concentration (lane 6, 7), 300 mM imidazole concentration (lane 8, 9), Protein molecular mass markers (lane 10).

Purification of protein was done using Ni-NTA affinity chromatography, as described earlier and in Materials and Methods. The total protein content of 1-, 2-, and 3- h induced samples from PlnE expressing strain was 8.5, 10, 11 mg/ml, respectively. In case of PlnJ, it was 8.0, 9.5, 10 mg/ml, respectively. The eluted protein samples were run on SDS-PAGE to resolve the recombinant plantaricins (Fig 2.28a and b).
Approximately 140 and 100 mg of fused PlnE and PlnJ proteins were obtained from 12 and 10 litre culture, respectively. The yield of purified fused proteins and cleaved peptides were found to be in the range of 10-12 mg/litre and 2-3 mg/litre, respectively, which were almost comparable to that of batch culture.

Fig 2.28b: SDS-PAGE analysis showing purification of fused Trx-(His)$_6$-PlnJ protein in Tris-Cl buffer using different imidazole concentrations. Lane 1: soluble fraction with induced PlnJ, lane 2: flow through of cell lysate, (lanes 3-8): eluted protein in different 10, 20, 50, 100, 200, 300 mM imidazole concentrations, respectively, protein molecular mass markers (lane 9).

Similar yield in batch culture and fermenter indicated the high stability of the recombinant plasmids, and amenability to increased production by increasing the production volume.

**Processing of Plantaricin:**

As described above, all the 4 genes coding for mature plantaricins, PlnE, -F, -J and –K could be successfully expressed in *E. coli* as TRX-(HIS)$_6$ – fusion peptides. Moreover, these fused peptides could be cleaved by enterokinase to release the active plantaricins. In nature, also these peptides are produced as pre-peptides, which are processed while being externalized. Though different transport systems have been identified for this purpose, many specially class IIb (to which plantaricins belong) employ an ABC transporter which recognizes a typical double glycine (G-G) motif in the leader sequence, where the cleavage occurs. Both cleavage and transport is mediated by the ABC transporter, the gene for which *plnG* is carried on the *pln* regulon.

In the present study, the processing function of ABC transporter was investigated upon. In a previous study (Nitika Ghosh, 2012 unpublished results), the transport operon was identified in *Lb. plantarum* LR/14 by PCR amplification, sequencing, and bioinformatic analysis. On the basis of sequence analysis of the PCR product of this
stretch, the previous study identified two ORF(s), labelled as $plnG_1$ and $plnG_2$. In order to check, whether this split actually exists or it was a sequencing error, ABC transporter gene ($plnG$) amplified from *Lb. plantarum* strain LR/14 as an ~ 2.1 kb amplicon was resequenced (Central Instrument Facility, University of Delhi, South Campus). As a control, DNA was also isolated from *Lb. plantarum* strain WCFS1, and the same $plnG$ gene was amplified and sequenced (Central Instrument Facility, University of Delhi, South Campus) (Fig 2.29).

Fig 2.29: Agarose gel electrophoresis to resolve PCR amplicons of $plnG$: lane 1- DNA of *Lb. plantarum* WCFS1, lane - 2: DNA of *Lb. plantarum* LR14, lane - 3: DNA size ladder (1kb).

After repeated sequencing, it was confirmed that the ~ 2.1 kb $plnG$ gene did not carry any internal chain termination codon. Sequence of *Lb. plantarum* LR/14 was compared with *Lb. plantarum* WCFS1 (accession no - AL935263.2). Nucleotide sequence alignment was done using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) and difference was observed only at two particular places (Fig 2.30).

**Sequence of $plnG$ of *Lb. plantarum* LR/14 (Accession no: KF471010)**
(Start and stop codons are underlined)

```
1   atgcattgga gaaactatgt tgcgcaagtt qacgagatgg actgtggttt gtcagcttta
61  gcatagttt tggaaatata tgttgcaca acatcattgg cctatctaaag aatatgtgct
121 aaacgagtt tagaaaggac taccggcatta ggttttagtaaa aacggtcga aagtttaggt
181 tttgaaaacca aagcattcca aagcagatatgt agtttttctg aggtacaaga tttaccattg
241 ccattcattg tgtctggtac caaaaatggg gatctacagc atttttatgt gtttctaa
301 acctcggaaa ccagcatgtctg agttgctgt cccggccccaa cagttgctgtg tattgctatg
361 tcagagagaa ggtttgaaag gcaataggtcc ggttggcctt ttttcttttgc acgcagacg
```

Contd...
gaatataagc cagttaagca agataaggcg tcactatggg gatttatcag aagcttatta
aagcagcgcc gacttgttat taatatgttg cttgtgtcacg tttataatgca gattattagt
atctgtggtt cttatcccc acaacggggtg atggatactt atatcccaaa taatatgcac
agtacattgg cagtgtggtgc cattgtgcta atcggttttt ttaaccttaca ggtctatcttt
aactattgcc agaatttttt attggcgggtt ttaggtcagcactttccat tgaattatt
ttaggctata ttcggcaagt ctttgaatttg ccaatgagtt tttttgccaac tctggcgagc
gggaaatttg ttccgaggtt taccggatgcc agtaagatta ttgtgcgtgt agtctgacca
attgggatgc gtttattttga tagtcgactc gttattttta tggggttaatc tcgagacatc
agctgtgtgt gattgttacccttta tgcattaoac acagtgcgtttt gagttttcagc gtttttattt
aatgttgaag ttgttatttt ggatctcgttg gatgactac gatgtgatgtc gatgtggtcag
gacaagtttta atgggattgtgt gacagcataat gcattaatagc gtttttaattt attttcccaac
aatattgatc ttcggcgggtt atgttattgg tagttattttt tggggtattc atgtgatgac
gattaattgtg aatgattgggt gatgattgtttt gagtttttt cgggacccat gcggggaggc
ttaatttgcg gcaagtttta atttttttttt ctgatgacggt gatggtttttt ctgttgacag
gatgttatttt aggatattttt actggattttt atgggattttt ctgagacagc
gactgtgatg ttgcatgttttc atgggtactgc tgggtattgc atggcgctatt ttggtgattgc
atgggattattt attgtgggttt ttttattttt gagtttattttc attttatattttta atttttttttt
aatgttgggtt atggtttttt gagtttttttt gcgggacccat gcggggaggc
ttttatggtg cagcagcttt cctgggcagc gcgttttttt cattgttattt atgggattttt
gagcagttt gccacccattt atggtttttt cgggacccat gcggggaggc
tgtgggttatttt gatgttatttt tgaattttttt tggggttattc atgggattttt
gagcagttt gccacccattt atggtttttt cgggacccat gcggggaggc
Comparison of \textit{plnG} sequence strain LR/14 and strain WCFS1

Contd…
Fig 2.30: Comparative analysis of \textit{plnG} sequence of \textit{Lb. plantarum} LR/14 with \textit{Lb. plantarum} WCFS1 by multiple sequence alignment using ClustalW2.
Cloning of plnG in pET 32a vector:

Fig 2.31 (a): Agarose gel electrophoresis showing PCR amplicon of plnG: lane 1: Lb. plantarum WCFS1, lane 2: Lb. plantarum LR14, lane 3: DNA size ladder (1kb); (b): Confirmation of clones carrying plnG in pET 32a vector by restriction digestion with BamHI and XhoI: lane 1 and 2 - digestion of pET32a carrying plnG (6 kb) and insert (2.1 kb) of Lb. plantarum WCFS1 and Lb. plantarum LR14, lane 4: DNA size ladder (1kb).

To validate the result further, it was decided to clone it in expression vector and to check the size of protein product formed. The PCR amplicons of plnG from both the strains (Fig 2.31a) were gel eluted, purified, and digested with BamHI and XhoI. This was then ligated into expression vector, pET 32a (+) (Novagen,USA) digested with the same enzymes in order to be cloned in frame with thioredoxin and (His)$_6$ tag. Ligated mixture was transformed into E. coli XL1-Blue competent cells (as described in Materials and Methods). The recombinant plasmid pET32aplG was confirmed by restriction digestion by BamHI and XhoI (Figure 2.31b), and by sequencing of plnG (Biochem Life Sciences, India).

Expression of plnG in E. coli

The recombinant plasmid was transformed in E. coli BL21 (DE3) as described in Materials and Methods. Various parameters, such as different temperatures and IPTG concentrations were tried to bring the protein in soluble fraction. Primary culture of pET32aplG recombinant strain was raised in LB broth containing ampicillin (50 µg/ ml) and incubated at 37°C with agitation. As the OD reached 0.4 at 600 nm, cultures were induced by different concentrations of IPTG (0.25, 0.5 and 1 mM) and grown further for 5 h at 18, 25, 30, and 37°C. Protein was accumulated in
pellet fraction under all the conditions though no induction was found at 0.25 and 0.5 mM IPTG concentrations. Further modification was done to bring the protein in soluble fraction. Primary culture raised at 25\(^0\)C, subcultured at 25\(^0\)C were induced by 1 mM IPTG. Such cells were harvested and processed to different fractions as described above (see also Materials and Methods).

For large scale purification, 2 litre culture was raised. After overnight induction by IPTG, cells were harvested by centrifugation (8000 g, 10 min, 4\(^0\)C) and cell pellets were washed with 15 ml of lysis buffer containing 20 mM Tris, 50 mM NaCl, 10 mM imidazole (pH 8). The resuspended cells were disrupted by sonication (60% amplitude) for 3 min. Sonication was done at room temperature but the samples were maintained on ice. Supernatant containing cell-free extract was collected by centrifugation (8000 g, 10 min, 4\(^0\)C) and was used as the soluble fraction. Pellets were dissolved in buffer containing urea and treated as insoluble fraction.

The soluble and insoluble fractions were analysed by glycine-SDS-PAGE, (Fig: 2.32). The results revealed that the fusion protein accumulated essentially as soluble material in the cytoplasmic fraction as well as in pellet fraction. The apparent molecular mass of the expressed fused peptide confirmed by SDS-PAGE was \(\sim 90\) kDa.

![](image.png)

**Fig 2.32: SDS-PAGE analysis showing expression of recombinant PlnG from pET32a: lane 1: uninduced sample, lane 2, 3 – Protein expressed in pellet fraction, 4, 5 - Protein expressed in soluble fraction 6 – protein molecular mass markers.**

**Purification of recombinant PlnG protein**

In order to purify the fusion TRX-(His)\(_6\)-PlnG protein, the soluble cytoplasmic
fractios was loaded onto Ni-NTA column (Qiagen, Germany). At first, 10 ml of lysis buffer containing 20 mM Tris and 50 mM NaCl was passed through the column. Then the column was washed by binding buffers containing 10, 20 and 50 mM imidazole, and then treated with a gradient of 100 mM to 500 mM imidazole. All the buffers were maintained at pH 8. The efficiency of purification procedure was checked by SDS-PAGE. The composition of all the buffers were mentioned in Annexure II.

In case of PlnG, maximum amount of protein was obtained in 50 mM imidazole, and rest was eluted at 100 mM imidazole. No protein was found to be eluted at higher imidazole concentrations. Since the yield of the protein was very less, it was first concentrated by passing through a column with a 10 kDa cut off (Pall Corporation, Pall Centrifugal Devices, USA). The concentrated protein was cleaved by enterokinase (as described in Materials and Methods). The purified and enterokinase-digested protein was run on SDS-PAGE (Fig 2.33 a and b).

![Image](a)

![Image](b)

Fig 2.33: SDS-PAGE analysis showing (a) purification of PlnG. Lane 1: molecular mass marker, lane 2: PlnG protein, (b) after enterokinase treatment: lane 1: molecular mass marker, lane 2 and 3: enterokinase treated PlnG.

Since the size of the protein was comparatively large (~75 kDa) with additional TRX tag (~14 kDa), non-specific cleavage was also observed after enterokinase digestion (Fig 2.33b). To get rid of this problem, pET32a (+) vector was replaced by pET28a (+), which does not contain TRX tag but contains only histidine tag. The insert was released from pET32a (+) and subcloned in pET28a (+) using same BamHI and XhoI restriction sites and the recombinant plasmid pET28aplntG was transformed in E. coli BL21 (DE3). The clones were confirmed by restriction digestion (Fig 2.34a) and sequencing (Biochem Life Sciences, India).
Fig 2.34 (a) Agarose gel electrophoresis showing restriction digestion of recombinant pET 28aplnG by BamH1 and XhoI to release insert. Lane 1 - 4: positive clones of plnG carrying vector backbone (5.3 kb) and insert (2.1 kb), lane 5: DNA size ladder (1kb). (b) pET28a (+) vector.

Expression of plnG was carried out using the same procedure and temperature conditions as described above. Soluble and insoluble fractions were resolved by SDS-PAGE (Fig 2.35). Purification of fused protein (~ 80 kDa) was done from the soluble fraction with the help of Ni-NTA column and maximum amount of protein was eluted in 50 mM imidazole concentration (Fig 2.36).

In order to show that the heterologously expressed and purified PlnG is functionally active, an activity assay was carried out for which the substrate needed to be provided. The latter was the full-length PlnE peptide (containing the leader sequence), as the PlnG is implicated in processing and transport of these peptides. The yield of total PlnG protein obtained from 1 litre culture was ~ 0.85 mg/litre.
Since the study described earlier dealt with mature plantaricins only, this part of the study was devoted to get the complete pre-PlnE protein. The experiment was also designed with a representative full-length PlnE on the premise that all the plantaricins pre-peptides carry leader sequence with G-G cleavage site and all are processed by the same ABC transporter protein.

**PlnE (with leader sequence):**

ORF *plnE* has a GC content of 38%. It translates into a pre-peptide made up of 56 amino acids with a molecular mass of ~6.2 kDa. It possesses 11 strongly basic, 2 strongly acidic, and 20 hydrophobic amino acids, with an isoelectric point of 11.2. PlnE is translated as pre-peptide with a double glycine in its leader sequence which is cleaved off by an ABC transporter during export to produce active peptide with 33 amino acids. The mature PlnE peptide is small peptide with molecular weight ~3.5 kDa and a pI of 11.0, as described above. The peptide has 5 strongly basic, 1 strongly acidic and 12 hydrophobic amino acids. The *plnE* gene (with leader) sequence was PCR amplified from genomic DNA of *Lb. plantarum* LR/14 based on the sequence similarity of this gene from strain LR/14 and the one derived from soil metagenome.

The PCR amplicon was gel eluted, purified, and digested with *Eco*RI and *Xho*I, and then ligated into expression vector, pET 32a (+) (Novagen,USA) digested with the same enzymes in order to be cloned in frame with thioredoxin and (His)_6 tag (**Fig 2.37a**). Ligated mixture was transformed into *E. coli* XL1-Blue competent cells (as
described in **Materials and Methods**). The recombinant plasmid was confirmed by restriction digestion with *EcoRI* and *XhoI* (**Figure 2.37b**) as well as by colony PCR (**Figure 2.36c**) and by sequencing. The sequence was submitted to the NCBI database under the accession number (**accession no- KF583753**).

![Figure 2.37](image)

**Fig 2.37 (a):** Agarose gel electrophoresis to resolve PCR amplicon of *plnE* full length pre-peptide gene: lane 1-DNA size ladder (100 bp), lane 2: amplicon from DNA of *Lactobacillus plantarum* LR14 (b): Restriction digestion by *EcoRI* and *XhoI* to release the cloned insert: lane 1: DNA size ladder (1kb), lane 2-7: positive clones carrying vector backbone (6 kb) and insert (171bp), lane 8: DNA size ladder (100 bp); (c): Confirmation of clone by colony PCR: lane 1-DNA size ladder(100bp), lane 2,3: amplicons of *plnE* gene.

**Sequence of full length plnE gene**

```
1  atgctacagt ttgagaagtt  acaatatccc aggttgcgccg  aaaaaaagct  tgccaaataa
61  tctggtggttt ttatcgggg  cggttataac  tttggtaaaaa  gtgttcgaca  tgttgttgtat
121  gcacctgtgtt  cagttgcagg  catctcggtt  attttgaata  ttaggggttaa
```

**Expression of plnE in E. coli**
The recombinant pET32a*plnE* plasmid was transformed into *E. coli* BL21 (DE3) as described in **Materials and Methods**. From the recombinant strain, primary culture
raised at 25°C, was subcultured at 25°C, and was induced with 0.25 mM IPTG, overnight at 18°C. The soluble and insoluble fractions prepared from such calls when resolved by SDS-PAGE showed that the protein was distributed in two fractions (Fig 2.38a) The preparation of different fractions and subsequent processing has been described in **Materials and Methods**. Purification of TRX-(His)₆-PlnE protein was carried out from the soluble fraction using Ni-NTA affinity chromatography (as described in **Materials And Methods**). The purified fractions were resolved on SDS-PAGE and it was found to be eluted in 100, 200, and 300 mM of imidazole concentration (Fig 2.38b).

![Figure 2.38a](image1.png)

![Figure 2.38b](image2.png)

**Fig 2.38** (a) SDS-PAGE analysis showing expression of PlnE protein from pET32a. lane 1 – protein molecular mass markers, lane 2 – protein expressed in soluble fraction (18°C), lane 3 - protein expressed in pellet fraction (18°C), lane 4 – protein expressed in soluble fraction (22°C), lane 5 – protein expressed in pellet fraction (22°C).

(b) SDS-PAGE analysis showing purification of fused PlnE pre-peptide in Tris-Cl buffer using different imidazole concentrations. lane 1, 2, 3, 4 – 500, 300, 200, 100 mM imidazole eluted protein, respectively; lane 5 – molecular mass markers; lane 6, 7 – 50 mM and 20 mM imidazole eluted protein, 8 – induced sample, 9 – uninduced sample.

After purification, the eluted TRX-(His)₆-Plantaricin fusion peptides were dialysed using buffer containing 20 mM Tris and 50 mM NaCl (pH 8) for overnight at 4°C, as described in **Materials and Methods**.

The activity of dialysed fused PlnE protein was checked against indicator organisms and it was found to be active only against *L. innocua*, but not against any of the tested lactic acid bacteria.

The fused PlnE pre-peptide was subjected to an overnight cleavage at 23°C with the help of enterokinase (NEB, USA) in the same buffer additionally containing 10 mM of CaCl₂ (as described in **Materials and Methods**). The total yield of fused PlnE was 9 mg/litre, and after enterokinase treatment, the yield was 2.2 mg/lit
**In-vitro Functional assay of ABC Transporter Protein**

Plantaricins as also several other bacteriocins are synthesized as precursor or pre-peptides containing N-terminal extensions (leader peptides) which are cleaved off during maturation. Most bacteriocins have leader peptides with the so called double glycine cleavage site, that is recognized and cleaved by an ABC transporter, as shown below.

\[
\text{PlnE: MQFEKLQYSRLPQKKLAKISGGF NRGGYNFGKSVRHVDAIGSVAGIRGILKSIR} \\
\text{PlnF: MKKFLVRDRELNAISGGVFHAYSARGVNRNYKSAVGPADWVIASAVRGFIHG} \\
\text{PlnJ: MTVNKMKDLDVDAFAPISNNKLNQVGGAWENFWSLROPYDGYDAGGRAIRR} \\
\text{PlnK: MKIKLTVINFEELTADAENISGGRRSRRKNGIGAYGAIFGAVERAVLLGDSRDNK}
\]

Several reports have shown that a dedicated ATP-binding cassette (ABC) transporter carries out a dual function. It not only proteolytically cleaves the pre-peptide (maturation), but also exports it across the cytoplasmic membrane.

![Diagram showing the in-vitro assay of ABC Transporter Protein](image)

Fig 2.39: Schematic representation of the *in vitro* assay for processing of the pre-peptide (Havastein *et al.*, 1995)

(Concentration of A – 500ng/µl, B – 500ng/µl, Na₂SO₄ – 250mM, DTT – 5mM)
Antimicrobial activity of the recombinant bacteriocins: *in vitro* assay to screen for leader peptidase activity

An *in vitro* assay was carried out to check the activity of ABC transporter in processing the PlnE pre-peptide as outlined in Fig 2.39.

As shown in Fig 2.39, the cleavage activity of ABC transporter on PlnE pre-peptide was checked and quantified with a microtiter plate assay (See Materials and Methods) using *En. casseliflavus* and *Lc. lactis lactis* 1821 as indicator. The cells of indicator strains derived from a fresh, overnight culture, were subcultured (initial OD₆₀₀ of 0.01) in 1 ml, and each was grown in 24-well microtitre plate along with different components of the reaction. Firstly, to eliminate the possibility that contaminating proteases from *E. coli* were responsible for processing of the pre-peptide, the proteins purified from *E. coli* cells with empty expression vector were tested. No activity was observed which ruled out the possibility of an *E. coli* protease, in activating the peptide.

In another, the PlnE pre-peptide, both as fusion protein or cleaved with enterokinase, also, did not show any antimicrobial activity on its own. In the third set, only ABC transporter protein and no pre-peptide was added, which also did not show any activity against the target bacteria. Finally, when ABC transporter (PlnG) was incubated with enterokinase-cleaved PlnE pre-peptide overnight at 37°C in the presence of Na₂SO₄ and DTT, strong antimicrobial activity was exhibited. Interestingly, this was comparable with the activity of enterokinase cleaved mature PlnE peptide (as described in the earlier sections of Chapter 2). Substrate (enterokinase cleaved pre-PlnE) and enzyme (ABC transporter) were incubated in 1:1 and 1:2 ratio (w/w) as described in Materials and Methods. Different parameters were standardized, and antimicrobial activity was detected only in fractions where 5 mM DTT and 250 mM Na₂SO₄ had been added.

By this *in vitro* assay, it can be assumed that PlnE pre-peptide has been cleaved by ABC transporter (Fig 2.40). Almost 50% inhibition against *Lc. lactis lactis* and *En. casseliflavus* was observed when 120 ng/ml and 100 ng/ml concentration of PlnE pre-peptide was used, (Fig 2.41). These two concentrations were determined as IC₅₀ concentration of mature PlnE against these two indicator organisms. In order to further confirm these results, the reaction products, as per fig 2.39, were resolved on
Tricine-SDS-PAGE. As shown in Fig 2.41, uncleaved PlnE pre-peptide showed a molecular mass of ~ 6 kDa, which upon treatment with PlnG released a peptide of ~ 3.5 kDa molecular mass, that was same as that of mature PlnE.

![Fig 2.40: A: PlnE pre-peptide, B: ABC transporter, C: Mature peptide](image)

Note: Inhibition was seen against *Lc. Lactis lactis* and *En. casseliftavus*.

![Fig 2.41: Tricine SDS-PAGE showing activity of ABC transporter (PlnG). Lane 1 – pre-PlnE uncleaved, lane 2 – molecular mass markers, lane 3 – PlnE cleaved by PlnG showing the release of PInE of ~3.5 kDa molecular mass](image)

**Role of accessory protein gene (plnH)**

Several studies have indicated the presence of an additional gene within bacteriocin operons, coding for the so called accessory protein (also accessory factor) that is required for the ABC-transporter-dependent function. In Gram-positive, unlike the Gram-negative bacteria, the function of the accessory factor is relatively unclear, since the processed product only needs to cross one membrane. Previous work in the lab on
*Lb. plantarum* LR/14 also identified *plnH* gene as part of the transport operon. In this study, the PCR amplicon (Fig 2.42a) of *plnH* gene derived from strain LR/14 was gel eluted, purified, and digested with *BamHI* and *XhoI*. This was then ligated into expression vector, pET 28a (+) (Novagen, USA) digested with the same enzymes in order to be cloned in frame with thioredoxin and (His)₆ tag. Ligated mixture was transformed into *E. coli* XL1-Blue competent cells (as described in Materials and Methods). The recombinant plasmid pET32aplnH was confirmed by colony PCR (Fig 2.42b), restriction digestion by *BamHI* and *XhoI* (Fig 2.42c), and by sequencing. The sequence was submitted to NCBI database under the accession number (accession number KF537532).

![Agarose gel electrophoresis](a)

![Agarose gel electrophoresis](b)

![Agarose gel electrophoresis](c)

**Fig 2.42:** Agarose gel electrophoresis (a) to resolve PCR amplicon of *plnH*. Lane 1-DNA size ladder (1 kb), lane 2- from genomic DNA of *Lb. plantarum* LR14 as template, (b) colony PCR confirming positive clones. lane 1- DNA size ladder (1 kb), lane 2-5- clones carrying no insert, lane 6, 7- positive clones carrying inserts (~1.4 kb), (c) restriction digestion by *BamHI* and *XhoI* of pET 28a-*plnH* transformant to release insert. lane 1, 2 - positive clones carrying vector backbone (5.3 kb) and insert (~1.4 kb), lane 3- DNA size ladder (1 kb).
Sequence of plnH (Accession no: KF537532):

1 atgagaaag acctgtgctga aagtgccgag ttttacggga ttcggtttca aaatatttca
61 acattactaa ttatcrocgt gacgctattg ttaatgggta cgttctgttt tctacgctga
121 gcgaagcgcg agatgtttat taatggtacc ggaactgttcc aaccgacccg aacggtttccg
181 gtgattcaag gcagtctgcca taatgcctaca aaaaagattt attatgtgta aggtgcgcga
241 gtgaagaagg gcacaaagct attagtttat actaacgttt ttaatcgcaa taatattacgc
301 gaagataatt tcaagcaacgc gccaagtgaa cagccttggag tcaaatattaaa
361 gatggcatca atactgcaag tgaatggtttt tccaacagcc aatgatattt ggacgagaaaa
421 ctattgccaat gtttatattaa acacgcttca aatctattttta cagagataaa aatgtggtca
481 gcgaatcgcg cgcagcttca tactaagaaa gccaagcttca cgaagacgcc gcaacaagtgc
541 gtatcaaatctc aacagccacct ctcggtattc tatcaacgct tatataacgctt aatgtggtcaat
601 ggtagcagct atgcacaaag tcgcaaatgc gggcgtcttt ttaatgagtt cgtcgcttaag
661 tcaaggaggg ccaaccaacgc aaacgataaa aaaaagattta aatctggcac aatgtggtca
721 attcaacgac agatagagttc gctctgctatcg caaatagttt cagcctaaag gcaggtgca
781 gaattacaag acctttggattc aatgtggtca ctaacaacc aataattggcc
841 atgttaaaga acgatcagtt aagcgaagtc gctgagaacc gaatgaaagc cgcagcagatag
901 ttacggaccg aagcgaagtc gctgagaacc gaatgaaagc cgcagcagatag
961 aacggcagcg aacgccgcttc cttacaaggt cgcagaacatc accgattggtg cagaatttggct
1021 agttcaggtag ctgaaatgcc aatctgagaa ccaacgcttac gctgtaggattc aatgtggtca
1081 gttaagtcat atatccggac ggcacagttt ccttcggttt aatgcggagcag ccattgaga
1141 tccaggtca gcgaaacgcc aacgaaagaattg tttggttggt ggctagagtcc
1201 ggggtttcctc aagtggagaa ggttaataac gcctttttatg aagtgacacgc gcacactgcgttgc
1261 attacagcgg ctacagccgca attatagcttg ttgggcaacgc ggcagttttagt
1321 acgccagaagg tggacttggttt ttatatttta aagataaggg ttcgccagtaa taattggca

(Start codon and stop codons are underlined)

The recombinant plasmid was transformed in *E. coli* BL21 (DE3) as described in Materials and Methods. Various parameters were tried with different temperatures and IPTG concentrations to bring the protein in soluble fraction. Primary culture of *E. coli* BL21 (DE3) carrying pET28aplnH recombinant plasmid was raised in LB broth containing ampicillin (50 µg/ml) and incubated at 25°C with agitation. When the
OD$_{600}$ reached 0.4, cultures were induced by 1 mM IPTG and further grown overnight at 22$^0$C. The overnight grown induced culture was centrifuged (8000 g, 10 min, 4$^0$C) and cell pellet was resuspended in 15 ml of buffer containing 20 mM Tris, 50 mM NaCl, 10 mM imidazole (pH 8). The cells were disrupted by sonication (60% amplitude) for 3 min. Sonication was done at room temperature keeping the samples on ice. Supernatant containing cell-free extract was collected by centrifugation (8000g, 10 min, 4$^0$C) and was loaded onto Ni-NTA column. Eluted samples were resolved by SDS-PAGE (Fig. 2.43).

![Fig 2.43: SDS-PAGE analysis showing purification of PlnH protein. Lane 1 - flow through after passing the cell lysate through the column, lane (2-7) eluted protein in different imidazole concentration, lane 8 – molecular mass marker, Prism Ultra protein ladder, Spain.](image)

The eluted 50 and 100 mM fraction was taken and dialysed in Tris-Cl buffer. The protein (~60 kDa) was run in SDS-PAGE (Fig 2.44). The total protein was quantified and it was found to be ~ 1 mg/litre.
To check the role of accessory protein in the cleavage of PlnE pre-peptide, an in vitro assay was carried out, as outlined in Fig 2.39. The pre-PlnE, ABC transporter and accessory protein were incubated in the ratio of 1:1:1, 1:2:1, and 1:1:2 (w/w) (as described above) and the activity was checked in microtitre plate assay (Materials and Methods) using En. casseli flavus and Lc. lactis lactis as indicator organisms. No enhancement of activity was observed by the addition of accessory protein (Fig 2.45). These results, therefore, suggested that accessory protein does not play a role in processing the pre-peptide to release the mature peptide.

The Structural Analysis of PlnG and PlnH

The nucleotide sequence of plnG and plnH was translated into respective amino acid sequence by expasy tool. The protein sequence was further analyzed for different structural and functional characteristics, using different bioinformatic tools:

1. Hydropathy Analysis

The amino acid sequence of PlnG and PlnH were used for hydropathy analysis according to Kyte and Doolittle [1982]. When the window size is 19, peaks with scores greater than 1.8 (red line) indicate possible transmembrane regions. However, when the window size is 9, strong negative peaks indicate possible surface regions of globular proteins (Figure 2.46). The Window Position values shown on the x-axis of the graph reflect the average hydropathy of
the entire window, with the corresponding amino acid as the middle element of that window.

Fig. 2.46: Kyte and Doolittle hydropathy plot of the putative protein sequence using a window size of 19 and 9: PlnG (a1 and a2), PlnH (b1 and b2).

2. Presence of transmembrane helices

The transmembrane helices in PlnG and PlnH were further validated by ‘TMHMM Server’ (version 2.0). As shown in the Fig 2.47, 5 transmembrane helices (TMH) were identified in PlnG and 1 in PlnH. This suggested that PlnG could be a membrane located protein.
3. Analysis of domains

Fig 2.47: The presence of transmembrane helices in the predicted (a) PlnG, (b) PlnH

Fig 2.48: Conserved domain analysis (a) PlnG, (b) PlnH
The homology search (Figure 2.48a) shows the protein to have Peptidase C 39B domain. This domain is characteristic of a sub-family of peptidase family C39. Peptidase family C39 mostly contains bacteriocin-processing endopeptidases from bacteria. The cysteine peptidases in family39 cleave at the "double-glycine" in the leader peptide from the precursors of various bacteriocins (mostly non-lantibiotics). The cleavage is mediated by the transporter protein, as part of the secretion process. The functional analysis of PlnH protein confirms the presence of bacteriocin secretion accessory protein family domain as shown in Figure 2.48b. Prediction of domains of important sites for functional analysis was done with the help of InterProScan software (www.ebi.ac.uk/interpro).

4. Prediction of secondary structure

Fig 2.49: Secondary structure of (a) PlnG and (b) PlnH
The secondary structure of putative PlnG and PlnH was predicted by PHYRE Protein Fold Recognition Server (www.sbg.bio.ic.ac.uk/phyre) (Figure 2.49a and b).

5. Prediction of tertiary structure

The tertiary structure of putative PlnG and PlnH was predicted by PHYRE Protein Fold Recognition Server (www.sbg.bio.ic.ac.uk/phyre) SWISS-PROT using the ‘Swissmodel Automated Web Server’ and. The model generated was viewed with the “pymol” software and is shown in Fig. 2.50.

![Fig 2.50: Tertiary structure prediction. (a) PlnG and (b) PlnH](image)

Based on this analysis both the proteins showed these characteristics structure required for the function/(s) assigned to them.

Summary

- Heterologous expression of individual pln E, F, J, and K genes was carried out from expression vector pET32(+) in E. coli BL21(DE3).
- These peptides were expressed as TRX-(His)$_{6}$-Pln fusion peptides.
- Purification of Pln E, F, J and K peptides was done using metal chelating affinity chromatography, and the fusion tag was removed by enterokinase treatment.
- Both ESI-MS and MALDI-TOF confirmed the mass of these peptides.
The antimicrobial activity of cleaved peptide was checked by viability assay. Cleaved peptides showed inhibition against a wide range of bacteria like *Enterococcus casseliflavus* NRRL B3502, *Lactococcus lactis* ssp. *lactis* NRRL 1821, *Micrococcus luteus* MTCC 106, *Lactobacillus curvatus* NRRL B4562, *Lactobacillus plantarum* NRRL B4496. However, other LAB strains, such as *Pediococcus acidilactici* 15958, *Leconostoc mesenteroides* ssp. *cremoris* 634 were insensitive to these peptides.

All four peptides were found to be active at nanomolar to micromolar range and their effects were found to be dose- and host-dependent.

PlnE and PlnF as well as PlnK and PlnJ showed synergistic activity against all these indicator organisms tested.

Fused peptides did not show activity against indicator organism (except *Listeria*) which signifies that free N-terminus is necessary for the activity of the peptides.

Hybrid peptides were constructed by swapping the N-terminal and C-terminal of PlnE and PlnF and such peptides found to be less active than individual PlnE and PlnF, indicating the requirement of both the components of the same peptide together. Fused PlnJ-K did not show any additive effect, and its activity was comparable with PlnJ.

The production of these peptides could be enhanced by increasing the production volume of the medium.

Comparative analysis of growth profile was done in LB and Terrific broth (TB). Expression and production of plantaricin (PlnE) was higher in LB compared to TB.

Large scale synthesis was demonstrated in a fermenter, where similar yield for PlnE and PlnJ was obtained as that in a batch culture. This indicated the high stability of recombinant plasmid. Thus, the heterologous production of
recombinant plantaricin peptides can be a successful route to obtain these useful molecules at a low cost and at large scale.

- The ABC transporter protein recognizes a typical double glycine (G-G) motif in the leader sequence of the pre-peptide of plantaricins, where the cleavage occurs. Cloning and heterologous expression of ABC transporter (PlnG) was done in *E. coli*. ABC transporter protein was purified and its role to cleave PlnE precursor peptide was illustrated with the help of an *in vitro* assay. The cleaved peptide showed the antimicrobial activity to the same extent as the mature peptide.

- Heterologous expression and purification of accessory protein (PlnH) was also achieved and *in vitro* activity assay was carried out along with purified ABC transporter and PlnE pre-peptide. However, no enhancement of ABC transporter cleavage activity was observed, suggesting its possible role in transport, but not in processing.

- Structural prediction and domain analysis of ABC transporter and accessory protein was done with the help of different bioinformatic tools.