Master project:

Engineering and Expression of Sactipeptide Constructs in *Lactococcus lactis*

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Abstract

Thurincin H, a ribosomally synthesised and post-translationally modified peptide with characteristic thiother bridges. Aim of this project is to modify six sactipeptide constructs by removing partial terminator in between structural and modification enzyme gene, and see how *thnB* enzyme works in *Lactococcus lactis* using *thnA* (Thurincin H structural gene) as substrate. The scope of the project is to use the sactipeptide modifications in combination with other modification systems in order to create new to nature molecules.

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Introduction:

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a rapidly growing group of natural products that vary in lengths. Precursor peptide formed from ribosomal synthesis consist of a leader and core section, after the post-translational modification the leader peptide will be cleaved off by respective peptidase. Extensive post-translational modifications of the core peptide results in structurally and chemically diverse groups possessing a wide range of bioactivities (Arnison, 2012).

Sactipeptides:

Sactipeptides contain characteristic thioether bridges, also known as sactionine bridges that are post-translationally installed. Sactipeptide biosynthesis requires a radical SAM enzyme, which contains multiple [4Fe-4S] clusters, to form the thioether bridges that crosslink the sulphur atoms of cysteine residues with the α-carbon of acceptor amino acids (Grove et al., 2017). So far five sactipeptides have been described, namely; Subtilosin A, Sporulation killing factor, thurincin H and thurincin CD. The first sactipeptide to be discovered was Subtulosin A derived from *Bacillus subtilis* 168 in 1985. It took around 18 years to solve its unusual structure comprising three thioether bridges by NMR spectroscopy (Flühe & Marahiel, 2013).

Sactipeptides are relatively hydrophobic, NMR examination shows that they have a structure resembles a hairpin with hydrophobic residue pointing to the exterior (subtilosin A and thurincin H) (Arnison, 2012). Maturation of sactipeptide is marked by formation of thioether bridges, catalysed by SAM enzyme (Flühe & Marahiel, 2013).

Thurincin H:

Thurincin H is produced by *thn* operon, comprises of three structural genes *thnA1*, *thnA2* and *thnA3* found in tandem repeats of chromosome and *thnB* gene encode an enzyme involves in the post-translational modification of precursor peptides (figure:1). The enzyme *thnB* generally consist of a typical CXXXCXXC binding motif for a [4Fe-4S] cluster. SAM enzyme reductively cleaves SAM into methionine and 5'-desoxyadenosyl radical and iron forms bond with sulphur of cysteine, by removing hydrogen as H^+ ion. 5'-desoxyadenosyl radical is highly unstable and reactive, it acquires a positive charge and become electrophile, results in the formation of thioether bond (Flühe & Marahiel, 2013).

The mature peptide is 31 amino acid long, and it is the only sactipeptide with four thioether bridge between four cysteine and the α-carbons of accepting amino acids. Thuricin H has a helical backbone which forms a hairpin structure, maintained by four pairs of thioether bridges. This hydrophobic peptide has a mode of action completely different from the generalized pore forming mechanism of lantibiotics. At an elevated level, it shows loss of cell integrity and rigidity (Wieckowski et al., 2015).

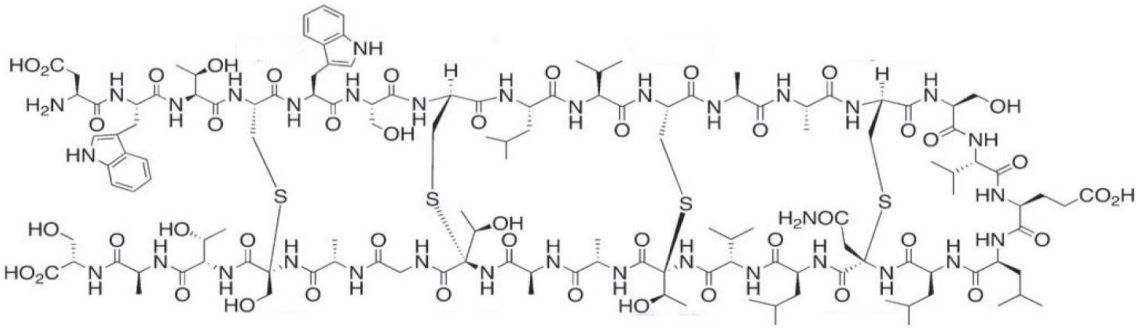
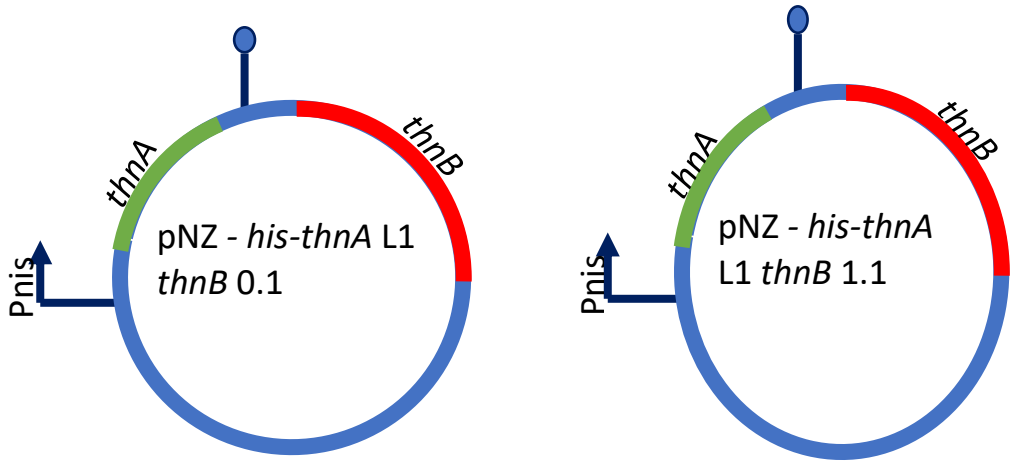


FIGURE 1: STRUCTURE OF THURINCIN H WITH FOUR THIOETHER BRIDGES BETWEEN SULPHUR ATOM OF CYSTEINE RESIDUE AND ALPHA CARBON OF ACCEPTING AMINO ACIDS

Project:

The goal of this project was to employ the radical SAM enzyme ThnB to modify ThnA and its derivatives in Lactococcus lactis. All six sactipeptide constructs, with partial terminator between structural and modification gene, cloned to plasmid pNZ8048 was provided. Plasmid consists of a nisin-inducible promoter upstream of the structural gene and a partial terminator from Lactococcus lactis in between the structural and enzyme genes (Figure: 2). Both the genes *thnA* and *thnB* are originally from *Bacillus thuringensis* SF361.



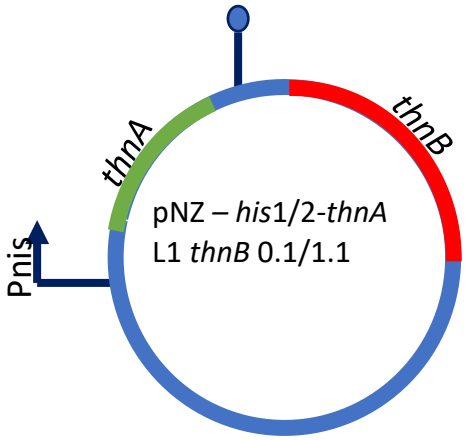


FIGURE 2: SACTIPEPTIDE CONSTRUCTS WITH PARTIAL TERMINATOR BETWEEN STRUCTURAL AND ENZYME GENE

Plasmids pNZ-*his-thnA L1 thnB* 0.1 and pNZ-*his-thnA L1 thnB* 1.1 have N-terminal his-tags, and 0.1 is wild type (WT) and 1.1 is codon optimized. Plasmids pNZ-*his1-thnA L1 thnB* 0.1./1.1 and pNZ-*his2-thnA L1 thnB* 0.1/1.1 have a C-terminal his-tags. His-tag one is slightly different from his-tag two.

Partial terminator: The role of partial terminator is to terminate the mRNA synthesis in between the template and modification enzyme with less than 100% efficiency.

Research Questions and hypotheses:

In this project, the activity of enzyme *thnB* on *thnA* structural gene in absence of partial terminator will be investigated. How the presence or absence of his-tag at C or N-terminal affect the binding of modification enzyme to the leader peptide will also be investigated. The resulting peptides will be purified in two steps: His tag purification and C 18 reverse phase chromatography respectively.

After the purification of peptides, it will be subjected to mass spectrometry analysis and activity testing. It is expected that the removal of partial terminator from the constructs will produce the peptide of interest with post translational modifications.

Materials and Methods:

Strains and Plasmids:

Lactococcus lactis strain used for the transformation of modified peptides is NZ9000, standard host strain for nisin regulated gene expression. The strain contains the regulatory genes nisR and nisK integrated into the gene. The plasmid used for this project is pNZ8048, with a chloramphenicol marker. It is a high copy number, rolling-circle-replicating plasmid. Detailed list of all the strains used in this experiment is provided in supplementary information.

Growth Conditions:

Lactococcus lactis was grown at 30^{o}C in M17 media with 0.5% glucose. When the strains contained pNZ8048 plasmid, 5 µg/ml chloramphenicol was added to the medium. Stocks were prepared by adding 0,8ml of overnight culture to 0.8 ml of 42.5% glycerol solution in a cryotube, and stored at -80^{o}C.

Preparation of competent cells:

Lactococcus lactis NZ9000 was made competent by growing overnight culture in 10 ml SMGG17 media (M17+ 0.5 M sucrose, 20% glycine and 0.5% glucose), and added to 100 ml fresh warm SMGG17 media. It was grown to an OD 0.3 to 0.45. This culture was split in to two 50 ml tubes and spun down at 7000 rpm for 8 minutes. The pellets were resuspended in 50 ml ice cold wash buffer (0.5 M sucrose and 10% glycerol) and spun down for 12 minutes at 7000 rpm. Washing step was repeated thrice and after the third wash, discarding the supernatant the pellets were resuspended in 0.5 ml of wash buffer each. The resuspended pellets were aliquoted in 40 µl, and frozen in liquid nitrogen. Aliquots were stored in -80^{o} C for later use.

Plasmid Purification:

Plasmids were purified using Macherey-Nagel Nucleospin Plasmid EasyPure kit, following the protocol given. After the very first centrifugation, the pellet was resuspended in a solution of lysozyme (10 mg/ml) and A1 buffer, incubated for 30 minutes at 37^{o}C. Concentration of purified plasmids were checked using a Implen N60 Nanophotometer and purity was checked by running the purified plasmids on 1% Agarose gel.

Agarose Gel Electrophoresis:

Agarose gel electrophoresis was used to separate and analyse DNA fragments from polymerase chain reactions, plasmid purifications, digests and restrictions.

1% agarose gel was used for this purpose. DNA samples were loaded after mixing with 6X loading dye which contains 60% w/v glycerol, 20 mM Tris-Hcl (pH 8), 60 mM EDTA, 0.48% SDS, 0.3% m/v Xylene cyanol, 0.3% m/v Bromophenol blue and 0.12% m/v Orange G.

Thermo Fisher Generuler1KB DNA ladder was used for running all the gels, each gel was run for approximately 45 minutes at 90 V (for smaller fragments; 30 min at 90 V). Ethidium bromide solution was used for staining gels and were photographed using Bio-Rad Gel Doc EZ-imager.

Polymerase Chain Reaction:

Round PCR:

- Phusion high fidelity DNA polymerase was used for all the round PCR's, in order to remove the partial terminator between structural and enzyme gene. In a master mix 1 ng of template was used for each reaction. The reaction volume in each tube was 20 µl and following protocol was used cycling 35 times from denaturation to elongation: Initial denaturation (95°C, 2 minutes); Denaturation (95°C, 45 seconds); Annealing (Specific temperature for each primer, 30 seconds); Elongation (72°C, Depend on the size of each constructs); Final hold (16°C). A schematic representation of PCR plan is provided in the supplementary information.

PCR of pNZ-his-thnA L1 thnB 0.1/1.1, pNZ -thnA his1 thnB 0.1/1.1 and pNZ -thnA his2 thnB 0.1/1.1:

- Phusion high fidelity DNA polymerase was used for all the round PCR's, each master mix contain 1 µl template of 1 ng concentration. The reaction volume was 20 µl each tube. The protocol was as follows with 35 cycling from denaturation to elongation: Initial denaturation (95°C, 2 minutes); Denaturation (95°C, 45 seconds); Annealing (54°C, 1 minute); Elongation (72°C, 8 minutes); Final elongation (72°C, 8 minutes); Final hold (16°C).

Clean-up and Extraction of PCR Products:

Both clean-up and extraction of PCR products from the gel were done using Macherey-Nagel Nucleospin Gel and PCR clean-up. Followed the protocol provided for the clean-up with an extra washing step. PCR gel extraction was done by soaking the gel in ethidium bromide bath for 15 minutes and visualizing

under UV transilluminator. Extracted gel was incubated at 55°C water bath in order to melt the gel.

Ligation of PCR Products:

All the six linear PCR products were ligated overnight using T4 DNA ligase and 10X buffer following protocol provided by the producer. Tubes with ligation mix was kept on ice overnight, cold condition ensured the finding of sticky and end and once the ice melts T4 DNA ligase was activated and ligation took place.

Transformation by Electroporation:

The overnight ligation was cleaned off charged particles by keeping on a 0.45 µm membrane filter floating in MQ for 15 minutes. The washed volume was transferred to cooled Cuvette. 40 µl of competent cells were added and electroporated at 2.5 V, 200 ohm and 25 µF capacitance. 1 ml of SMG17MC (M17, 0.5M sucrose, 0.5% glucose,20mM $MgCl_2$ and 2mM $CaCl_2$) media to the cuvette. The volume was resuspended and incubated for 2 hours at 30° C. After incubation tube was spun down at 6500 rpm for two minutes, discarded 90 % of the supernatant. Pellet was resuspended in the remaining and plated out on SM17 agar plates with 5 µg/ml chloramphenicol. The transformants were grown on 30°C

Colony PCR:

- For colony PCR the master mix same as for the PCR was used, colonies appeared on the selection plates were picked up using a sterile tooth pick and used as template to run a colony PCR. Primers 3, 4 and 5 were used to verify the deletion of partial terminator. Both positive and negative controls were used for all four colony PCRs. Pfux7 polymerase was used instead of Phusion high fidelity polymerase. The protocol used as follows with 30 cycling from denaturation to elongation: Initial denaturation (95°C, 3 minutes); Denaturation (95°C, 1 minutes); Annealing (Specific temperature for each primer, 45 seconds); Elongation (72°C, Depend on the size of DNA fragments); Final hold (16°C).

Colony PCR of pNZ-his-thnA L1 thnB 0.1/1.1, pNZ -thnA his1 thnB 0.1/1.1 and pNZ -thnA his2 thnB 0.1/1.1:

- Protocol used was as follows with 30 cycling from denaturation to elongation: Initial denaturation (95°C, 3 minutes); Denaturation (95°C, 1 minutes); Annealing (52°C, 45 seconds); Elongation (72°C, 2 minutes); Final hold (16°C).

Primers and Sequencing:

The primers for round PCR and colony PCR were designed using Clone Manager software. One of the primers, either forward or reverse for round PCR was phosphorylated, so that it can be circularized by overnight ligation. Primers were provided by Biolegio in 100 mM concentration, from that a working solution of 10 mM for round and colony PCR, and 5 mM for sequencing were made. Both the stock and working solutions were stored at -20^{o}C. Sample sequencing was done by Macrogen EZ-sequencing. A list of all primers used is provided in the supplementary information (Table: S1).

Nisin Induction and Protein Purification:

Fermentation and Harvesting

For nisin induction 12 ml of overnight culture of *Lactococcus lactis* with modified plasmids were added to 800 ml of GM17 (0.5% glucose and M17) media with chloramphenicol (5 ng/ml). The cells were grown up to OD_{600} equals to 0.4. To that culture 160 µl of 5000X nisin was added and incubated for 4 hours. Each sample were divided in to two 400 ml centrifuge bottles and spun down for 30 minutes at 7000 rpm,4^{o}C after carefully balancing it. Supernatant was discarded and resuspended the pellet in 20 ml (20x2=40 ml) pre-cooled BB (Binding Buffer- 20 mM H_2NaPO_4 + 0.5 M NaCl + 20 mM imidazole). Resuspended pellet was transferred to 50 ml Greiner tubes, spun down for 15 minutes at 7000 rpm, 4^{o}C. Supernatant was discarded the pellets were stored at -20^{o}C.

Obtaining Cellular Fraction

Cell pellets were dissolved in 8 ml of lysis buffer (BB + 10 mg/ml lysozyme + protease inhibitor pill + Triton X-100), and incubated for 60 minutes at 37^{o}C.

Soluble fraction

Dissolved samples were sonicated for 20 minutes, 10 seconds on/ 10 seconds off at 70% amplitude. Sonicated samples were spun down at 8000 rpm for 30 minutes. The supernatant was poured into a 10 ml syringe and filtered into a Greiner tube using a 0.4 µm syringe filter.

His-tag Purification

His-tag column was prepared by adding 1 ml of Ni-NTA to an empty column with a column volume of 0.5 ml. Colum was washed with 5 CV (Column Volume) MQ followed by 10 CV of BB. Samples were run through the columns followed by 10 CV BB, 5 CV WB (Wash Buffer) and finally 5 CV EB (Elution Buffer).

- Protocol used for the purification was as follows: Wash the column with 10 CV BB; Sample (collect 1.2 ml sample, rest was allowed to flow through); Wash the column again with 10 CV BB; Wash buffer (collected 1.8 ml wash, rest was allowed to flow through); Elution buffer (Collected 1.8 ml Elution, rest was allowed to flow through

After use columns were washed with 10 CV 2M NaCl, 5 CV MQ and 5 CV 20% ethanol. Columns were filled with 20% ethanol, sealed airtight and stored at -4^{o}C for further use.

Reverse Phase Chromatography (C18)

For desalting and further purification C18 reverse phase chromatography was used. Column was prepared by adding 300mg of C18 material by Sigma Aldrig, column was made wet by adding 2ml of solution 1 (50% Acetonitrile + 0.1% TFA). For reverse phase chromatography to work the samples should have a pH below 4, which was obtained by adding 0.2% of TFA solution to the samples. To introduce an extra purification step, elution was done using a gradient of acetonitrile concentrations (30% and 50%). After equilibrating the column by adding 4 ml of solution 2 (0.1% TFA) samples were purified.

Protocol used for C18 reverse phase chromatography was as follows: Sample (collected 1.2 ml and rest was allowed to flow through); 8 ml of Solution 2 (collected 1.8 ml wash and rest was allowed to flow through); 3 ml of Solution 1 (collected 1.8 ml elution and rest was allowed to flow through)

The columns were stored airtight at -4^{o}C with solution 1.

C18 Tips

Elution's from his tag purification was desalted using C18 tips. It is similar to desalting by reverse phase chromatography. The only difference was, instead of using a column, a C18 tip was used. The C18 tips were made wet by pipetting Solution 1 up and down, and tips were equilibrated in a similar way using

Solution 2. The protocol used was as follows: Sample (pipetted 20 times up and down using the tip); Elution (pipetted up and down using the tip in 20µl of 30% elution buffer followed by 35µl of 50% elution buffer).

Matrix Assisted Laser Desorption/Ionization Time -Of-Flight Mass Spectrometry (MALDI TOF):

A matrix was prepared for the MALDI chip by adding α-cyano-4-hydroxycinnamide in 50% acetonitrile + 0.1% TFA. Target was loaded with 1 µl of concentrated samples and was allowed to dry. After drying, 0.8 µl of matrix was loaded on the samples.

Results:

Preparation of Expression Plasmids

All six sactipeptide constructs with partial terminator between structural and enzyme gene was sequenced and ready to use for round PCR. List of primers used for PCR and sequencing is provided in the supplementary information.

Modification of pNZ-*his-thnA L1 thnB* 0.1 and pNZ -*his-thnA L1 thnB* 1.1

For pNZ-*his-thnA L1 thnB* 0.1/1.1, round PCR was done to remove the partial terminator between Structural gene *thnA* and enzyme *thnB*. List of all primers used is provided in the supplementary information (Table: 1). Similar protocol was used to run PCR for both the plasmids, and agarose gel results are shown in figure: 3.

The bands on the gels were found to be of expected length. The original plasmid with partial terminator should have a length of 4962 bp, the modified ones were expected to have a band size of 4914. The agarose gel, which was the first indication that the round PCR was successful looked clean and promising. The PCR products were then extracted from the gel, cleaned using PCR clean-up kit, ligated overnight and transformed *Lactococcus lactis* with the same.

Colonies appeared on the GM17 agar plate with chloramphenicol antibiotic was checked by colony PCR to ensure the whether the transformation was successful. Primer 3 and 4 for pNZ-*his thnA thnB* 0.1 NT and primer 3 and 5 for pNZ-*his thnA thnB* 1.1 NT were used to verify the deletion of partial terminator from both the plasmids. The primers were designed in such a way that it binds to the region close to were the deletion should have taken place.

The agarose gel with colony PCR products are shown in figure 4. There was both positive control and negative control on the gel, positive control being the

original plasmid and negative control being the PCR mix without any template. All the six colonies had a band of size smaller (234 bp) than the original plasmid (282 bp). Colony PCR results confirmed that the desired constructs were created successfully. All the verified transformants were stored in -80°C for future use as glycerol stocks.

pNZ-*his-thnA thnB* 0.1

pNZ-*his-thnA thnB* 1.1

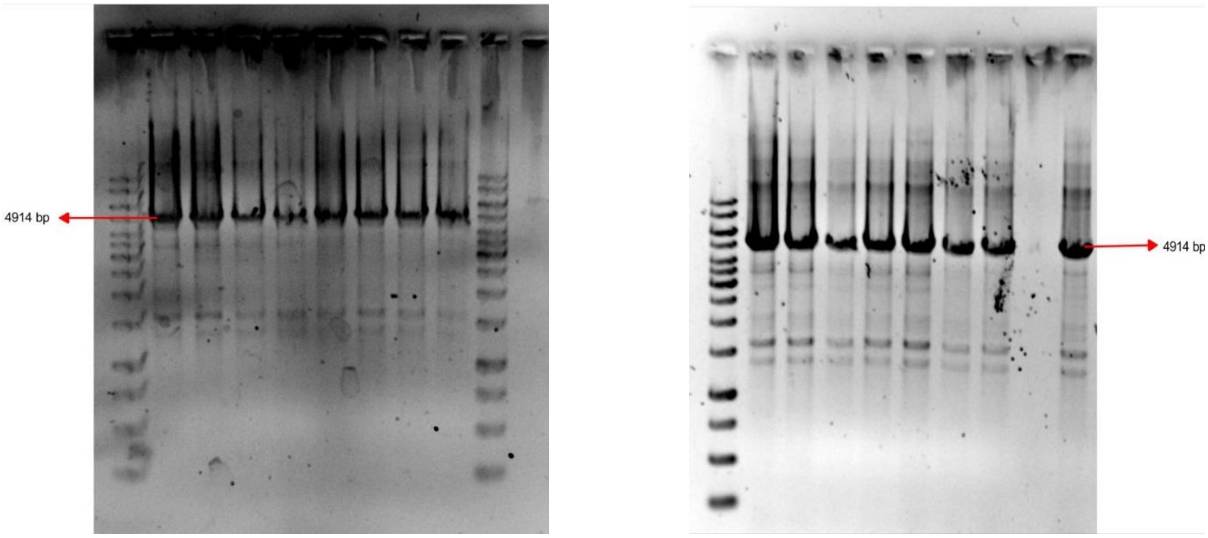


FIGURE 3: ROUND PCR RESULTS CONFIRMED THAT THE MODIFICATION WAS A SUCCESS. PLASMID WITH PARTIAL TERMINATOR SHOULD BE 4962 BP AND MODIFIED ONE WAS EXPECTED TO BE 4914 BP LONG

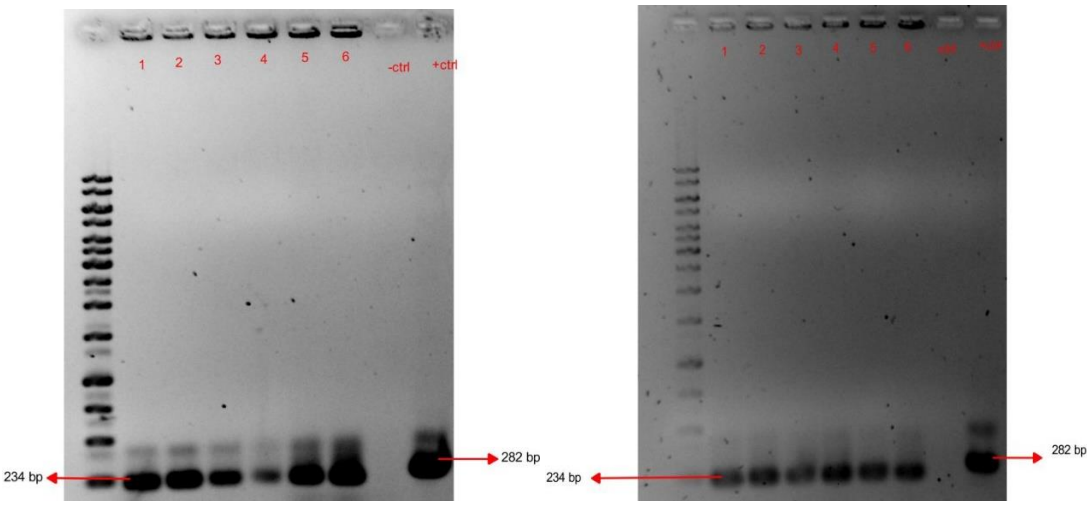


FIGURE 4: COLONY PCR RESULTS CONFIRMED THAT THE DESIRED CONSTRUCTS WERE CREATED SUCCESSFULLY. MODIFIED PLASMIDS (234 BP) WAS EXPECTED TO BE SMALLER THAN THE ORIGINAL CONSTRUCT (282 BP)

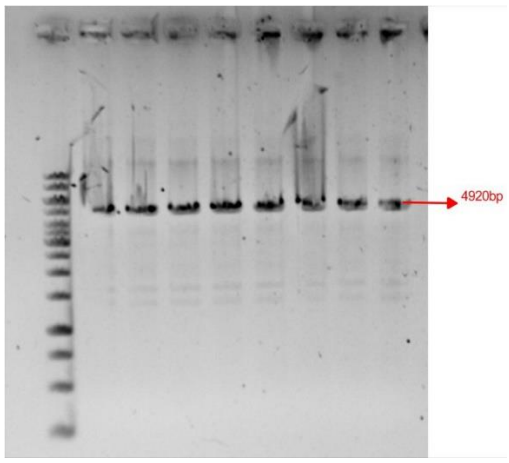
Modification of pNZ-*his 1 thnA L1 thnB* 0.1/1.1 and pNZ-*his 2 thnA L1 thnB* 0.1/1.1

Instead of an N-terminal his-tag all the four constructs had a C-terminal his-tag. Round PCR was used to remove the partial terminator between structural gene and enzyme. As mentioned above same PCR protocol was used for all four constructs.

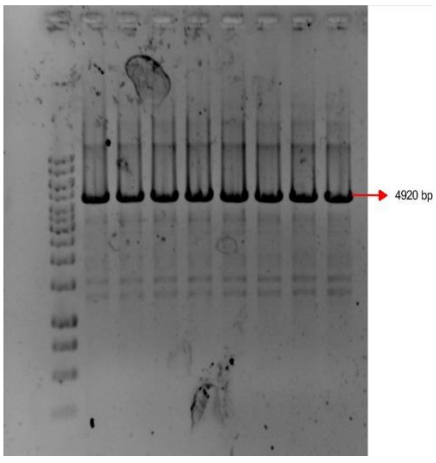
The original constructs should be of band size 4968 bp and the modified plasmids were expected to be of size 4920 bp. The agarose gel results are shown in the figure x. The PCR products were extracted, cleaned-up, ligated, and transformed *Lactococcus lactis* with the same.

The agarose gel image for the colony PCR is shown in figure: 6. All the six colonies that was picked up from the plate had the correct band size of 340bp and the positive control which is original plasmid had a band size of 388 bp. This indicate that cloning was a success and all the verified transformants were stored in -80^{o}C for future use.

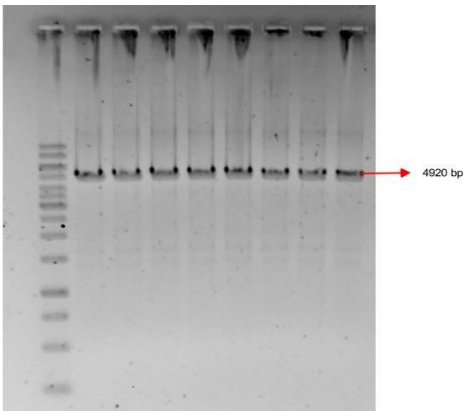
pNZ-*his1-thnA thnB* 0.1



pNZ-*his1-thnA thnB* 1.1



pNZ-*his2-thnA thnB* 0.1



pNZ-*his2-thnA thnB* 1.1

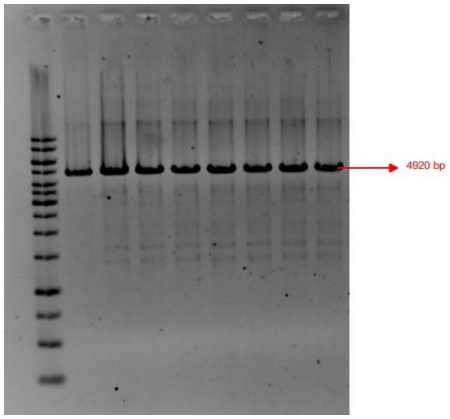
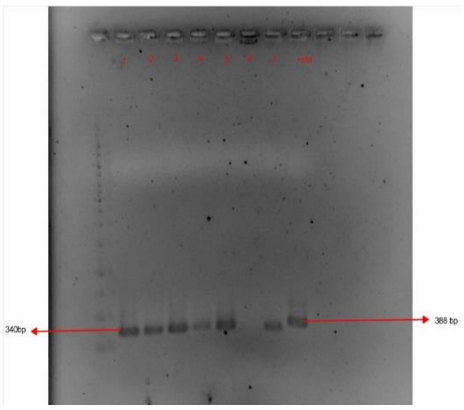
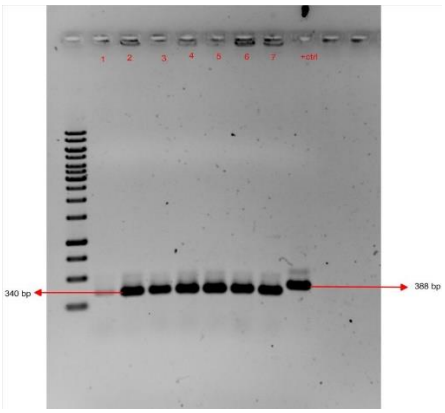
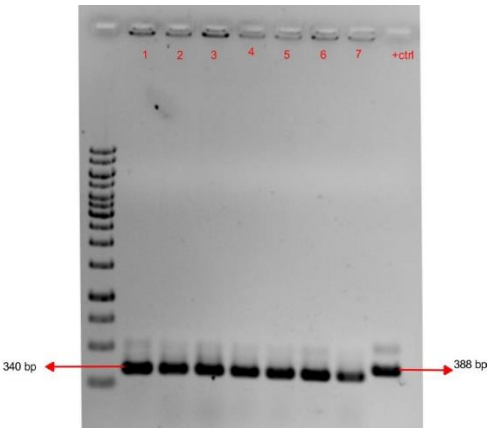


FIGURE 5: ROUND PCR RESULTS CONFIRMED THAT THE MODIFICATION WAS A SUCCESS. PLASMID WITH PARTIAL TERMINATOR SHOULD BE 4968 BP AND MODIFIED ONE WAS EXPECTED TO BE 4920 BP LONG







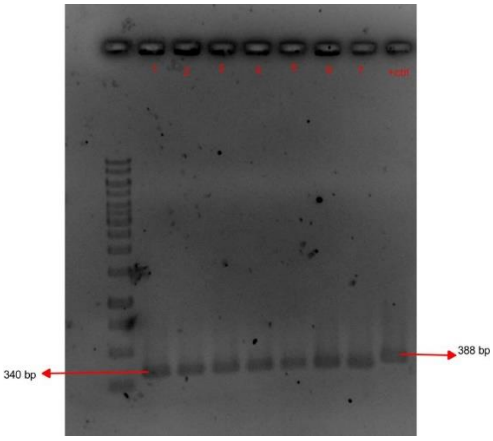


FIGURE 6: COLONY PCR RESULTS CONFIRMED THAT THE DESIRED CONSTRUCTS WERE CREATED SUCCESSFULLY. MODIFIED PLASMIDS (340 BP) WAS EXPECTED TO BE SMALLER THAN THE ORIGINAL CONSTRUCT (388 BP)

Sequencing

The plasmids were purified and send for sequencing to verify that the round PCR was successful. Not all of the colonies gave a good sequencing result;

SACTIPEPTIDE CONSTRUCTS	VERIFIED COLONY
pNZ-*his thnA thnB* 0.1 NT	Colony 2
pNZ-*his thnA thnB* 1.1 NT	Colony 3
pNZ-*his1 thnA thnB* 0.1 NT	Colony 2
pNZ-*his1 thnA thnB* 1.1 NT	Colony 2
pNZ-*his2 thnA thnB* 0.1 NT	Colony 5
pNZ-*his2 thnA thnB 1.1* NT	Colony 2

TABLE 1: LIST OF SACTIPEPTIDE COLONIES WITH MODIFIED PLASMIDS

The sequencing data confirmed that the partial terminator was deleted without any other mutations in thnB for both pNZ-his thnA thnB 0.1/1.1 NT. In case of pNZ-his1/2 thnA thnB 0.1/1.1 the sequencing information confirmed that the partial terminator was successfully deleted from the constructs. The stocks of verified transformants were kept for later use and the remaining ones were discarded.

Nisin Induced Expression and Protein purification

The cultures with all six transformants were induced by 5000X nisin to produce Thurincin H. Cultures having transformants without modification enzymes were used as controls. Proteins were purified from the pellet of cultures. Soluble fractions were subjected to his-tag purification followed by C18 tip purification. The samples obtained after desalting and concentration were used for MALDI-TOF.This desalted and concentrated samples were used for MALDI-TOF

MALDI-TOF

Matrix Assisted Laser Desorption/Ionisation Time of Flight mass spectrometry was used to check the presence of either modified or unmodified peptides produced by nisin induced hosts. There are variety of masses that we can expect, since the nine amino acid long leader peptide can be cleaved off or not, the methionine at N-terminal could be cleaved off. Also, there is the possibility that there could be some problem with the domains of thnB, which affect the maturation of thnA.

Thurincin H constructs	Expected Mass (Da)	FOUND MASS (Da)
pNZ-his thnA thnB 0.1 NT	4934.7 Da	
pNZ-his thnA thnB 1.1 NT	4934.7 Da	
pNZ-his1 thnA thnB 0.1 NT	5078.8 Da	4286.98
pNZ-his1 thnA thnB 1.1 NT	5078.8 Da	
pNZ-his2 thnA thnB 0.1 NT	5062.8 Da	
pNZ-his2 thnA thnB 1.1 NT	5062.8 Da	
pNZ-his1 thnA Ctrl	5209.8 Da	
pNZ-his2 thnA Ctrl	5193.8 Da	

TABLE 2: EXPECTED AND FOUND MASS OF PEPTIDES

Soluble fractions were purified and concentrated for mass spectrometry. Peaks were observed only in 50% elution. Most of the samples didn't show any distinct peaks or the one which has peaks didn't shown any sign of modification. Spectra taken for all the samples are given in supplementary information.

pNZ-his-thnA thnB 0.1 50% elution

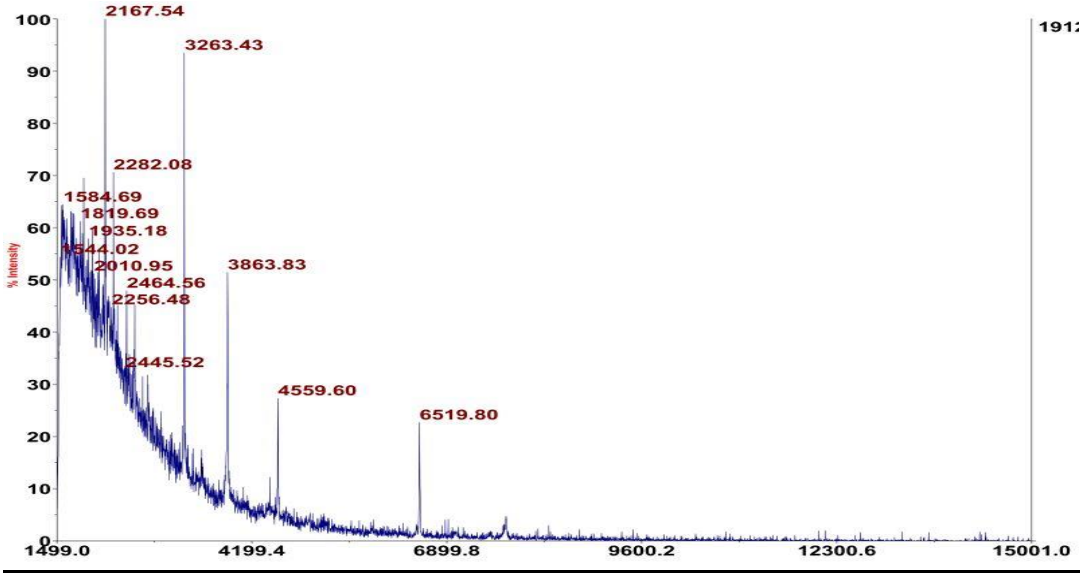


FIGURE 7: THERE ARE NO SIGNIFICANT PEAKS TO BE SEEN, EXPECTED TO SEE A PEAK OF 4934.7 DA, AND THE PEAK ON THE RIGHT MOST END SEEMS TO BE DOUBLE CHARGED

pNZ-*his1-thnA thnB* 0.1 50% elution

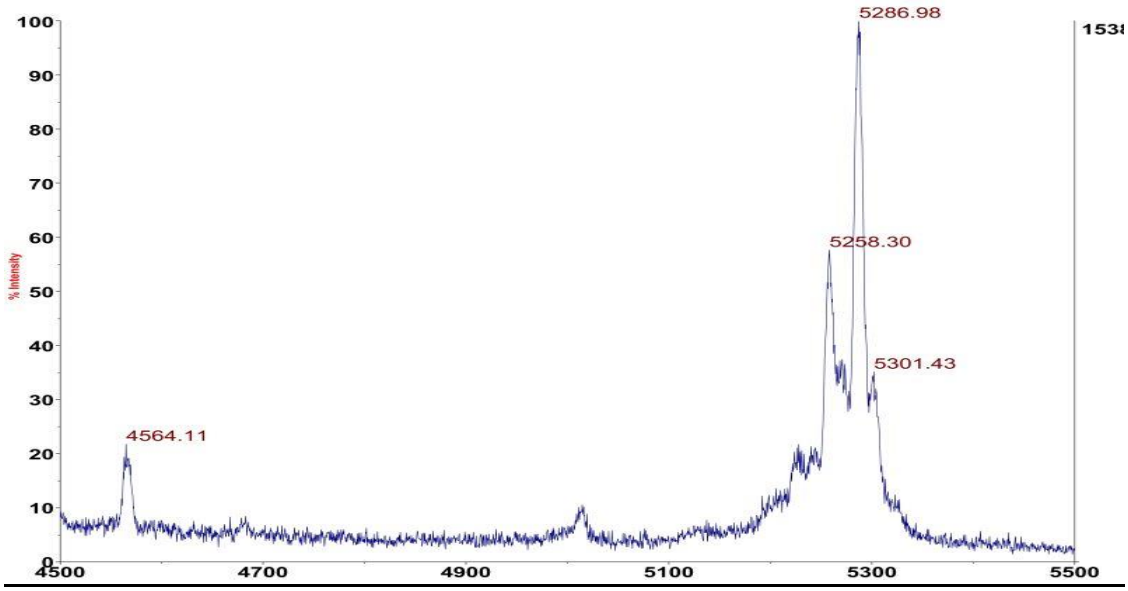


FIGURE 8: EXPECTED TO SEE A PEAK OF 5078.8 DA, BUT OBSERVED A PEAK AROUND 5286.98 DA

The spectrum of pNZ-*his-thnA thnB* 0.1 50% elution did show any significant peaks. The peak on the right most end of the spectrum seems to be double charged. The peak of interest was expected to be appeared between 4559.60 Da and 6519.80 Da. On the other hand, the spectrum results of pNZ-*his1-thnA thnB* 0.1 50% elution had a peak of 5286.98 Da. The peak is 208.18 Da heavier than the expected peak size of 5078.8 Da.

Discussion:

Aim of this experiment was to see the working of thnB enzyme in *Lactococcus lactis* using *thnA* as substrate. The mass spectrometry results didn't show presence of peptide of our interest, since most of the spectrum results were empty including the controls. But the presence of peak 5286.98 Da in pNZ-*his1-thnA thnB* 0.1 NT 50% elution and absence of any peaks in the control spectrum without thnB gene is interesting.

Nisin induction was done a second time due to the absence of any relevant peaks. The sequencing results of modified plasmids shows no sign of point mutation or mismatches. During the second induction, C18 tips were used for C18 column to perform desalting.

The production of proteins of biological interest in a host depends on several parameters such as production time, culture conditions and growth temperature. It is a good option to repeat the induction with some changes in the protocol. The main aim behind modification of Sactipeptide constructs by deleting partial terminator was to ensure the production of more *thnB* enzyme.

There is a possibility for the formation of disulfide bonds instead of thioether bridge (Grove et al., 2017). More studies to be done why the peptides are not being produced in the host (*Lactococcus lactis*) environment. Sequencing results confirmed that the desired constructs were created successfully without any mutations.

The presence of at least unmodified peptide in mass spectrometry will verify the plasmid modification was a success and the problem lie in the modification enzyme. Presence of peptides after expression of original constructs with partial terminator makes it difficult to reach a conclusion.

Conclusion:

In this project, the sactipeptide constructs were modified for expression in *Lactococcus lactis*. The mass spectrometry results didn't show any presence of peptide of interest, thurincin H, the sequencing result of modified plasmids confirmed that cloning was successful. Absence of any significant peaks could be due to one or more step gone wrong during purification. The peptide production and purification can be repeated with some modifications to the protocol.

Future Perspective:

The enzyme thnB and its activity is import because once we know how it works in *Lactococcus lactis* using its natural substrate thnA, we can use that modification in combination with other modification systems to create new to nature molecules. By using a different protein purification method and host, this experiment can be repeated for better yielding of peptides. The resulting peptides can be subjected to activity test. Also, it an option to try the expression of modified peptides in *E.coli.*

Literature:

- Arnison, P. G. (2012). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.*, *30*(1), 108–160. https://doi.org/10.1039/c2np20085f.Ribosomally
- Flühe, L., & Marahiel, M. A. (2013). Radical S-adenosylmethionine enzyme catalyzed thioether bond formation in sactipeptide biosynthesis. *Current Opinion in Chemical Biology*, *17*(4), 605–612. https://doi.org/10.1016/j.cbpa.2013.06.031
- Grove, T. L., Himes, P. M., Hwang, S., Yumerefendi, H., Bonanno, J. B., Kuhlman, B., … Bowers, A. A. (2017). Structural Insights into Thioether Bond Formation in the Biosynthesis of Sactipeptides. *Journal of the American Chemical Society*, *139*(34), 11734–11744. https://doi.org/10.1021/jacs.7b01283
- Igor, M., & Michiel, K. (2005). 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied Microbiology and Biotechnology*, 68: 705–717.
- Paul, M., Scott, E., Sungwon, Hwang., & Albert, A. (2016). Production of Sactipeptides in *Escherichia coli:* Probing the Substrate Promiscuity of Subtilosin A Biosynthesis. *American Chemical Society,* 11 (6), pp 1737–1744.
- Patricia, A., Manuel, M., Dongdong, Mu., & Oscar, k. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Applied Microbiology and*

Biotechnology, 100(7), pp 2939-2951. https://link.springer.com/article/10.1007/s00253-016-7343-9

- Wieckowski, B. M., Hegemann, J. D., Mielcarek, A., Boss, L., Burghaus, O., & Marahiel, M. A. (2015). The PqqD homologous domain of the radical SAM enzyme ThnB is required for thioether bond formation during thurincin H maturation. *FEBS Letters*, *589*(15), 1802–1806. https://doi.org/10.1016/j.febslet.2015.05.032
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