SUPPLEMENTARY- 2

MEASUREMENTS

Understanding HPLC and Its Selection for the Detection and Quantification of Iturin A

High Performance Liquid Chromatography (HPLC) is a technique that separates compounds (analytes) dissolved in a liquid, mobile phase by using their specific interaction with a stationary phase. It operates on the principle of affinity driven partitioning of compounds between the mobile phase and the stationary phase. The K value or partition coefficient for a particular compound is directly proportional to the affinity of the analyte to the stationary phase. On injection into the column, the compounds with a higher affinity and thus K value for the stationary phase are eluted more slowly than those with less as the interactions between said compound and the stationary phase hinder their movement.

HPLC is advantageous over other chromatographic techniques mainly due to its sensitivity and selectivity. HPLC can detect even trace amounts of the analyte, from nanograms to picograms. HPLC is also beneficial for its versatility as it can separate a variety of products ranging from polar and nonpolar compounds, acidic and basic compounds, and large and small molecules.

From literature surveys it was found that Reverse Phase HPLC is the standard method used for the identification, quantification and purification of Iturin A and its isoforms. The specifics of HPLC for Iturin A have been specified in various papers. However, the conditions to obtain a proper chromatogram for your sample through HPLC is extremely system specific, therefore, the conditions needed for our elution of Iturin A in our HPLC system had to be optimised. This is known as the method file construction and was done via multiple iterations.

Method File Construction

- Preparation of standard samples

    1) A 1:1 dilution series of pure Iturin A samples was prepared in the range 100µg/ml to 1.5625µg/ml. A dilution range with lower concentration values was preferred for the standard curve as the concentration of Iturin in our samples will be lower. The specifics of the preparation of the dilution series are shown in the table below:

Table 1: Dilution series of pure Iturin A

Sample Number	Concentration	Total volume	Iturin stock Volume	Methanol	Remaining
			(200 g/ml)	800	500
9	100	1 ml	500 µl	500	nil
8	50	1 ml	500 µl	500	nil
7	25	1 ml	500 µl	500	nil
6	12.5	1 ml	500 µl	500	nil
5	6.25	1 ml	500 µl	500	nil
4	3.125	1 ml	500 µl	500	nil
3	1.5625	1 ml	500 µl	500	500 µl
2	Blank - 1ml of crude methanol				
1	Blank - 1ml of HPLC grade methanol				

- Preparation of Instrument
    1) A C-18 column with 5µm pore size was fitted with a guard column at the bottom and the entire column was fitted into the oven of the HPLC machine.
    2) All the mobile phase solvents mentioned in literature– 0.1%TFA in water, acetonitrile and methanol – were prepared/obtained and ultrasonicated before connecting with the pumps.
    3) The instrument was washed with water and acetonitrile before starting with our method file development.
- HPLC runs to obtain the Ideal Method File
    1) 100µl of each sample from the Iturin dilution was added into the vials and loaded onto the HPLC autosampler.
    2) The concentration of 12.5 µg/mL was primarily selected for method development, as it represents a median value that is neither too high nor too low.
    3) Nine HPLC runs were performed using the 12.5µg/ml sample and three were performed using the 25µg/ml sample. The 25µg/ml was tested for three runs to check if the instrument is detecting only higher concentrations of Iturin A.
    4) The parameters were set on the computer system connected to the instrument.
    5) Various concentrations of methanol, acetonitrile and 0.1%TFA in water were tested for mobile phase.
    Note: The column is purged if the mobile phase is changed between each run. Before purging, the solvent is put through an ultrasonicator to remove any air bubbles invisible to the eye that may cause blockage in the column if not taken care of.
    6) Two flow rates were tested out – 0.8ml/min and 1ml/min.
    7) Various iterations of parameters were run to obtain the ideal method file.

A method file was developed with the following parameters as they provided optimal peak separation:

- 40% to 80% acetonitrile gradient
- 0.8 mL/min flow rate
- 25°C Temperature
- Pressure is a gradient as the mobile phase is a gradient.

The table below depicts the 12 runs conducted with the observation and inference learnt from each:

Table 2: Observations and inferences of various runs to finalize the specifications to achieve optimal peak separation

Run Number	Specification	Observation	Inferences
1.	Starting pressure =10.6MPa Pump B (ACN) CONCEN = 60 % Flow Rate = 0.8 ml/min Temp = 25°C	A high intensity cluster of peaks was eluted out in the first 3 to 4 mins A small peak at about 11 minutes	It is possibly methanol getting eluted. The mobile phase used was concentrated with a value of 60% ACN, the Iturin could've been eluted out all at once in

		2 more small peaks at 15 and 18 minutes.	the very beginning as it is quite soluble in ACN.
2.	Starting pressure = 13.7 MPa Pump B (ACN) = 40% Flow Rate = 0.8 ml/min Temp = 25°C	The peaks observed were similar to the trend in the previous run, however, the intensity of the peaks was lower than that of the first run.	The MP was not as concentrated as the first run, with just 40% ACN, which might have led to less interaction of Iturin with the mobile phase, thus giving us peaks of less intensity.
3.	0.1%TFA Pump B = 50% Flow Rate = 0.8 ml/min Temp = 25°C starting pressure = 12.3 MPa	The peaks were not eluted in any particular trend and were of very low intensity.	The sporadic peaks could be due to the sudden change in pH as an acidic solvent was used.
4.	Methanol - Water Pump B = 50% Flow Rate = 0.8 ml/min Temp = 25°C starting pressure = 25.9 MPa	Only one high intensity peak was observed in the beginning at the 5-minute mark.	This could be a result of isoforms not splitting due to higher solubility in methanol in comparison to ACN or this could have been methanol getting eluted out. This could've been methanol getting eluted out.
5.	Methanol - Water Pump B = 10-90% Flow Rate = 1.0 ml/min Temp = 25°C starting pressure = 26.7 MPa	A good cluster of peaks were observed between the 19th and 23rd minute when the methanol conc. was between 70% to 80% The peaks were not separated very well but six separate peaks were observed.	The Iturin A is eluting out at higher methanol concentrations. Therefore, the next run will be done in a gradient with a higher methanol concentration range of 50% to 90%
6.	Methanol - Water Pump B = 50-90% Flow Rate = 1.0 ml/min Temp = 25°C	The results were similar to the previous run however, the peaks started forming during the middle of the run and more peaks started to appear after 20mins. The peaks were still not well-defined.	In the next run, the reverse of this gradient will be tested to check if the change in interaction of the Iturin A with the mobile phase helps us get more separated peaks.

	Pressure gradient		
7.	Methanol - Water Pump B = 90% to 50% Flow Rate = 1.0 ml/min Temp = 25°C Pressure Gradient	More separated peaks were observed however the peaks were observed only in the beginning indicating that the elution of the solute happened early. Therefore, we concluded that 90% might be too high a methanol conc. for Iturin A, causing it to elute faster.	A gradient with a lower methanol concentration of 80% to 50% will be tested.
8.	Methanol - Water Pump B = 80% to 50% Time = 45mins Flow Rate = 1.0 ml/min Temp = 25°C Pressure Gradient	Some peaks were observed in the beginning, however, after that point, the peaks were not resolved. For this run, a 45 min run time was kept allowing the line to fully flatten out before starting the next run. This was done as in previous washing steps a lot of the solute was eluted during the wash which we assumed indicated that the solute required a longer run time to properly elute.	In the next run we will try a gradient of 50% to 80%. This gradient goes from more polar to less polar thus changing the interactions between the solute and mobile phase which might give us better results.
9.	Methanol - Water Pump B = 50% to 80% Flow Rate = 1.0 ml/min Temp = 25°C Pressure Gradient	Though peaks were eluted, resolved peaks were not obtained.	In the next run we will be switching solvents to acetonitrile as the methanol gradient was not yielding good results and an acetonitrile gradient has not been tried.
10.	ACN - Water Pump B = 50% to 80% Flow Rate = 0.8ml/min Temp = 25°C Pressure Gradient	Peaks were eluted early with only slight resolution.	The opposite gradient will be tried in the next run to obtain better resolved peaks.

11.	ACN - Water Pump B = 80% to 50% Flow Rate = 0.8ml/min Temp = 25°C Pressure Gradient	The peaks obtained were less resolved compared to the previous run.	Another acetonitrile gradient will be tested out in the next run.
12.	ACN - Water Pump B = 40% to 80% Flow Rate = 0.8ml/min Temp = 25°C Pressure Gradient	Resolved peaks of good intensity were obtained.	This was finalised as the method file.

Chromatograms

The following chromatograms were obtained because of our HPLC experiment:

Standard Curve

HPLC was performed for the following concentrations of pure Iturin to obtain an ideal standard curve: 100 µg/ml, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/ml.

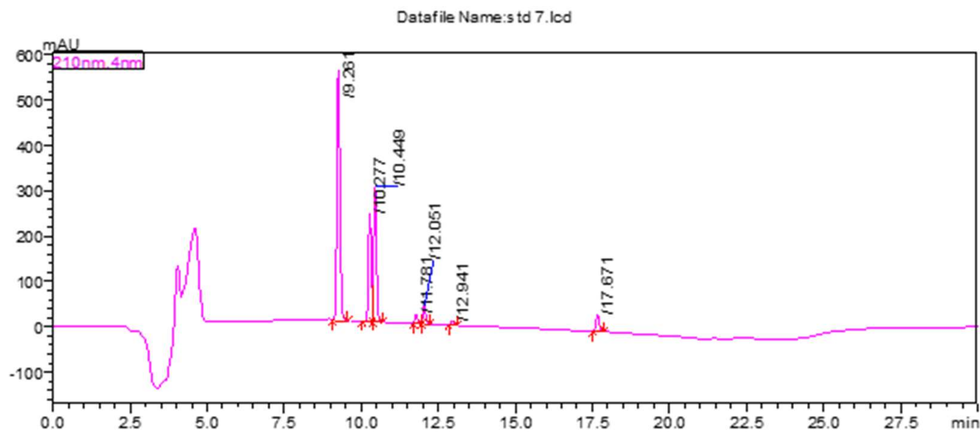

Figure 1: 100µg/ml Standard Curve

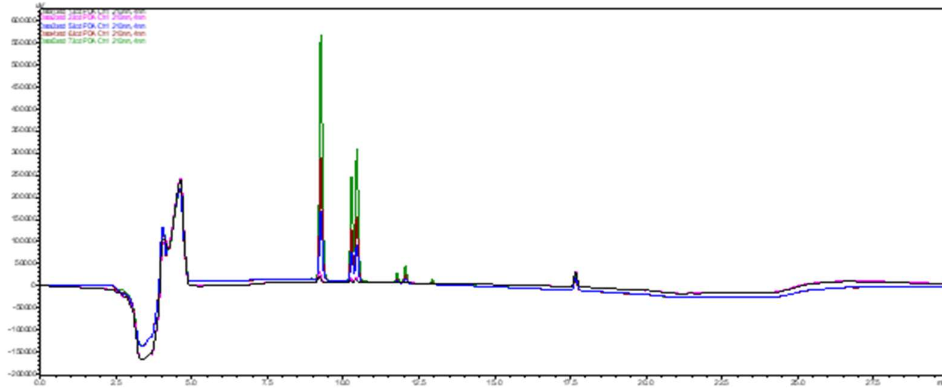

Figure 2: Cumulative standard curve incorporating the standard curve of each concentration of pure Iturin

Test samples:

- LB

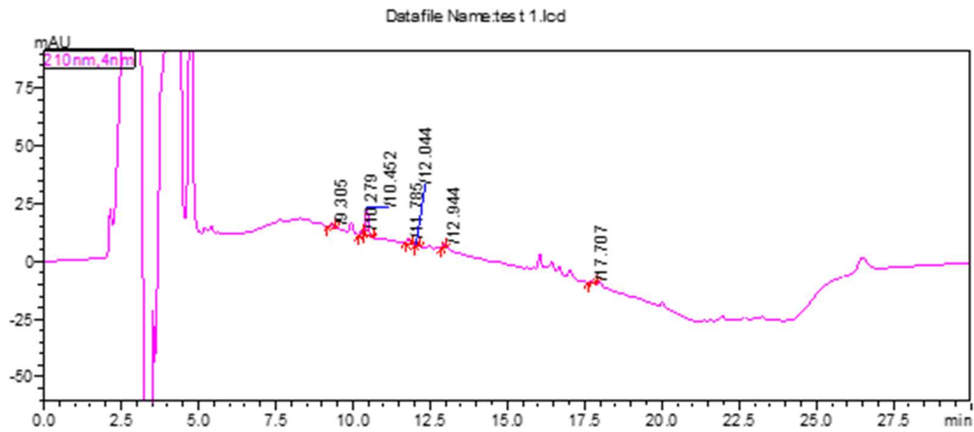

Figure 3: Graph showing Iturin peaks in our sample extracted from cells grown in LB

- PDB

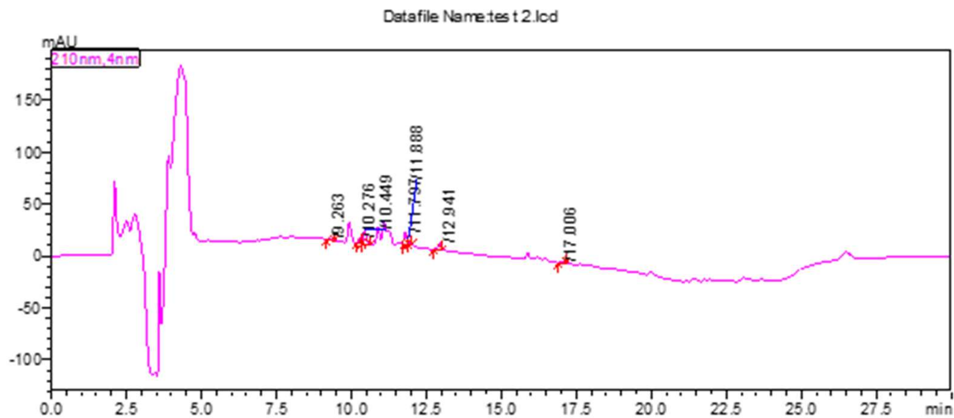

Figure 4: Graph showing Iturin peaks in our sample extracted from cells grown in PDB

Blank:

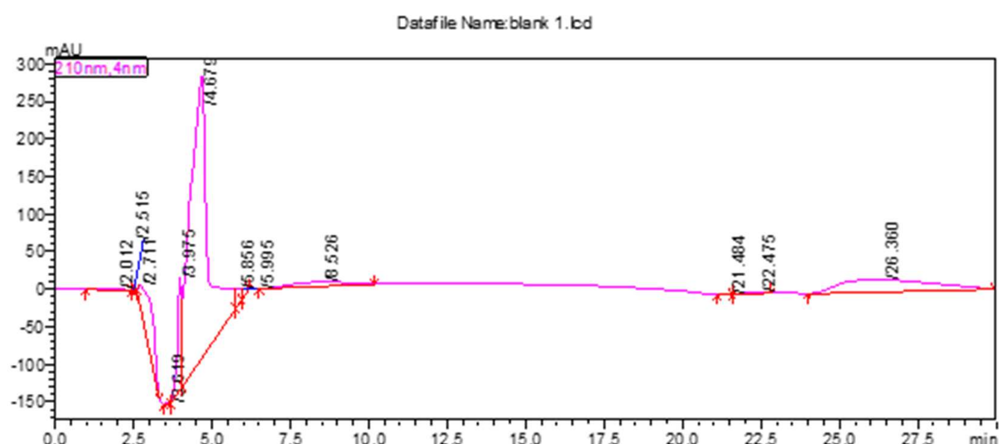

Figure 5: The graph shows the methanol peak, which was used as the blank since both our Iturin A standards and test samples were dissolved in methanol.

The blank helps us understand which peak is methanol and which peak is Iturin, thus helping us identify the right peaks. From the blank we were able to conclude that the initial cluster of peaks that eluted before the dead time were methanol peaks and therefore need not be considered.

Calculation of Area Under the Peaks

The table below depicts the area under each peak in each standard curve:

Table 3: Area under each peak in Standard Curve

Standard Conc. (µg/ml)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Cumulative Area
100	3876505	1532067	1833313	128625	232490	62592	271091	7936683
50	1937832	756545	951640	64932	116880	32414	188259	4048502
25	1074970	418753	517632	35919	64978	17703	207034	2336989
3.125	156370	58850	74599	5251	9038	2752	257202	564062
1.5625	86100	32015	41195	2942	4978	1236	243346	411812

The concentrations of 12.5 and 6.25 were not considered due to the following reasons:

- The 6.25µg/ml did not yield properly eluted or separated peaks.
- The volume of the 12.5µg/ml sample was exhausted in performing multiple HPLC runs to obtain the ideal method file. Therefore, there was not enough volume left to obtain properly eluted and separated peaks.

Baseline errors were subtracted while calculating the area under each peak.

The Standard Curve (Cumulative Area vs Concentration) obtained from the above data is as follows:

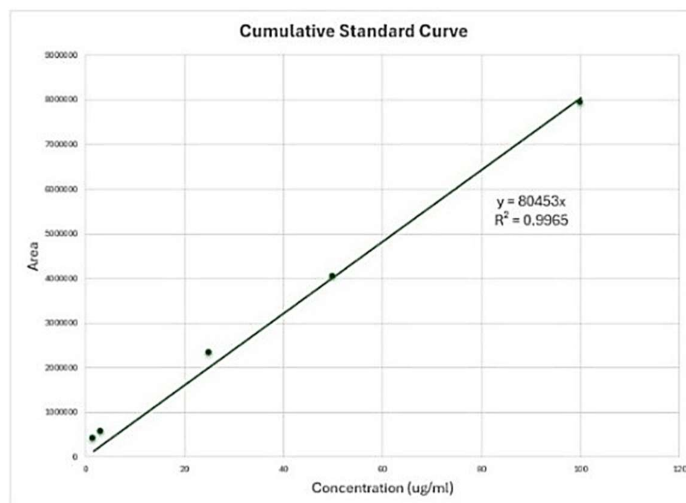

Figure 6: Standard Curve - Cumulative Area vs Concentration

The cumulative area for each test sample was obtained as follows:

Table 4: Cumulative area of test sample from LB media

LB	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Cumulative Area
Unknown	7196	9473	69523	13443	2835	9821	6174	118465

Table 5: Cumulative area of test samples from PDB media

PDB	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Cumulative Area
Unknown	14512	35229	82946	67258	14539	44585	9992	269061

Result

The concentration of Iturin A in each sample was calculated using the calibration curve as follows:

- LB

$$y = mx$$

$$y = 80453x$$

$$118465 = 80453x$$

$$x = 118465/80453$$

$$x = 1.472 \mu g/ml$$

The concentration of Iturin A present in the sample extracted from cells grown in LB is 1.472µg/ml.

- PDB

$$y = mx$$

$$y = 80453x$$

$$269061 = 80453x$$

$$x = 269061/80453$$

$$x = 3.344\mu g/ml$$

The concentration of Iturin A present in the sample extracted from cells grown in PDB is 3.344µg/ml.

Comparison of Test samples with the Standard samples

- Black depicts standard chromatogram
- Pink depicts test sample chromatogram

- **LB**

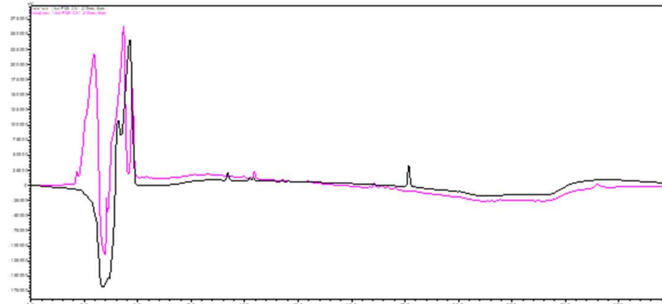

Figure 7: Comparison of the peaks between the extracted LB sample and the standard samples

- **PDB**

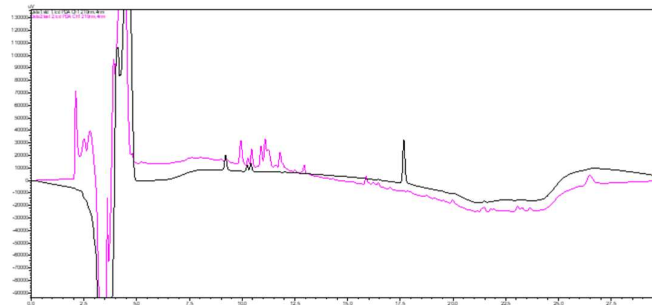

Figure 8: Comparison of the peaks between the extracted PDB sample and the standard samples

From the above chromatograms it is seen that the sample extracted from PDB produces higher peaks than the sample extracted from LB. This indicates that it contains a higher concentration of Iturin A which is reflected in our calculations.

From the above result we have quantified the amount of Iturin A produced by the natural promoter P_{itu}.

Further iterations of HPLC will be performed with varying purity levels of extracted Iturin to identify a constant concentration of Iturin produced by our strain (the amount of Iturin produced in different media will be different as demonstrated in our result above).

Notes:

- In our standard and samples, seven major peaks were identified as opposed to the eight peaks that are supposed to elute for the eight isoforms of Iturin A.
- For the calculation of Iturin A concentration, the isoforms of iturin A were not differentiated. The area under every peak was combined to get the final cumulative area as Iturin A is composed of all eight isoforms whose activities and properties are similar.

PROTOCOLS

Overexpression of Iturin A by Promoter Insertion by Homologous Recombination

I. Revival of Gene Fragments and Primers

AIM: To revive the DNA fragments, P_{bacA} and *P43* along with their primers for experimentation.

Procedure:

1. Add 100 µL of autoclaved Milli-Q water to the lyophilized gene fragments. (The volume of water to be added for the primers was determined in accordance with the primer data sheet provided)
2. Invert the tube a few times.
3. Vortex the sample for 1 second.
4. Centrifuge the sample at 11,000 rpm for 60 seconds (1 minute), then place it on ice immediately.
5. Add 25 µL of this mixture to 75 µL of Milli-Q water.
6. Place the mixture on ice.
7. Store the mixture at -20°C.

II. PCR

AIM: To amplify specific DNA fragments from *Bacillus subtilis* or other organisms using polymerase chain reaction for genetic analysis.

Procedure:

1. Reaction Setup:

- In a sterile PCR tube, add the following components:

- 1-2 µL of DNA template (10-100 ng)

- 1 µL of Forward primer (10 µM)

- 1 µL of Reverse primer (10 µM)

- 1 µL of dNTP mix (10 mM)

- 0.25 µL of Taq DNA polymerase (5 U/µL)

- 2.5 µL of PCR buffer (10X)

- 0.75 µL of MgCl2 (if not included in the buffer)

- Nuclease-free water to make the total volume up to 25 µL

2. Mixing:

- Gently mix the reaction by tapping the tube or using a pipette.

- Briefly spin down the PCR tube in a microcentrifuge to ensure the solution is at the bottom.

3. PCR Cycling Conditions:

- Place the PCR tubes into the thermal cycler and run the following program:

1. Initial Denaturation: 95°C for 2 minutes.
2. Denaturation: 95°C for 30 seconds.
3. Annealing: 50-65°C (depending on primer Tm) for 30 seconds.
4. Extension: 72°C for 1 minute per kb of target DNA.
5. Repeat steps 2-4 for 25-35 cycles.
6. Final Extension: 72°C for 5-10 minutes.
7. Hold: 4°C indefinitely.

4. Post-PCR Analysis:

- After the PCR is complete, take 5-10 µL of the PCR product and run it on an agarose gel.

- Stain the gel with ethidium bromide or SYBR Safe to visualize the DNA bands.

- Use a UV transilluminator to check the presence and size of the amplified fragments.

III. Homologous Recombination

AIM: to introduce a specific DNA sequence into the genome of *Bacillus subtilis* through homologous recombination for genetic modification.

Procedure:

Day 1
Streak out cells on appropriate selective media for single colonies.

Day 2
Use a single, fresh (no more than 18 hour old) colony to inoculate 1 mL of 1X MC tryptophan (trp) and phenylalanine (phe) with 3mM MgSO4 in a 15mL culture tube (900 µL H2O, 100 µL 10X MC, 3

µL 1M $MgSO_4$, 4 µL trp, and 4 µL phe).
Place on roller drum at 37°C for 4 hours.

At 4 hours prepare 5 mL culture tubes with 100µl of DNA (not concentrated). If the DNA has been concentrated add 50µl.

At 5 hours (1 hour after adding DNA) add 250 µL cells to each tube with DNA.

Roll all tubes (including no DNA control) for 2 hours. (One hour is often sufficient).
At this time pre-warm plates to 37°C.

Plate on LB media and visualize GFP.

Preparation of the required media and solutions:

10X MC

(50ml preparation)

- Add 5.35g of potassium phosphate dibasic.
- Add 2.6g of potassium phosphate monobasic.
- Add 10g of a carbon source (we added dextrose)
- Add 0.44g of sodium citrate dihydrate.
- Add 0.5ml of the prepared 2.2% stock of 1000X Ferric Ammonium Citrate.
- Add 0.5g of Casein Hydrolysate (Oxoid).
- Add 1.1g of potassium glutamate monohydrate.
- Add 50ml of MiiliQ water.

Further Instructions:

- Mix everything using half the final volume of water (we first mixed all the dry components with 25ml of MilliQ water).
- Once everything is dissolved adjust to the appropriate final volume.
- Filter sterilizes using screw cap filter and appropriately sized bottle.
- Distribute into 10mL aliquots (in 15mL conical tubes) using sterile technique.
- Label tubes “10X MC”.
- Store in door of −20°C freezer.

1000X Ferric Ammonium Citrate
(100 mL preparation – 2.2% stock)

- Add 2.2g of Ferric Ammonium Citrate
- Add ddH_2O to make up to 100 mL
- Filter sterilizes using screw cap filter and 125 mL bottle.
- Wrap in foil (as the salt is light-sensitive).

1M Mg (SO_4) solution

(1ml preparation)

- Add 0.3075g of Magnesium sulphate heptahydrate to 1ml of MilliQ water.

Tryptophane and Phenylalanine

- Prepare 1mg/ml stock solution.

IV. GFP Detection

AIM: To detect the expression of Green Fluorescent Protein (GFP) in genetically modified *Bacillus subtilis* or other organisms.

Procedure:

1. Place the genetically modified bacteria culture in a gel dock, G:Box Chemi XRQ
2. Configure the Gene Sys software for GFP visualization, ensuring the emission peak is set at 509 nm (emission wavelength of GFP).
3. Visualize the result and capture the image in the software.

Acknowledgements

I. Homologous Recombination and Growth curve - Dr Sabari Sankar Thirupathy, Assistant Professor Grade I (Biology), IISER Trivandrum.

RESULTS

1) Gram Staining

Bacillus subtilis ATCC 13952 cells were gram stained and violet colour cells with rod-like morphology was observed.

The bacteria were confirmed to be gram positive *Bacillus subtilis*.

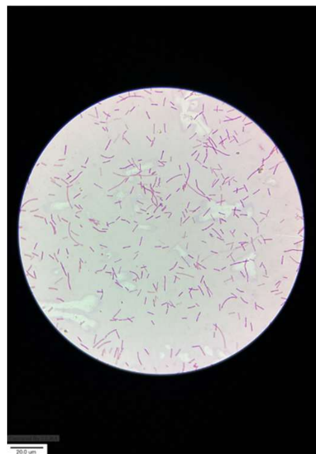

Figure 9: Gram-staining-100x

2) Hot Spore Staining

As *Bacillus subtilis* is a spore forming bacteria, the bacteria were stained to visualize the endospores. An overgrown culture was used for this stain as bacteria tend to form spores under stress such as lack of nutrients. Endospore formation was observed.

Faint green endospores were visualized which proved the spore forming nature of our bacteria.

3) Indole Test
Spot indole test was conducted and a yellow colour was observed.

A negative indole test confirmed that the organism cannot breakdown tryptophan to produce indole thus categorizing it with most other *Bacillus* species.

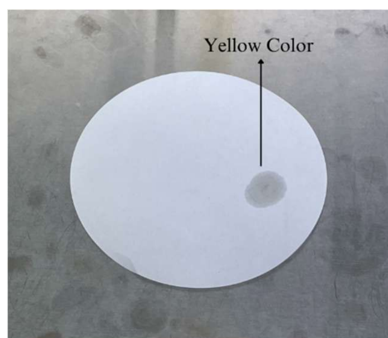

Figure 10: Indole Test

4) MR-VP Test

Methyl Red test: Negative

The test showed no colour change

Voges-Proskauer: Positive

The test showed yellow colour with no colour change.

The bacteria does not produce stable acidic end products from glucose fermentation and uses butylene glycol pathway to produce neutral end products like actoin. This confirms that the bacteria are *Bacillus subtilis.*

Figure11: Methyl Red (MR) Test

Figure 12: Voges-Proskauer (VP) Test

5) Oil Emulsification Test

Upon addition of 100 µl of broth onto the plate containing petrol, the Biosurfactant activity of the Iturin A in the broth created a zone of clearance was created due to the interaction.

100µl of broth supernatant, Extracted Iturin A and pure Iturin A was added to each test tube containing water and petrol and were shaken for 5 minutes and observed for emulsification.

All these tubes showed emulsification with highest activity from the Pure Iturin A sample and least in the test tube containing the broth supernatant.

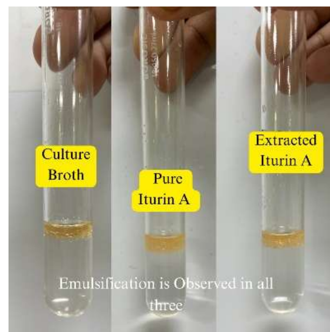

Figure 13: Oil Emulsification Test

6) Growth Curve

24-hour growth curve was first experimented with an inoculation volume of 100 µl of broth. However, the growth curve observed was not very distinct with respect to the various stages of growth. Diauxic growth curve was observed in the 24-hour growth as well.

Upon consultation with Dr. Dr Sabari Sankar Thirupathy, we obtained the optimized protocols to add the 0.7 OD culture in a 1:1000 dilution factor and run the growth curve for 6 hours. However, there was hardly any growth in the medium for the first 4 hours and hence the graph plotted was inconclusive.

Our 4th trial based on 1:500 dilution and a 12-hour long growth, gave a distinct and accurate graph.

The bacteria showed Diauxic growth initially due to the presence of multiple sugars in the LB medium. Upon exhaustion of these sugars, we observed an exponential phase spanning over 3 hours and then proceeded into stationary phase.

Bacillus subtilis growth aligns with the various growth curves as mentioned in many literatures.

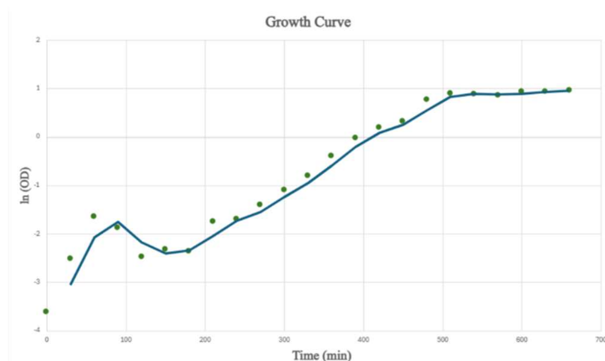

Figure 14: 12-Hours-Growth-Curve

7) Iturin A Extraction from LB (Luria-Bertani) media and PDB (Potato Dextrose Agar) media

Iturin A was extracted from two sets of growth media – LB and PDB giving us a comparative analysis of which medium is better for extraction; this was analyzed using HPLC.

The total volume of extracted sample was 8ml in methanol which was reduced to 4 ml upon filtration.

Figure 15: Iturin A Extraction, Centrifuged pellets

8) HPLC (High Pressure Liquid Chromatography) of Extracted Iturin A and Pure Iturin A

Standard of pure Iturin A sample was first run at variable concentrations ranging from 100µg/ml to 1.5625µg/ml and multiple peaks of Iturin A isomers were obtained. 7 isomer peaks were observed.

A standard calibration curve was made by calculating the cumulative area of all the peaks at various concentrations and were observed to be linear.

LB gave sharper peaks due to lesser media complexity in terms of the various elements present in the media like beef extract, peptone etc. as compared to PDB which is highly complex in its composition leading to unnecessary peaks or impurities.

Despite the lesser sharper peaks of the Iturin A was extracted in PDB and the quantity of extraction was greater than in LB as more area was calculated in PDB as compared to LB.

Using the calibration curve, the concentration of the crude extracted Iturin A was estimated. The extract's concentration was low due to high dilution in methanol during the extraction and filtration process. Giving a total concentration of 1.472 µg/ml in LB and 3.344µg/ml in PDB, respectively.

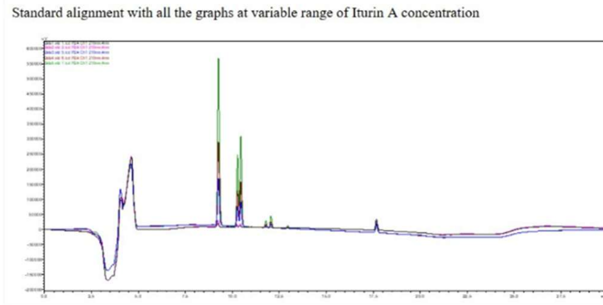

Figure 16: Standard Alignment

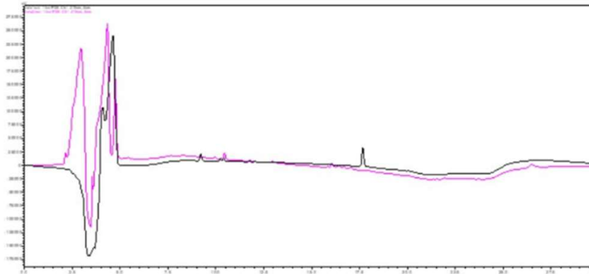

Figure 17: Standard vs LB-extracted-iturin-A

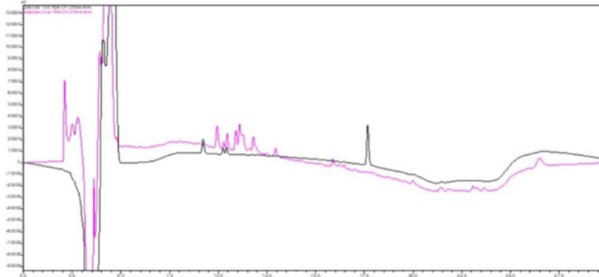

Figure 18: Standard vs PDB-extracted-iturin-A

In the above-mentioned images the Black color graph is the standard while the purple graph is the test graph.

9) Antifungal Assay

Unfortunately, there was no zone of clearance observed due to the culture being too overgrown. The experiment will be repeated on a freshly cultured fungal lawn to test the antifungal activity of Iturin A and the extracted Iturin A samples.

10**) Fragment Amplification**

The fragments were amplified, and our results were as follows:

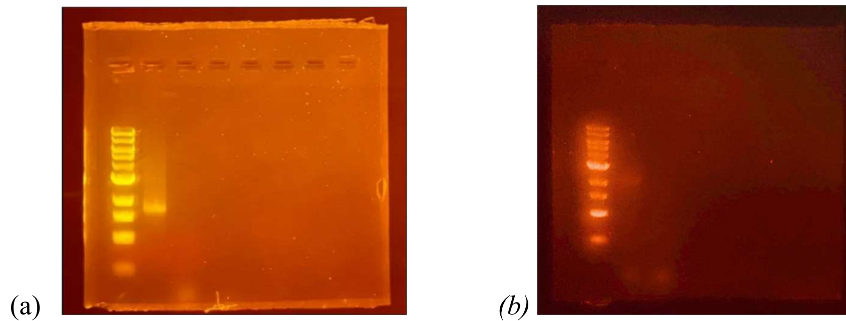

Figure 19: Amplified Fragments (a) P_{bacA} (b) P43

The amplification results demonstrate that the P_{bacA} fragment was successfully amplified, while the *P43* fragment produced only a faint band. This can be attributed to the suboptimal performance of the primers designed for the *P43* fragment, which was affected by the following factors:

1. Many of the primer combinations formed hairpin structures and primer dimers.
2. The ΔG (Gibbs free energy) values of the primers were slightly lower than the optimal range.

The most favorable primer combination was selected to move our project forward. However, it remained suboptimal and resulted in weak amplification.

The primers used are specified in the table below:

Table 1: Primer Data Sheet

Fragment	Primer	Primer Sequence (5’-3’)	Number of Bases	GC Content	Tm value (°C)
P_{bacA}	Forward	CGCCGCTTACAAGTGTAAC	19	55	59.3
	Reverse	GACGGTTTTCAAGGAATTTACG	22	41.7	59.3
P43	Forward	CACAGCTTGCCGGTGTC	17	43	58
	Reverse	GGGGGCTTCACAATGATTTATG	23	45.5	58

11) Homologous Recombination (HR)

HR was performed using protocols obtained from Dr. Sabari Sankar Thirupathy, a professor at IISER Trivandrum. These protocols were optimized for our lab, and all precautionary measures were taken before experimenting to ensure the best results.

Our selectable marker was GFP, and this was visualized in the transformed colonies at an emission wavelength of 509 nm using a Gel dock.

The HR done with our P_{bacA} fragment yielded successful results with a clear demarcation of green fluorescent colonies, whereas *P43* did not yield successful results.

The results are as follows:

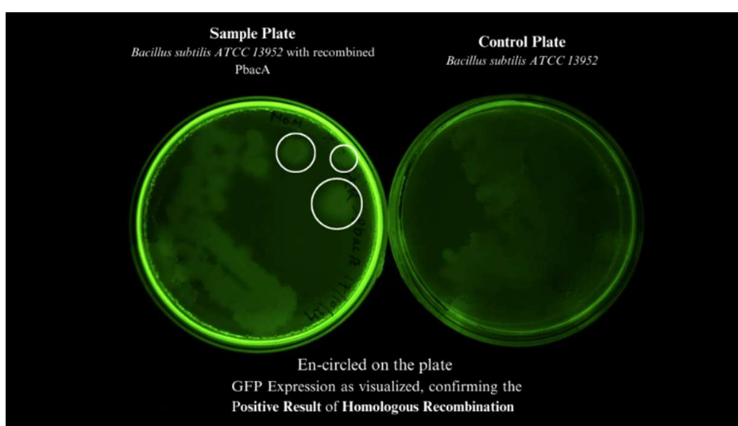

Figure 20: Homologous Recombination GFP result - P_{bacA}

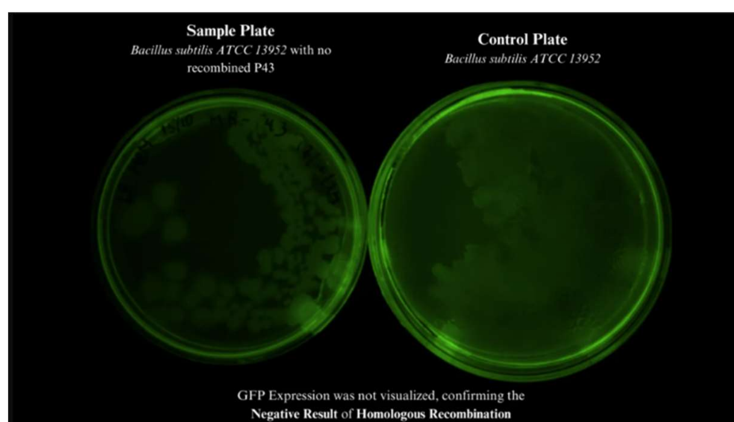

Figure 21: Homologous Recombination GFP result - P43

What we learnt from the HR experiments we conducted are as follows:

1. The negative HR result for our *P43* fragment was due to its low amplification even after several tries.

2. Potassium L-glutamate monohydrate is a component that increases the efficiency of HR. The amount we added to the HR media (1X MC) was insufficient, and by using a higher concentration, we could have potentially increased the probability of HR occurring and observed more fluorescent colonies.

LAB SAFETY

Our team places utmost importance on safety and security, prioritizing the well-being of our laboratory members, the integrity of our experiments, and environmental protection. With strong institutional backing, we have embraced our responsibility to maintain strict biosafety standards. Every experiment was carefully planned and executed with a focus on ethical practices, ensuring no harm to the environment or us. To ensure full transparency and accountability, we consulted extensively with our principal investigator before proceeding with any experiments, performing them only after receiving her approval. This approach has allowed us to uphold the highest levels of safety throughout our project.

1. Laboratory Overview

The wet lab members of our team conduct experiments in the Department of Biotechnology's Project Lab, which is designated as Biosafety Level 2 (BSL-2). Our laboratory adheres to the biosafety and biosecurity guidelines outlined by the Indian government and the Centre of Bioethics at Manipal Academy of Higher Education (MAHE).

2. Training and Risk Assessment

All members of the wet lab subsystem have received extensive training in safe laboratory practices from our Principal Investigator (PI), Dr. Ritu Raval, and our advisors prior to commencing work in the lab. This training includes:

- Risk Assessment: An assessment of the reagents in use.
- Waste Management: Proper identification, management, and safe disposal of biological and chemical hazardous waste and lab consumables.
- Equipment Familiarization: Training on the functioning and safe usage of all available equipment, including centrifuges, UV-visible spectrophotometers, and autoclaves.

3. Safety Equipment and Emergency Protocols

Lab members have been made familiar with:

- Emergency Equipment: The locations of safety showers, eyewash stations, first aid kits, fire extinguishers, and emergency evacuation routes.
- Biosafety Cabinet Training: Training sessions conducted by advisors and PhD scholars Mr. Rajesh M. Gowda and Mrs. Atheena PV on working within the biosafety cabinet to avoid contamination.

4. Safety Practices in the Laboratory

The following safety practices are strictly enforced within the laboratory:

- Prohibitions: Eating and drinking are strictly prohibited in the lab.

- Personal Protective Equipment (PPE): All members are required to wear lab coats, protective goggles, and nitrile gloves as necessary while working.
- Sterilization: Proper sterilization protocols are followed before working in the BSL-2 biosafety cabinet.
- Equipment Maintenance: All laboratory equipment, including centrifuges, spectrophotometers, cold storage units, and autoclaves, are handled with care and regularly maintained.
- Fumigation: The laboratory is fumigated every 3-4 weeks for additional safety.

5. Use of Harmful Reagents and Procedures

While some experimental procedures involve harmful chemicals and reagents, all necessary safety measures are taken to prevent any harm. Below are the details of specific hazardous chemicals and their implications:

a. Human Health and Safety Hazards:

- Highly Flammable Chemicals:
- Ethyl Alcohol: Used as a solvent and for sterilization; poses a fire hazard during storage and handling.
- Isopropanol: Also, highly flammable and used for sanitation; must be stored away from fire sources.

b. Acids and Corrosive Chemicals:

- Hydrochloric Acid: Strong acid that can cause severe burns; risk of skin, eye, or respiratory irritation during buffer preparation or pH adjustments.

c. Other Toxic Chemicals:

- Iturin A: Maximum of 5 mg used; negligible toxicity at low concentrations.
- Acetonitrile: Extremely toxic; used as a solvent for HPLC. Inhalation can cause severe symptoms, while ingestion is highly unlikely.

d. Environmental Hazards

- *Hemileia vastatrix*: Pathogenic spore-forming fungus; potential risk of spores dispersing outside the lab.
- *Candida albicans*: Can contaminate surfaces and enhance resistance to antifungal agents.

6. Antifungal Safety Assessment

Iturin A is not a hazardous substance or mixture according to Regulation (EC) No 1272/2008 (Sigma Aldrich) [4]. A preclinical safety assessment of the consumption of Iturin A conducted acute (7 days, 5000mg/kg BW) and subacute (28 days, 200mg/kg BW) toxicity tests through daily intragastric administrations of the stipulated amounts. The results showed no significant damage to the small intestine, liver or kidney. Another study compared the effect of Iturin A in comparison to amphotericin B in treating 24-hour old *Candida albicans* infections via intraperitoneal injections. Initial clinical trials on animals and humans showed that iturin A was effective as an antifungal, with low allergic effects and no discernable toxic effects from topical applications.

In the case of any overdose on Iturin A, potential treatments can be provided before permanent damage occurs through adding magnesium ions to or raising cholesterol levels in the bloodstream to inhibit Iturin A's activity.

Iturin does not affect *D. melanogaster* (common fruit fly), honeybees and other organisms commonly found in the coffee microbiota, and it causes no harm to coffee plantations. It is larvicidal to *Aedes aegypti* mosquito larvae.

7. Regulatory Compliance

Our project complies with the following regulations:

- The Environment (Protection) Act, 1986: Provides guidelines for disposal of synthetic biology-related products and handling of hazardous substances.
- Rules for the Manufacture, Use/Import/Export, and Storage of Hazardous Micro-Organisms/Genetically Engineered Organisms or Cells (Rules 1989): Oversee safe practices in experimentation and waste disposal.

8. Laboratory Facilities and Safety Equipment

We have access to the following facilities:

- BSL-1 Laminar Air Flow Hood
- BSL-2 Biosafety Cabinet
- Chemical Fume Hood
- Emergency installations include eyewash stations, full-body showers, and fire extinguishers to minimize risks to lab members.

9. Waste Disposal and Inactivation

Waste management protocols include:

- Waste Segregation: Different colored bins for general waste, plastic/gloves, tissue paper, and glass syringes/needles.
- Liquid Chemical Disposal: Reserved containers for liquid chemicals.
- Decontamination Procedures: Used media and glassware are carefully packaged and autoclaved. Lab technicians ensure proper disposal and cleaning before reusing glassware.

References:

1. M. K. Gupta et al., "A comparative review on High-Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC) & High-Performance Thin Layer Chromatography (HPTLC) with current updates," Current Issues in Pharmacy and Medical Sciences, vol. 35, no. 4, pp. 224–228, Dec. 2022, doi: 10.2478/cipms-2022-0039.
2. H. Calvo, I. Mendiara, E. Arias, D. Blanco, and M. E. Venturini, "The role of iturin A from B. amyloliquefaciens BUZ-14 in the inhibition of the most common postharvest fruit rots," Food Microbiol, vol. 82, pp. 62–69, Sep. 2019, doi: 10.1016/j.fm.2019.01.010.
3. F.-C. Hsieh, T.-C. Lin, M. Meng, and S.-S. Kao, "Comparing Methods for Identifying Bacillus Strains Capable of Producing the Antifungal Lipopeptide Iturin A," Curr Microbiol, vol. 56, no. 1, pp. 1–5, Jan. 2008, doi: 10.1007/s00284-007-9003-x.
4. J. Yuan, W. Raza, Q. Huang, and Q. Shen, "Quantification of the antifungal lipopeptide iturin A by high performance liquid chromatography coupled with aqueous two-phase extraction," Journal of Chromatography B, vol. 879, no. 26, pp. 2746–2750, Sep. 2011, doi: 10.1016/j.jchromb.2011.07.041.
5. "Iturin | Sigma-Aldrich." Accessed: Sep. 08, 2024. [Online]. Available: https://www.sigmaaldrich.com/IN/en/search/iturin?focus=products&page=1&perpage=30&sort=relevance&term=iturin&type=product

6. H. Zhao et al., “Potential of iturins as functional agents: safe, probiotic, and cytotoxic to cancer cells,” Food Funct, vol. 9, no. 11, pp. 5580–5587, Nov. 2018, doi: 10.1039/C8FO01523F.
7. G. Dey et al., “Pre-clinical risk assessment and therapeutic potential of antitumor lipopeptide ‘Iturin A’ in an in vivo and in vitro model,” RSC Adv, vol. 6, no. 75, pp. 71612–71623, 2016, doi: 10.1039/C6RA13476A.
8. S. Lei et al., “Capability of iturin from Bacillus subtilis to inhibit Candida albicans in vitro and in vivo,” Appl Microbiol Biotechnol, vol. 103, no. 11, pp. 4377–4392, Jun. 2019, doi: 10.1007/S00253-019-09805-Z/FIGURES/10.
9. J. P. CLAIRBOIS and L. DELCAMBE, “[Clinical and biological trials on iturin, a new antifungal drug].,” Arch Belg Dermatol Syphiligr, vol. 14, no. 1, pp. 63–82, Mar. 1958.
10. S. BLOCQUIAUX and L. DELCAMBE, “[Treatment of dermatomycoses with iturin].,” Arch Belg Dermatol Syphiligr, vol. 12, no. 2, pp. 224–7, Sep. 1956.
11. F. Besson, F. Peypoux, G. Michel, and L. Delcambe, “Mode of action of iturin A, an antibiotic isolated from Bacillus subtilis, on Micrococcus luteus,” Biochem Biophys Res Commun, vol. 81, no. 2, pp. 297–304, Mar. 1978, doi: 10.1016/0006-291X(78)91532-2.
12. M. J. Quentin, F. Besson, F. Peypoux, and G. Michel, “Action of peptidolipidic antibiotics of the iturin group on erythrocytes,” Biochimica et Biophysica Acta (BBA) - Biomembranes, vol. 684, no. 2, pp. 207–211, Jan. 1982, doi: 10.1016/0005-2736(82)90007-4.
13. L. Assié et al., “Insecticide activity of surfactins and iturins from a biopesticide Bacillus subtilis Cohn (S499 strain),” Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, vol. 67, no. 3, pp. 647–655, 2002, Accessed: Oct. 01, 2024. [Online]. Available: https://orbi.uliege.be/handle/2268/4444
14. G. Hertlein et al., “Biological Role of Paenilarvins, Iturin-Like Lipopeptide Secondary Metabolites Produced by the Honeybee Pathogen Paenibacillus larvae,” PLoS One, vol. 11, no. 10, p. e0164656, Oct. 2016, doi: 10.1371/JOURNAL.PONE.0164656.
15. K. Revathi, R. Chandrasekaran, A. Thanigaivel, S. A. Kirubakaran, S. Sathish-Narayanan, and S. Senthil-Nathan, “Effects of Bacillus subtilis metabolites on larval Aedes aegypti L,” Pestic Biochem Physiol, vol. 107, no. 3, pp. 369–376, Nov. 2013, doi: 10.1016/J.PESTBP.2013.10.005.
16. Double-strand break repair in bacteria:
Kang, Y., & Blaser, M. J. (2011). Double-strand break repair in bacteria: A view from *Bacillus subtilis*. *FEMS Reviews*, 35(6), 1055-1070. https://doi.org/10.1111/j.1574-6976.2011.00270.x
17. Bacillus subtilis genome editing using ssDNA:
Datta, S., & Bhan, A. (2011). *Bacillus subtilis* genome editing using ssDNA with short homology regions. *Journal of Biological Engineering*, 7(29). https://doi.org/10.1186/1754-1611-7-29
18. Metzker, M. L., & Caskey, C. T. (2009). Polymerase chain reaction (PCR). In *Major Reference Works*. Wiley Online Library. https://doi.org/10.1002/9780470015902.a0000998.pub2
19. iGEM MIT-MAHE. (2023). *MIT- MAHE Revival protocol*. Retrieved from https://2023.igem.wiki/mit-mahe/experiments.