ISOLATION, PRODUCTION, AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM *LACTOBACILLUS SPP.*

Submitted in partial fulfillment of the
requirements for the degree of

Bachelor of Technology
In
Biotechnology

By

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Vellore Institute of Technology
(Deemed to be University under section 3 of UGC Act, 1956)

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO SCIENCES AND TECHNOLOGY

June 2020

DECLARATION

I hereby declare that the thesis entitled "**Isolation, Production and Characterization of bioactive peptides from *Lactobacillus* spp.**" submitted by me, for the award of the degree of B.Tech., in Biotechnology to VIT University, is a record of bonafide research work carried out by me under the supervision of Dr. Subathradevi. C

I further declare that the work reported in this thesis has not been submitted and will not be submitted, either in part or full, for the award of any other degree or diploma in this institute or of any other institute or university.

Place: VIT Vellore

Date: 08/06/2020

Signature of the Candidate

CERTIFICATE

This is to certify that the dissertation entitled "**Isolation, Production and Characterization of bioactive peptides from *Lactobacillus* spp.**" submitted by Sathyanarayanan.V (16BBT0255) to the VIT University, for the award of the degree of B.Tech., in Biotechnology, is a record of bonafide work carried out by him under my supervision, as per the VIT code of academics and research ethics.

The contents of this report have not been submitted and will not be submitted either in part or in full, for the award of any degree or diploma in this institute or any other institute or university. The thesis fulfills the requirements and regulations of the University and in my opinion meets the necessary standards for submission.

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THESIS APPROVAL FORM

This thesis, entitled

ISOLATION, PRODUCTION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM LACTOBACILLUS SPP.

and authored by Sathyanarayanan.V (16BBT0255), is hereby accepted and approved

External Examiner(s) Head of Department Dean SBST

ABSTRACT

Bioactive peptides are multifunctional as they can have different functions such as anti-inflammatory, antihemolytic, antioxidant, antimutagenic, ACE inhibitory and antimicrobial. These bioactive peptides are identified and isolated from 4 strains of Lactic acid bacteria (LAB) *that are Lactobacillus acidophilus, fermentum, plantarum* and *VITSE07*. In this study, the bioactive peptides are screened for antimicrobial, and ACE inhibitory properties. It was found that *Lactobacillus plantarum* from MRS medium has the highest ACE inhibitory and antimicrobial activity due to its consistent zone of inhibition but only targeted specific pathogens while the *Lactobacillus spp*. from casein medium didn't have antimicrobial peptides due to absence of zone of inhibition. The *Lactobacillus plantarum* from MRS medium was found to show this activity at the 71st h of the growth curve for both ACE Assay and Antimicrobial assay. The growth kinetics for *Lactobacillus plantarum* was taken till 71st h and it still hadn't reached death phase of the growth curve. This could be due to the high doubling time, which means slower growth and more consistent production. However, it isn't optimal for bacterial growth due to more time into production. This suggested the potential for production over 71st h of the growth kinetics and which could have higher activity of the biological functions of the peptides. In Antimicrobial assay from MRS medium, Lactobacillus plantarum had the highest and most consistent inhibitory activity among all the cultures at 71st hour of growth kinetics and the average zone of inhibition for *Lactobacillus plantarum* at the 71st hour was 0.6 x 0.7 cm. In ACE assay, *Lactobacillus plantarum* has the highest ace inhibition at OD of 0.082 with 182% inhibition, *Lactobacillus fermentum* has the highest ace inhibition at OD of 0.253 with 105% inhibition, Captopril control has the highest ace inhibition at OD of 0.143 with 168% inhibition.

ACKNOWLEDGEMENT

I would like to use this opportunity to express my deepest gratitude to Dr. Subathradevi C, Ph.D. Scholars, and lab assistants for helping and guiding me throughout my project. This experience improved my understanding and complimented my theoretical knowledge with practical skill.

I also thank the HOD and coordinators of SBST for arranging the reviews with notion for each student. I also thank our caterer, hostel office, and my friends, because they played a big role in my life every day which helped me progress even better in my project.

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I look at this opportunity as a big milestone in my educational development. I will strive to use the attained skills and knowledge in the best possible way, and will continue to work on their improvement, to attain my desired career objectives.

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SYMBOLS & NOTATIONS

spp.	Species
ACE	Angiotensin Converting Enzyme
µl	Microlitre
L.B	Lactobacillus
OD	Optical Density
w/o	without
LP	Lactobacillus plantarum
LF	Lactobacillus fermentum
LA	Lactobacillus acidophilus
L07	Lactobacillus VITSE07
HHL	Hippuryl-L-Histidyl-L-Leucine
MRS	De Man, Rogosa and Sharpe agar
MHA	Mueller-Hinton agar
h	Hours
min	Minutes

CHAPTERS

1. INTRODUCTION

Protein is an organic compound made up of amino acids which are considered as the material basis of life as all life and life events have protein participation. Food proteins are studied nowadays due to their effective impact on human health based on the specific sequences encrypted into the native protein, called bioactive peptides (BAPs) (Koshland & Haurowitz, 2020).

These specific sequences are usually inactive when present in the parental protein but can be released after hydrolysis of the protein during gastrointestinal digestion, in vitro enzymatic hydrolysis, or microbial fermentation (Hernández-Ledesma, Amigo, Ramos & Recio, 2004).

Bioactive peptides also shortened to BAPs have interesting biological functions such as angiotensin-converting enzyme (ACE) inhibition, mineral binding, antidiabetic, or antimicrobial activities (Raveschot et al., 2018).

In every bacteria, there is bacteriocins which are basically peptides and they have biological functions, these peptides are released from the bacteria during a period of their growth. Using growth kinetics, this phase of release of peptides can be found. Usually, it is based on doubling time of the bacteria. If the doubling time is high then late release of peptides, and if the doubling time is low then early release of peptides. Every bacteria and its corresponding strain can have different doubling time, it can be based on the medium of growth, its sub-cultured medium or even its specificity for growth.

Industrial applications are emerging such as the production of functional foods or the production of peptides to serve as active ingredients for pharmaceuticals or dietary supplement products.

Bioactive peptides (BAPs) research is a unique field of research with high potential as it has ACE inhibitory properties as this property reduces blood pressure and has many applications as a remedy for cardiovascular diseases (CVD) (Ebrahimi, Ai, Alizadeh & Shariaty, 2017; Pihlanto, Virtanen & Korhonen, 2010; quan, Tsuda & Miyamoto, 2008).

As this research in this field is still incomplete, we cannot correlate the structure with the function of the bioactive peptides. However, we know that the structure of the bioactive peptides is between 2-20 amino acids in length and they have hydrophobic amino acids like proline, lysine, or arginine groups (Koshland & Haurowitz, 2020).

Bioactive peptides are considered as the new generation of biologically active regulators that can prevent oxidation and microbial degradation. Hence, it can be used in the food industry to increase the shelf life of food. They can also be used to prevent certain diseases, as from this study, we observed that they showed antimicrobial properties only to specific pathogens unlike general antibiotics (Raveschot et al., 2018).

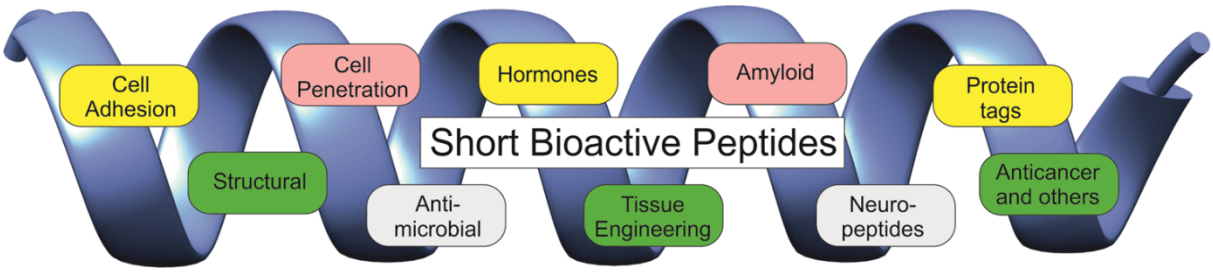

Figure 1. Classes of bioactive peptides (Hamley, 2017).

The bioactive peptides are amino acid sequences that have biological functions. The number of functions is based on the length of the peptides. If they are long peptides, then they will have multiple biological functions and short peptides that are less than 5 kDa will only have one function. Short peptides are more useful as a biomaterial for products as they have uniform functions (Agyei, Ongkudon, Wei, Chan & Danquah, 2016).

2. SURVEY OF LITERATURE

Title of Paper	Journal and Year	Description
I. Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific *Lactobacillus plantarum* strains	® American Dairy Science Association ®, 2017.	The milk-derived peptides from *Lactobacillus Plantarum* have only single activity, and those peptides under 3 kDa had even higher activity in anti-inflammatory of 2112.3 & 1631 µg/mL of DS (sodium diclofenac as reference drug). In anti-hemolytic the activity was higher from 3-10kDa than <3kDa, and antioxidant activities had higher activity in <3kDa of 234.1µmol of Trolox equivalents and 3-10 kDa had 210 µmol of Trolox equivalents. The reason why the peptides show the higher activity of biological functions in different kDa is still unknown.
II. Isolation and identification of novel casein-derived bioactive peptides and potential functions in fermented casein with *Lactobacillus helveticus*	Food Science and Human Wellness 8, 2019	The journal provides an effective method of isolation, purification, identification of intracellular and extracellular peptides. *Lactobacillus helveticus* was incubated for 7 h in casein medium and they subjected to extraction of both intracellular and extracellular peptides. They used LC MS/MS for characterization of the bioactive peptides. About 241 peptide sequences were identified from casein and *lactobacillus helveticus* proteins

			which were further subdivided by their biological functions. They also found that in both intracellular and extracellular peptides there was the recurring sequence 'DELQDKIHPF'. DELQ sequence is said to aid immune system activity. The concentration of the recurring sequence was 23.1 ng/mL in extracellular peptides and 9.76 ng/mL in intracellular peptides which was quantitively analyzed by Ultra Performance Liquid Chromatography. This work provides useful information that can help improve and aid further research in bioactive peptides.
III.	Angiotensin I - converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk	International Dairy Journal 20, 2010	In this journal, ACE inhibitory activity and antihypertensive effect of 25 Lactic Acid Bacteria was studied. The ACE inhibitory activity of *Lactobacillus Casei* 17 was 74% which is the highest and following that is the *Lactobacillus Jenseneii ATCC 25258* which had an activity of 72%. The antihypertensive effect was studied in the spontaneously hypertensive rat (SHR) essential in studies of cardiovascular diseases. *Lactobacillus jensenii, acidophilus*, and *Leu. mesenteroides 358.* The SHRs were injected milk fermented with *Lactobacillus jensenii* for 7 days. It

		showed a transient reduction in blood pressure and a maximum reduction of 90 mm Hg after 1.5 h of administration. Most of the *lactobacillus* strains that produced ACE inhibition and has strong proteolytic activity usually produces ACE inhibitory and antihypertensive peptides.

IV.	Angiotensin I-converting enzyme inhibitory peptides in skim milk fermented with *Lactobacillus helveticus* 130B4 from camel milk in Inner Mongolia, China	Journal of the Science of Food and Agriculture, 2008	In this journal, they fermented camel milk with *Lactobacillus helveticus.* The whey fraction in camel milk determined the ACE inhibitory activity and it had IC50 inhibitory concentration. The ace inhibitory peptide was isolated with the help of Sephadex G25 and then eluted into reverse-phase column. The amino acid sequence identified was Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp which is commonly found in bovine casein. The activity also should be preserved while digestion. Invitro digestion and heat treatments were performed and

		inspite of successive treatments, the peptide preserves its activity until it degrades into individual amino acids.
V. Imminent angiotensin-converting enzyme inhibitor from microbial source for cancer therapy	International Journal of Preventive Medicine, 8(1), p.80, 2017	Bacterial strains were isolated from cow milk collected then substrates were screened for ACE inhibitor production by the fermentation with the isolated strain. The ACE inhibitor was purified by ethanol precipitation, ion exchange column chromatography and gel filtration column chromatography. The molecular mass was determined by SDSPAGE and anticancer properties were determined using breast cancer MCF-7 cell lines. The isolate coded as BUCTL09 was selected and identified as Micrococcus luteus. Fermented beef extract broth showed the highest inhibition of 79% and was reported as the best substrate. The peptide was purified, and molecular mass was determined. The IC50 value of peptide was found to be 59.5 µg/ ml. The results of this study revealed that Peptide has been determined as an active compound that inhibited the activity of ACE.

VI.	Identification of Antimicrobial Peptides from Novel Lactobacillus fermentum Strain	The Protein Journal 39, 2020	In this journal, the *Lactobacillus fermentum* strain HF-D1 from the human gut producing AMPs which prevents the growth of *P. aeruginosa* and *S. marcescens* is isolated using MRS agar then antagonistic activity was tested on various pathogens. In this experiment, agar spot assay was used and incubated for 24 h in anaerobic conditions to develop spots. After that, the bacterial zone of inhibition was measured then the highest zone of inhibition bacteria was considered, and it was isolated. Then MALDI-TOF mass spectrometry was performed on the bacterial isolate for identification. After identification range of 3 which is high was observed. Hence, the DNA extraction kit was used to isolate the genomic DNA of bacteria for a sequence. The sequence was amplified to an observable level using PCR. Then blast algorithm and NCBI was used to identify the LAB isolate as fermentum. Then peptide extraction is done using MRS broth which was set to gut PH and is incubated for 24 h for LB fermentum to grow. Then centrifuged at 2000 rpm for 20 mins, the supernatant is collected and is further of 80% was purified with

			ammonium sulphate precipitation overnight. After series of centrifugation, the precipitate is dissolved in 10mM PBS. Then LC-MS/MS is performed. Then by analysis, 1111 peptides were identified using in silco analysis such as ADAM, CAMPR3, and AMPA prediction servers. Then using novel bioinformatics algorithms, they determined that, the HF-D1 *Lactobacillus fermentum strain* produces antimicrobial peptides that targets *P. aeruginosa* and *S. marcescens.*
VII.	Isolation, Identification, and Characterization of Small Bioactive Peptides from Lactobacillus GG Conditional Media That Exert Both Anti-Gram-negative and Gram-positive Bactericidal Activity	Journal of Pediatric Gastroenterology and Nutrition, 2009	In this journal, Lactobacillus rhamnosus GG (LGG) is isolated, identified and characterized for various probiotic and antimicrobial activities. LGG was cultured in MRS broth and kept in culture shaker for 24 h. Then diluted to 12th dilution and spread onto MRS agar plates for 24 h. Then it is centrifuged and extracted using ion exchange chromatography and SDS-PAGE. LC/MS analysis for analysis of peptides derived from proteins in the medium. LC/MS scans were searched against Lactobacillus database (IPI) using SEQUEST search algorithm.

		They identified 7 small peptides from LGG cultured media, 2 of which are NPSRQERR and PDENK, retained the antibacterial activity detected with LGG conditional media. The antibacterial activity was exerted against both Gram-negative (E. coli EAEC 042 and S. typhi) and, with less potency, Gram-positive (Staphylococcus aureus) bacteria. These peptides show various degrees of antibacterial activity against infectious diseases.
VIII. Isolation and characterization of antibacterial peptides derived from the f (164–207) region of bovine αS2-casein	International Dairy Journal, 2005	The aim of the journal is to isolate and characterize antibacterial peptides from chymosin digests of bovine casein. The bovine milk proteins were digested by reducing the PH to 3.0 with chymotrypsin and HCl. Then heated at 80 ºC for 15 mins. After heating, using NaOH, the PH was brought to 7.0 and then centrifuged. The supernatant was tested for antimicrobial assay against *L. innocua.* The zones of inhibition were examined then RP-HPLC was done to fractionalize the solution. The fractions from RP-HPLC were subjected to N-terminal amino acid sequence analysis. The Five antibacterial peptides, Cr1, Cr3, Cr4, Cr5 and Cr7 corresponding to amino acid residues 181–207, 180–207,

		175–207, 164–207 and 172–207 of bovine aS2-casein were isolated. They were then checked for antagonistic activity against gram positive and negative bacteria where they found that Cr1, Cr4 and Cr5 had ineffective in comparison to lactoferricinn B against Gram positive bacteria while the peptides Cr1, Cr4 and Cr5 are highly effective against Gram negative bacteria. The partially purified chymosin digest of sodium caseinate (CrMIX), containing the antibacterial peptides Cr1, Cr4 and Cr5, had high amounts of peptides and had good activity against *B.subtilis* even after heating for 15 mins at high temperature. The CrMIX showed good activity against S.typhimurium in 0.1% peptone medium but on adding skimmed milk to this, declined its antimicrobial activity. There was no antibacterial activity observed when tested in skimmed milk at the same concentration as peptone medium.

3. MATERIALS AND METHODS

3.1. Materials used in sub-culturing Lactobacillus spp.

- **Chemicals/ Culture/ Medium:** 6.75 gram of Man, Rogosa and Sharpe (MRS) Agar, Distilled Water, 70% ethanol – Disinfectant, 1 gram of Agar-agar, and Mother Culture for *Lactobacillus spp.* (*plantarum*, *fermentum*, *acidophilus* and *VITES07*).
- **Glasswares/ Equipments:** 100 ml Conical flask, 5 x Petri dishes, Autoclave Cooker, 200 - 100 µl micropipette, Micropipette Tips for 200 - 1000 µl, Non-absorbent Cotton, Culture incubator, Parafilm tape, Bunsen Burner and L-shaped cell spreader rod.

3.2. Protocol for sub-culturing Lactobacillus spp.

The protocol for sub-culturing *Lactobacillus spp.* is as follows:

- In this experiment four species of Lactic acid bacteria were used which are *plantarum*, *fermentum*, *acidophilus* and *VITES07*.

A. 6.7 gram of MRS agar and 1 gram of agar-agar is added to 100 ml of distilled water in a 100 ml conical flask.
B. The conical flasks are mixed, plugged with non-absorbent cotton and sealed with paper.
C. Micropipette tips, non-absorbent cotton, 5 x petri dishes, and 100 ml MRS agar is kept for sterilization in the autoclave cooker for 35-40 min.
D. Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.
E. After sterilization, MRS agar is poured onto the five petri dishes in which one petri dish is for control and rest is culture petri dishes.
F. The petri dishes with agar is left to cool for 15 – 20 min approximately.
G. After the agar solidified, the *Lactobacillus spp.* mother culture was inoculated 200 µl with micropipette and then spread with L-rod onto each corresponding petri dish.
H. The petri dishes are wrapped with parafilm tape to prevent any form of cross contamination during the incubation period.
I. The petri dishes are labelled for identification.

J. The petri dishes are kept in the culture incubator for 24 – 48 h based on the progress of the growth.

- After incubation, the following are the pictures of the sub-cultured *Lactobacillus spp*. (*plantarum, fermentum, acidophilus* and *VITSE07*) which were spread with L-rod.

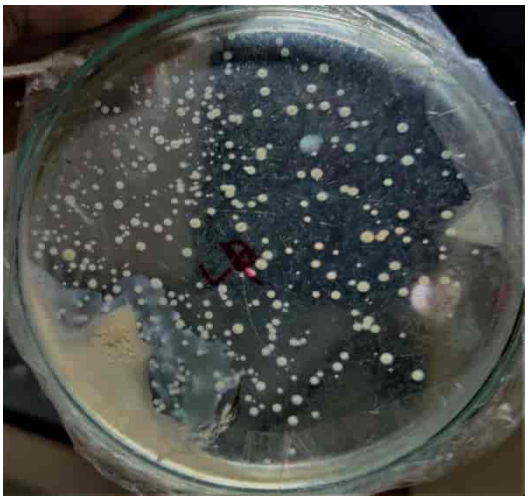

Figure 2: *Lactobacillus Plantarum* spread with L rod

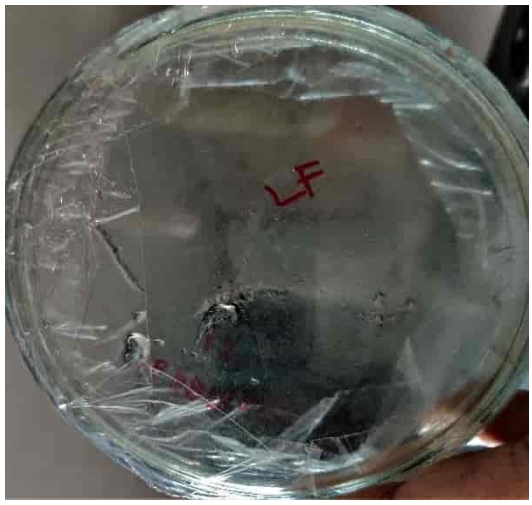

Figure 3: *Lactobacillus Fermentum* spread with L rod

Figure 4: *Lactobacillus Acidophilus* spread with L rod

Figure 5: *Lactobacillus VITSE07* spread with L rod

3.3. Materials used for isolating Lactobacillus spp.

- **Chemicals/ Culture/ Medium:** 6.75 gram of Man, Rogosa and Sharpe (MRS) Agar, Distilled Water, 70% ethanol – Disinfectant, 1 gram of Agar-agar, and Subcultured *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*).
- **Glasswares/ Equipments:** 100 ml Conical flask, 5 x Petri dishes, Autoclave Cooker, Non-absorbent Cotton, Inoculation loop, Culture incubator, Parafilm tape, and Bunsen Burner.

3.4. Protocol for isolating Lactobacillus spp.

The protocol for isolating *Lactobacillus spp.* is as follows:

- The sub-cultured species are in a mixed population along with other bacteria that grows in the MRS agar medium. Hence, in order to isolate the population of the desired bacteria alone, hence perform quadrant streaking or quaternary streaking.

A) 6.7 gram of MRS agar and 1 gram of agar-agar is added to 100 ml of distilled water in a 100 ml conical flask.

B) The conical flask is mixed, plugged with non-absorbent cotton and sealed with paper.

C) Non-absorbent cotton, 5 x petri dishes, and 100 ml MRS agar is kept for sterilization in the autoclave cooker for 35-40 min.

D) Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.

E) After sterilization, MRS agar is poured onto the five petri dishes in which one petri dish is for control and rest is culture petri dishes.

F) The petri dishes with agar is left to cool for 15 – 20 m approximately.

G) After the agar solidified, the *Lactobacillus spp.* culture was screened, then a colony is pricked from the sub-cultured petri dish and then inoculated with an inoculation loop and streaked into each corresponding petri dish via quadrant streak.

H) The petri dishes are wrapped with parafilm tape to prevent any form of cross contamination during the incubation period.

I) The petri dishes are labelled for identification.

J) The petri dishes are kept in the culture incubator for 24 – 48 h based on the progress of the growth.

- After incubation, the following are the pictures of the isolated *Lactobacillus spp*. (*plantarum, fermentum, acidophilus* and *VITSE07*) which were quadrant streaked.

Figure 6: *Lactobacillus Plantarum* spread with inoculation loop via quadrant streak

Figure 7: *Lactobacillus Fermentum* spread with inoculation loop via quadrant streak

Figure 8: *Lactobacillus VITSE07* spread with inoculation loop via quadrant streak

Figure 9: *Lactobacillus Acidophilus* spread with inoculation loop via quadrant streak

3.5. Materials used for preparing Lactobacillus mother culture

- **Chemicals/ Culture/ Medium:** 5.5 gram of Man, Rogosa and Sharpe (MRS) broth, Distilled Water, 70% ethanol – Disinfectant, and Isolated *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*).
- **Glasswares/ Equipments:** 100 ml Conical flask, 5 x 10 ml - Test tubes, Autoclave Cooker, Non-absorbent Cotton, Inoculation loop, Culture incubator, Parafilm tape, Beaker and Bunsen Burner.

3.6. Protocol for preparing Lactobacillus mother culture

- In order to obtain pure *Lactobacillus* mother culture for future sub-culturing, this protocol is used.

A) 5.5 gram of MRS broth is added to 100 ml of distilled water in a 100 ml conical flask.

B) The conical flasks are mixed, and then the mixture is poured into 5 test tubes. The test tubes are sealed with cotton and paper.

C) Non-absorbent cotton, and the 5 test tubes are kept for sterilization in the autoclave cooker for 35-40 min.

D) Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.

E) After sterilization, the isolated *Lactobacillus spp.* culture is pricked and inoculated with an inoculation loop into the corresponding test tubes (1 control and 4 culture).

F) The mouth of the test tubes are sterilized using Bunsen burner before incubation.

G) The test tubes are wrapped with parafilm tape to prevent any form of cross contamination during the incubation period. The test tubes are placed inside a beaker of 250 ml and wrapped with paper.

H) The test tubes are labelled for identification.

I) The test tubes are kept in the culture incubator for 24 – 48 h based on the progress of the growth.

3.7. Materials used for cryopreserving Lactobacillus mother culture

- **Chemicals/ Culture/ Medium:** 5.5 gram of Man, Rogosa and Sharpe (MRS) broth, Distilled Water, 70% ethanol – Disinfectant, Glycerol and *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*) sub-cultured in MRS Broth.
- **Glasswares/ Equipments:** 100 ml Conical flask, 5 x 10 ml - Test tubes, Autoclave Cooker, Non-absorbent Cotton, Inoculation loop, Culture incubator, Parafilm tape, Beaker, Micropipette 200 – 1000 µl, Micropipette tips for 1000 µl, Bunsen Burner and Refrigerator (2 – 5 ºC).

3.8. Protocol for cryopreserving Lactobacillus mother culture

- In order to use the mother culture for sub-culturing in the future. It is required to be cryopreserved using glycerol stock.

A) Glycerol stock is prepared, where 25ml of glycerol is added to 25 ml of distilled water in a beaker.

B) The test tubes with sub cultured *Lactobacillus spp.* after 48 h incubation is taken, and 4 ml of each *Lactobacillus spp.* is taken and added into fresh 10 ml falcon tubes.

C) The glycerol stock prepared is also added 4 ml to the each of the 10 ml falcon tubes.

D) The falcon tubes are mixed and then labelled properly in order to correctly identify the preserved culture.

E) The glycerol preserved mother culture is now kept in the refrigerator for cryopreservation until it is required.

- If the cryopreserved mother culture needs to be sub-cultured, it is to be cultured into petri dishes and then into broth.
- The cryopreserved culture needs to be thawed before its used, otherwise the cells won't grow in a linear and uniform manner as some cells would still be frozen and will have slower growth.

3.9. Materials used in production of peptides with Lactobacillus in casein medium

- **Chemicals/ Culture/ Medium:** Distilled Water, 70% ethanol – Disinfectant, 8 grams of Casein, 6 grams of Lactose, 0.5 gram of sodium chloride (NaCl) and *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*) sub-cultured in MRS Broth.
- **Glasswares/ Equipments:** 200 ml Conical flasks, Autoclave Cooker, Non-absorbent Cotton, Parafilm tape, Beaker, Micropipette 200 – 1000 µl, Micropipette tips for 1000 µl, Bunsen Burner, Dropper, PH meter, Culture shaker and Water bath.

3.10. Protocol used in production of peptides with Lactobacillus in casein medium

A. In a 200 ml conical flask, 8 grams of casein, 6 grams of lactose and 0.5 gram of sodium chloride is added and to that, 100 ml of distilled water is added.
B. The conical flasks are kept in a water bath set at 65ºC in order to dissolve the casein and the medium is to be calibrated to a PH of 6.5.
C. The PH meter was used, and it was calibrated with the PH 7.0 and PH 5.0 from the PH calibration kit. 4 gram/ 100 ml NaOH was added with a dropper and the medium was brought to around 6.5 PH.
D. The casein medium was kept in the water bath for 1 h to dissolve it and to bring PH to 6.5 for optimal growth.
E. After medium calibration, the conical flasks are mixed, plugged with non-absorbent cotton and sealed with paper.
F. Micropipette tips, non-absorbent cotton, and conical flasks with casein medium is kept for sterilization in the autoclave cooker for 25 min, in order to prevent denaturation of casein protein.
G. Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.
H. The conical flasks with casein medium are left in the laminar air flow to cool to room temperature.
I. Then the sub-cultured *Lactobacillus spp.* in MRS broth is added 1 ml through micropipette into each corresponding conical flask with casein medium.

J. The mouth of the conical flasks are sterilized using Bunsen burner before incubation.

K. The conical flasks are plugged with non- absorbent cotton and covered with paper to prevent any form of cross contamination during the incubation period.

L. The conical flasks are labelled for identification.

M. The conical flasks are then incubated in a culture shaker for 48 h based on the consistency of the medium.

N. After incubation period, they are poured from conical flasks into 50 ml falcon tubes in the laminar air flow.

O. The 50 ml falcon tubes with casein induced culture are then centrifuged at 4000 rpm, 25ºC. The supernatant is collected and is later subjected to several assays.

Figure 10: Supernatant of *L. VITSE07* from casein medium

Figure 11: Supernatant of *L. fermentum* from casein medium

Figure 12: Supernatant of *L. plantarum* from casein medium

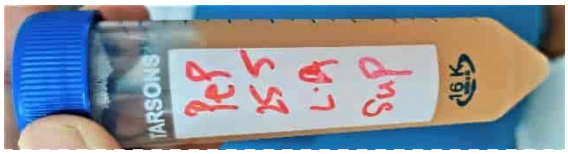

Figure 13: Supernatant of *L. Acidophilus* from casein medium

Figure 14: Calibration of PH meter

Figure 15: PH adjustment in water bath

3.11. Materials used in production of peptides with Lactobacillus in MRS medium

- **Chemicals/ Culture/ Medium:** Distilled Water, 70% ethanol – Disinfectant, 5.5 gram of Man, Rogosa and Sharpe (MRS) broth and *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*) sub-cultured in MRS Broth.
- **Glasswares/ Equipments:** 200 ml Side arm flasks, Autoclave Cooker, Non-absorbent Cotton, Parafilm tape, Beaker, Micropipette 200 – 1000 µl, Micropipette tips for 1000 µl, Bunsen Burner, Thermocol culture incubator, and Spectrophotometer.

3.12. Protocol for production of peptides with Lactobacillus in MRS medium

A) 5.5 gram of MRS broth is added to 100 ml of distilled water in a 200 ml side arm flask and was repeated for five 200 ml side arm flasks.
B) The side arm flasks are mixed and sealed with cotton and paper.
C) Non-absorbent cotton, micropipette tips and 5 side arm flasks are kept for sterilization in the autoclave cooker for 35-40 min.
D) Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.
E) After sterilization, the MRS broth in the side arm flask is left to cool to room temperature.
F) Then the sub-cultured *Lactobacillus spp.* in MRS broth is added 1 ml through micropipette into each corresponding side arm flasks.
G) The mouth of the side arm flasks are sterilized using Bunsen burner before incubation.
P. The side arm flasks are then plugged with non-absorbent cotton and covered with paper to prevent any form of cross contamination during the incubation period.
H) The side arm flasks are labelled for identification.
I) The side arm flasks are kept in the Thermocol incubator for 24 - 48 h based on the progress of the growth as growth kinetics is studied.
J) In growth kinetics, each incubated sample is measured at an optical density of 600 nm using a digital colorimeter.

K) Growth kinetics is plotted in order to find out the time of release of the peptides and the time at which there is higher activity of biological functions of the bioactive peptides.

L) After incubation period, they are poured from conical flasks into 50 ml falcon tubes in the laminar air flow.

M) The 50 ml falcon tubes with MRS medium induced culture are centrifuged at 4000 rpm, 25ºC. The supernatant is collected and is later subjected to several assays.

Figure 16: Recording optical density readings with the digital colorimeter for growth kinetics

Figure 17: Calibrating digital colorimeter with control

Figure 18: Incubating side arm flasks with *Lactobacillus spp*. in a Thermocol culture incubator.

3.13. Materials used in Antimicrobial Assay

- **Chemicals/ Culture/ Medium:** Distilled Water, 70% ethanol – Disinfectant, 3.8 gram of Mueller-Hinton agar, antimicrobial disks – Gentamicin, & Erythromycin, Pathogen cultures (*E. coli, Salmonella T, Bacillus S, Pseudomonas A*) and *Lactobacillus spp.* (*plantarum, fermentum, acidophilus* and *VITES07*) sub-cultured in MRS medium/Casein medium (in flask – side arm/conical flask).
- **Glasswares/ Equipments:** 200 ml Side arm flasks, 5 x Petri dishes, Autoclave Cooker, Non-absorbent Cotton, Parafilm tape, Beaker, Micropipette 200 – 1000 µl, Micropipette tips for 1000 µl, Well borer, Forceps, Bunsen Burner, Culture incubator, and cotton swabs.

3.14. Protocol for Antimicrobial Assay

A) 3.8 gram of Mueller-Hinton agar (MHA) is added to 100 ml of distilled water into 100 ml conical flasks.

B) The conical flasks are mixed, plugged with non-absorbent cotton and sealed with paper.

C) Micropipette tips, non-absorbent cotton, 5 x petri dishes, and 100 ml MHA agar is kept for sterilization in the autoclave cooker for 35-40 min.

D) Laminar air flow is cleaned with 70% ethanol – disinfectant and UV light are switched on for 10 min.

E) After sterilization, MHA agar is poured onto the five petri dishes in which one petri dish is for control and rest is culture petri dishes.

F) The petri dishes with agar is left to cool for 15 – 20 min approximately.

G) After the agar solidified, using the micropipette, pathogen culture is added roughly 150 – 200 µl and is swabbed throughout the plate.

H) After cotton swabbing the plate, using a well borer, 4 holes were punched towards the corner of petri dish with equal distance between each hole.

I) The supernatant from *Lactobacillus spp.* is added to these wells.

J) Then using forceps, antibiotic disks are added to the center of the petri dish

K) The petri dishes are wrapped with parafilm tape to prevent any form of cross contamination during the incubation period.

L) The petri dishes are labelled for identification and are kept in the culture incubator for 24 h in order to observe the zone of inhibition by the supernatant which indicates the presence of antimicrobial peptides.

Figure 19: Antimicrobial assay in laminar air flow

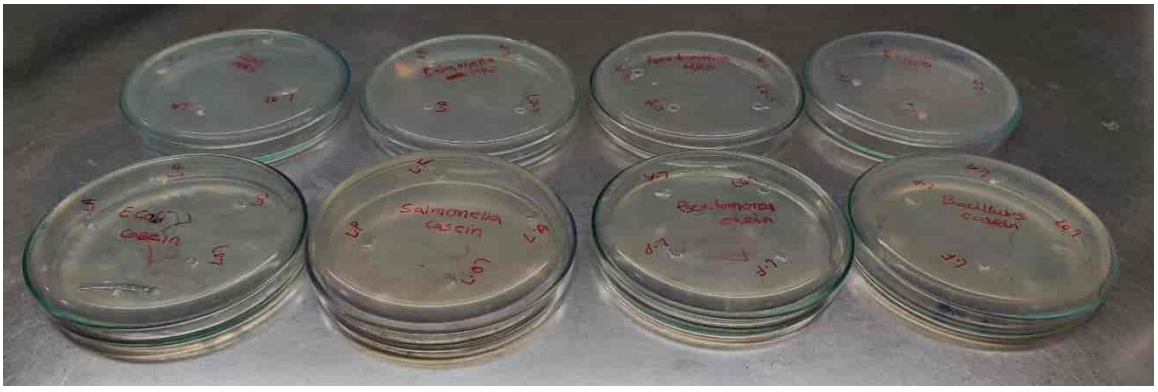

Figure 20: Petri dish with MHA is swabbed with pathogen culture and agar well is cut

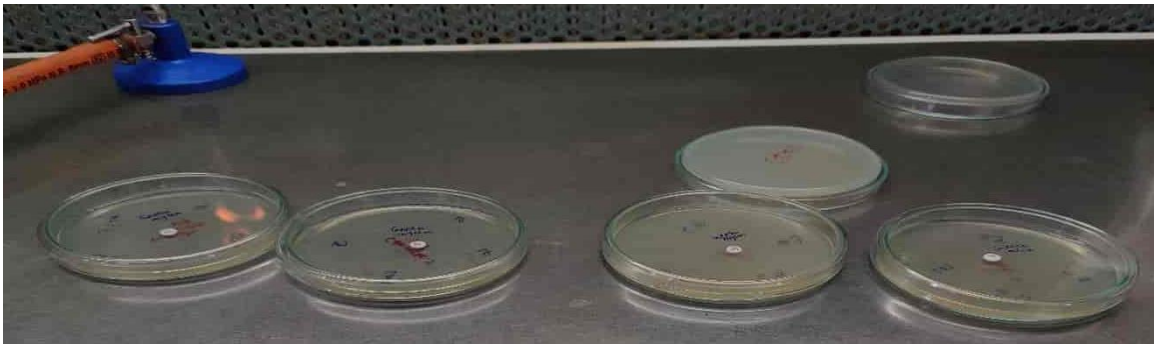

Figure 21: MHA well filled with supernatant and the antibiotic disks are placed as positive control

Figure 22: Antibiotic disks used for antimicrobial assay which is used as a positive control.

3.15. Materials used in Angiotensin Converting Enzyme (ACE) inhibition Assay

- **Chemicals/ Culture/ Medium:** Distilled Water, 70% ethanol – Disinfectant, *Lactobacillus spp.* (*plantarum, & fermentum)* supernatant from MRS medium, Captopril ACE inhibitor drug (Control), Hippuryl-L-Histidyl-L-Leucine Solution (HHL), Angiotensin converting enzyme (ACE), and 50mM Sodium Borate buffer.
- **Glasswares/ Equipments:** Tray, Quartz cuvette, Eppendorf tubes, Eppendorf stand, spectrophotometer, Non-absorbent Cotton, Beaker, Micropipette 200 – 1000 µl, Micropipette tips for 20 µl and 1000 µl pipettes, and Micropipette 0.5 – 20 µl.

3.16. Protocol for Angiotensin Converting Enzyme (ACE) inhibition Assay

- The supernatant used in this experiment was in the 71st h of the growth kinetics.

1. **Case (A):** With both Supernatant and ACE enzyme

   A) 25 µl of Hippuryl-L-Histidyl-L-Leucine Solution (HHL) is added to 10 µl of supernatant (*LB plantarum & fermentum*)/Captopril control in an Eppendorf tube.
   B) It is incubated for 10 min at room temperature (~ 37ºC).
   C) To the Eppendorf tube, add 10 µl ACE enzyme and the reaction starts. It is then incubated for 30 min at room temperature (~ 37ºC).
   D) 200 µl of 1 N HCl is added to stop the reaction.
   E) Optical density is taken at 228 nm using a quartz cuvette for accurate reading.

2. **Case (B):** With Supernatant and no ACE enzyme

   A) 25 µl of Hippuryl-L-Histidyl-L-Leucine Solution (HHL) is added to 10 µl of supernatant (*LB plantarum & fermentum*)/Captopril control in an Eppendorf tube.
   B) It is incubated for 10 min at room temperature (~ 37ºC).
   C) 200 µl of 1 N HCl is added to increase the volume of the solution.
   D) Optical density is taken at 228 nm using a quartz cuvette for accurate reading.

3. **Case (C):** No Supernatant and with ACE enzyme [control]

   A) 25 µl of Hippuryl-L-Histidyl-L-Leucine Solution (HHL) is added to an Eppendorf tube and is incubated for 10 min at room temperature (~ 37ºC).
   B) To the Eppendorf tube, add 10 µl ACE enzyme and the reaction starts. It is then incubated for 30 min at room temperature (~ 37ºC).
   C) 200 µl of 1 N HCl is added to stop the reaction.
   D) Optical density is taken at 228 nm using a quartz cuvette for accurate reading.
   E) The Optical density is taken with taking blank as the control (case c).
   F) After the optical density for each case is collected, they are tabulated and then calculated to understand the inhibition percentage.

- Case A and B are repeated for each supernatant (*Lactobacillus plantarum and fermentum*) and a positive control drug (Captopril – ACE inhibitor).

Figure 23: Ace assay in Eppendorf stand where A, B and C are the various cases in order to calculate the inhibition

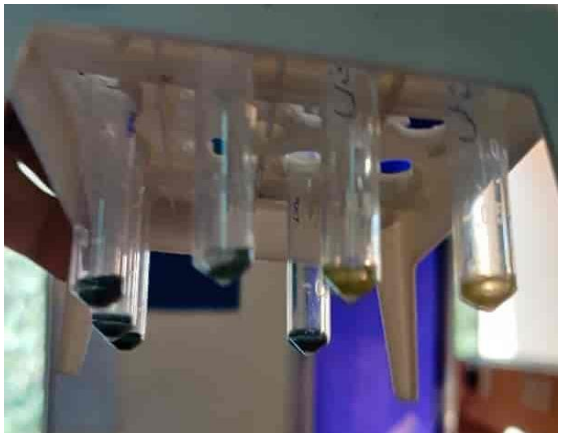

Figure 24: Ace assay color change in Eppendorf tubes from stopping the reaction using HCl

4. OBSERVATIONS

4.1. Observations of Growth kinetics

4.1.1. Table for Growth kinetics at 26 h

Table 1

Time(hrs)	LF	LP
0	0.05	0.07
4.5	0.15	0.19
22	0.68	0.69
26	1.24	1.29

4.1.2. Graph for Growth kinetics at 26 h

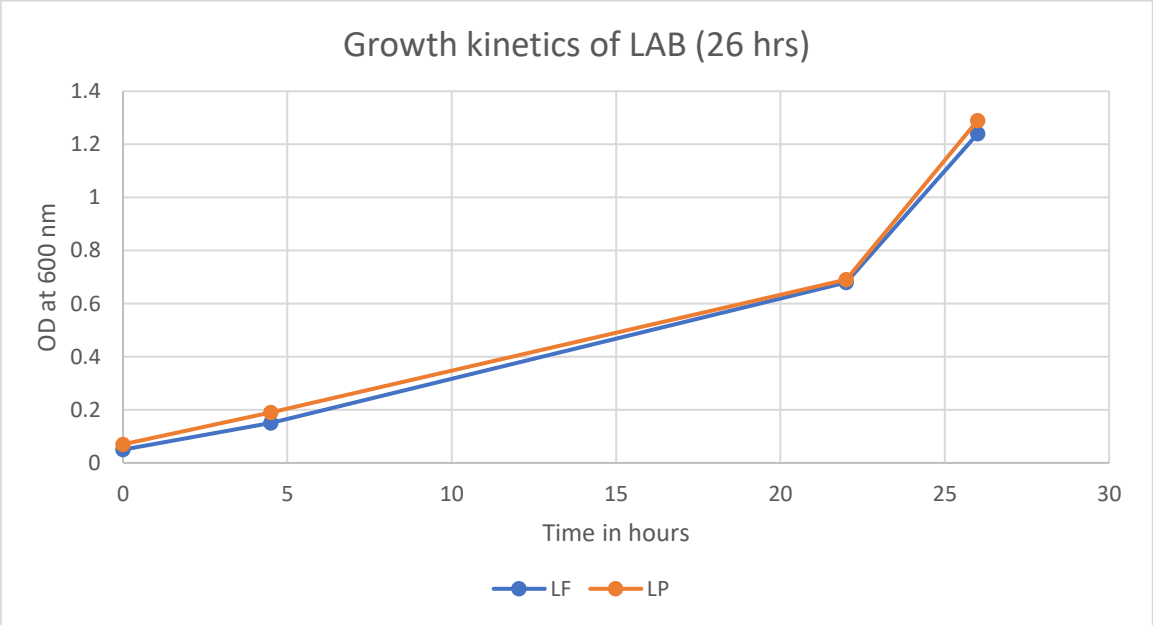

Figure 25: Growth kinetics of LAB at 26 h

- From the graph it is visible that it is still in the acceleration and growth phase. So, the peptide activity is very low at 26 h and below.

4.1.3. Table for Growth kinetics at 34 h

Table 2

Time(hrs)	LP
0	0.03
28.5	0.84
34	0.85

4.1.4. Graph for Growth kinetics at 34 h

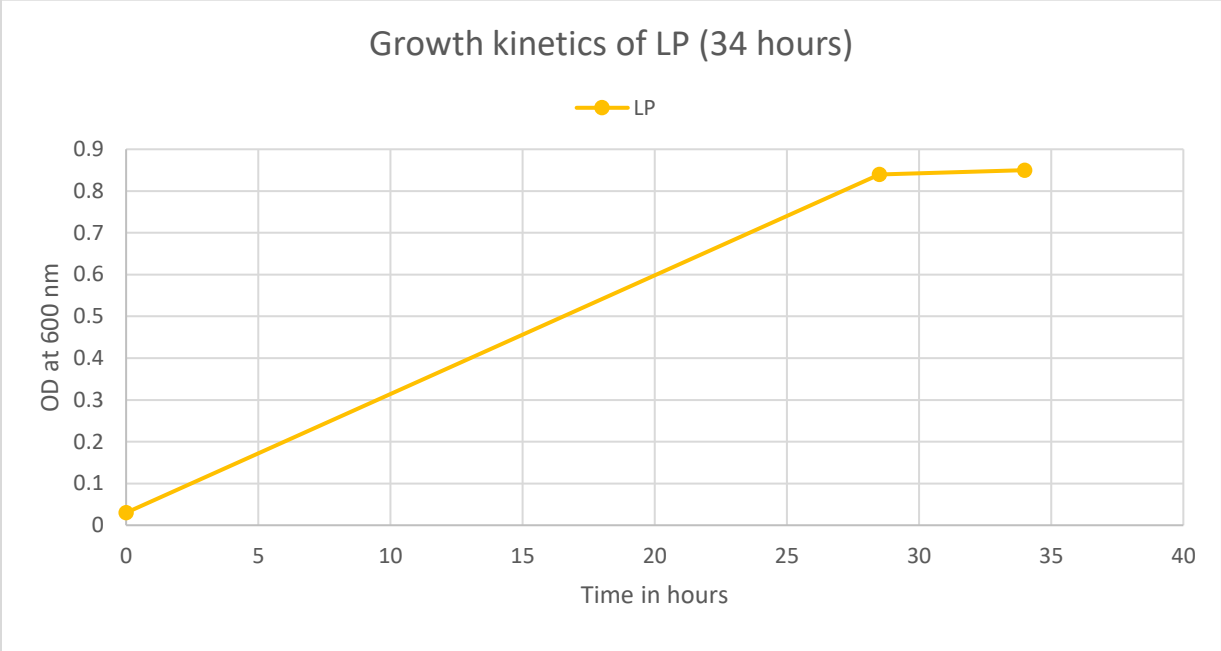

Figure 26: Growth kinetics of LAB at 34 h

- From the graph it is visible that, by the 30th h it has reached stationary phase where there is lower acceleration and growth.

4.1.5. Table for Growth kinetics at 49 h

Table 3

Time(hrs)	LP
0	0.03
21	0.8
25	0.84
27	0.85
30	0.86
49	0.89

4.1.6. Graph for Growth kinetics at 49 h

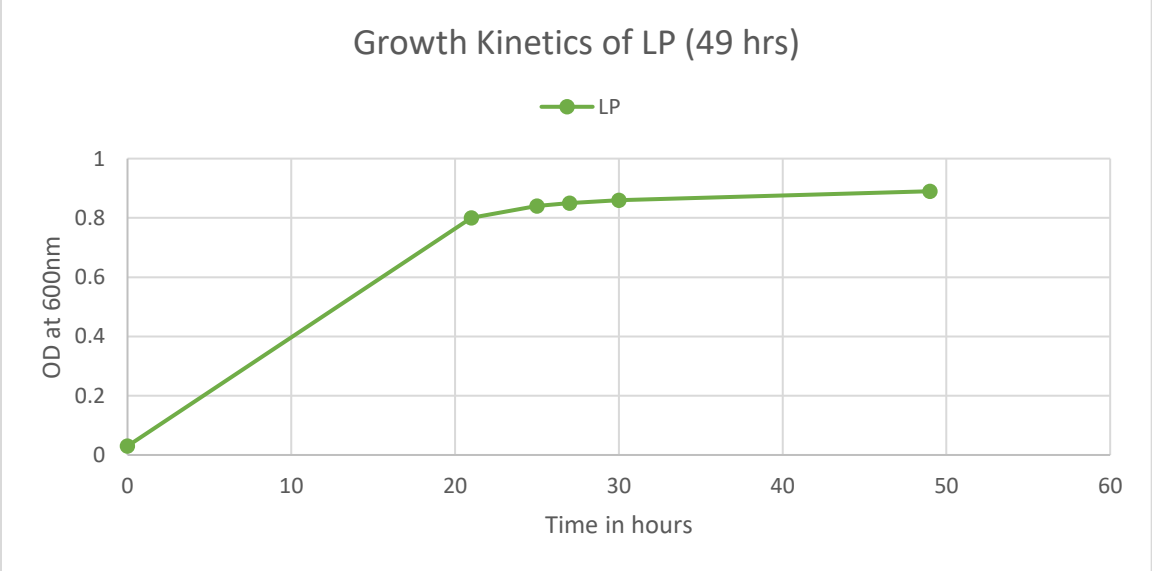

Figure 27: Growth kinetics of LAB at 49 h

- From the graph it is visible that, it is still in the stationary phase and hasn't reached death phase even at the 49th h. Hence, the increment in the activity of peptides is potent with more time provided.

4.1.7. Table for Growth kinetics at 71 h

Table 4

Time(hrs)	LA	LF	L07	LP
0	0.01	0.01	0.04	0.03
5.5	0.02	0.07	0.06	0.14
22	0.67	0.68	0.7	0.68
26.5	0.72	0.72	0.74	0.72
29	0.93	0.93	0.96	0.91
71	0.95	0.95	0.96	0.95

4.1.8. Graph for Growth kinetics at 71 h

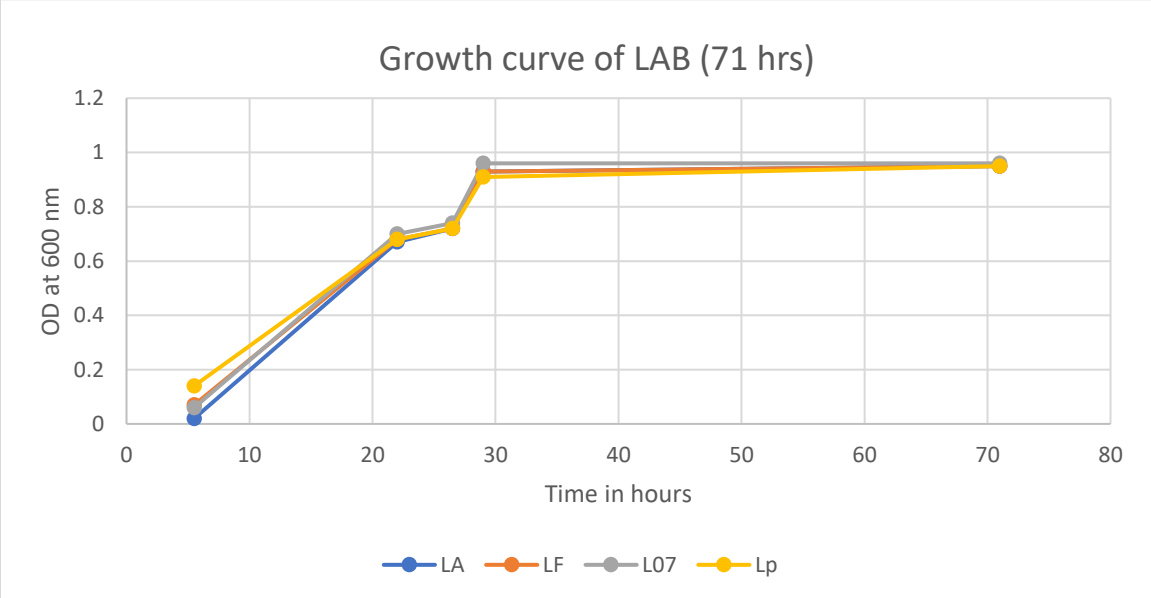

Figure 28: Growth kinetics of LAB at 71 h

- From the graph it is visible that, it is still in the stationary phase and hasn't reached death phase even at the 71st h. Hence, the increment in the activity of peptides is potent with more time provided.

4.2. Observations of Antimicrobial Assay

4.2.1. Observations from Antimicrobial assay of Lactobacillus in casein medium

- Observations from antimicrobial assay at **48th** h:

Figure 29: *Salmonella typhi* swabbed plate without any zone of inhibition by the *Lactobacillus spp*. supernatant from casein medium at 48th h

Figure 30: *Pseudomonas aeruginosa* swabbed plate without any zone of inhibition by the *Lactobacillus spp.* supernatant from casein medium at 48th h

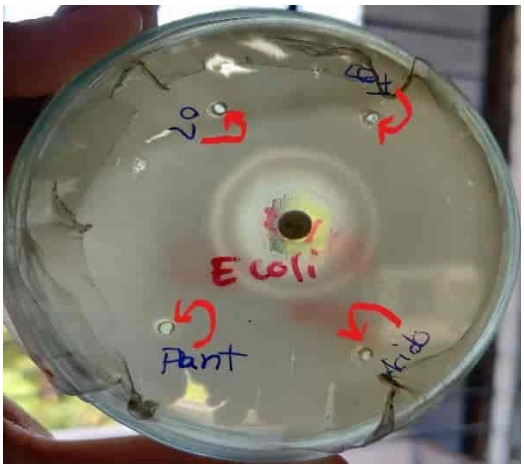

Figure 31: *Escherichia coli* swabbed plate without any zone of inhibition by the *Lactobacillus spp.* supernatant from casein medium at 48th h

Figure 32: *Bacillus subtilis* swabbed plate without any zone of inhibition by the *Lactobacillus spp.* supernatant from casein medium at 48th h

4.2.2. Observations from Antimicrobial assay of Lactobacillus in MRS medium

- Observations from antimicrobial assay at **34th h** of growth kinetics:

Figure 33: *Bacillus subtilis* swabbed plate with small zone of inhibition by the *Lactobacillus plantarum* supernatant from MRS medium at 34th h of growth kinetics

Figure 34: *Escherichia coli* swabbed plate without any zone of inhibition by the *Lactobacillus plantarum* supernatant from MRS medium at 34th h of growth kinetics

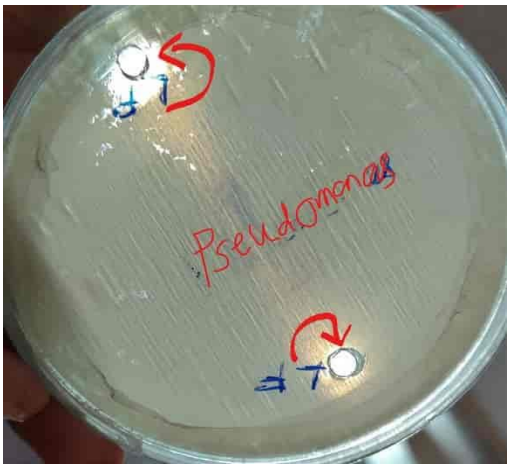

Figure 35: *Pseudomonas aeruginosa* swabbed plate with an observable zone of inhibition by *Lactobacillus plantarum* from MRS medium, while no zone of inhibition by the *Lactobacillus fermentum* from MRS medium at 34th h of growth kinetics

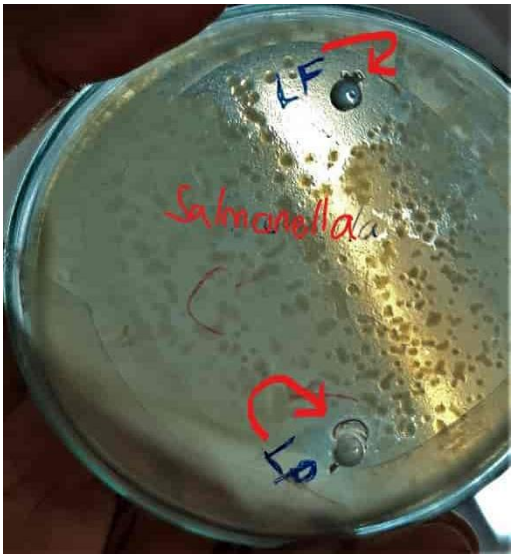

Figure 36: *Salmonella typhi* swabbed plate with observable zone of inhibition by *Lactobacillus fermentum* and *plantarum* from MRS medium at 34th h of growth kinetics

- Observations from antimicrobial assay at **71st h** of growth kinetics:

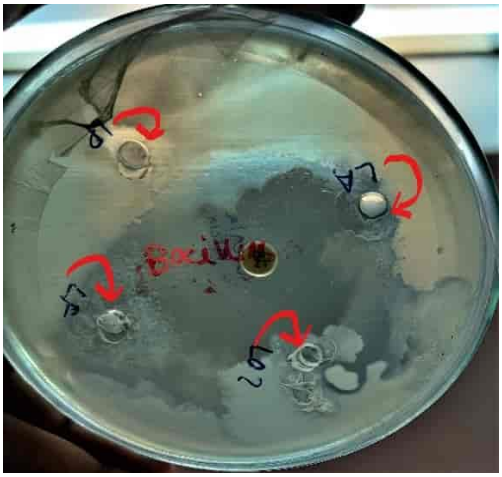

Figure 37: *Bacillus subtilis* swabbed plate with observable zone of inhibition by the *Lactobacillus acidophilus and plantarum* supernatant while it isn't clear for *fermentum* and *VITSE07* from MRS medium at 71st h of growth kinetics

Figure 38: *Escherichia coli* swabbed plate without any zone of inhibition by the *Lactobacillus spp.* supernatant from MRS medium at 71st h of growth kinetics

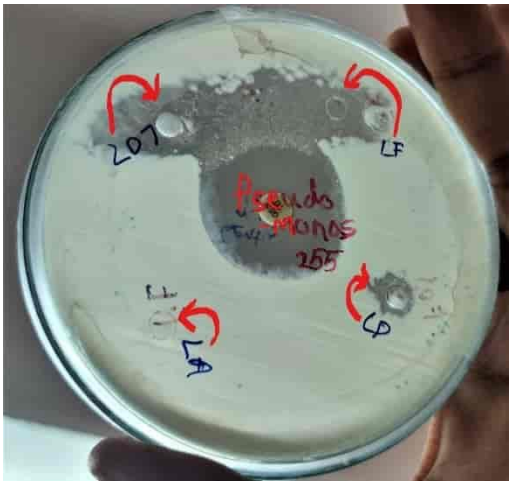

Figure 39: *Pseudomonas aeruginosa* swabbed plate with an observable zone of inhibition by *Lactobacillus plantarum, fermentum and VITES07* from MRS medium, while no zone of inhibition by the *Lactobacillus acidophilus* from MRS medium at 71st h of growth kinetics

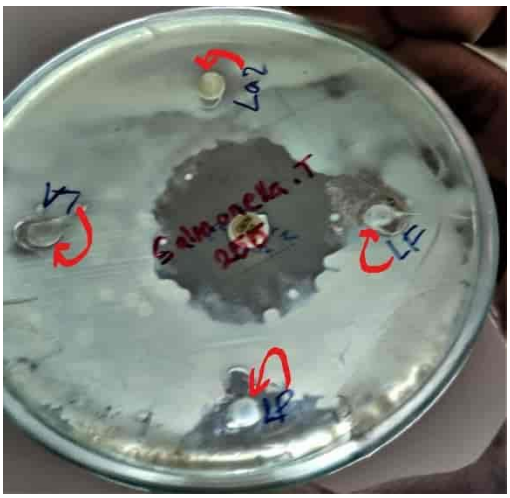

Figure 40: *Salmonella typhi* swabbed plate with an observable zone of inhibition by *Lactobacillus plantarum, fermentum and acidophilus* from MRS medium, while no zone of inhibition by the *Lactobacillus VITES07* from MRS medium at 71st h of growth kinetics

4.2.2.1. Table for Zone of inhibition at 34 h

Table 5

Pathogens	LP
E. Coli	-
Salmonella T	0.55 x 0.66 cm
Bacillus S	0.64 x 0.7 cm
Pseudomonas A	0.62 x 0.7 cm

4.2.2.2.Table for Zone of inhibition at 71 h

Table 6

Pathogens	LP	LA	L07	LF	Gentamycin Ab
E. Coli	-	-	-	-	2.0 x 2.0 cm
Salmonella T	1.5 x 2.1 cm	0.6 x 0.6 cm	0.55 x 0.55 cm	0.5 x 1.0 cm	3.0 x 3.0 cm
Bacillus S	0.9 x 0.9 cm	0.7 x 0.7 cm	-	-	4.0 x 4.0 cm
Pseudomonas A	0.9 x 0.8 cm	-	1.1 x 2.0 cm	1.0 x 2.0 cm	2.8 x 2.6 cm

4.3. Observations of ACE Assay

- The readings were taken 3 times due to technical errors. The readings were taken at 228 nm in a Spectrophotometer.

  A = Optical density in presence of ACE enzyme and supernatant

  B = Optical density without supernatant [Control]

  C = Optical density without ACE enzyme

4.3.1. Table 1 for Ace Assay with OD at 228 nm

Table 7

	LF	LP	Captopril - Ctrl
A	0.0006	0.0297	0.0475
B	0.2545	0.2545	0.2545
C	0.0127	0.0629	0.0723

4.3.2. Table 2 for Ace Assay with OD at 228 nm

Table 8

	LF	LP	Captopril - Ctrl
A	0.026	0.1	0.039
B	0.182	0.182	0.182
C	0.031	0.137	0.097

4.3.3. Table 3 for Ace Assay with OD at 228 nm

Table 9

	LF	LP	Captopril - Ctrl
A	0.064	-0.026	0.085
B	0.144	0.144	0.144
C	-0.051	-0.029	-0.003

5. RESULT AND DISCUSSION

5.1. Results for Growth kinetics

5.1.1. Table for Rate of growth and Doubling time at 26 h

Table 10

	LF	LP
Rate of growth (r)	0.08636	0.07369
Doubling time (d)	8.02625	9.40625

5.1.2. Table for Rate of growth and Doubling time at 34 h

Table 11

	LP
Rate of growth (r)	0.11691
Doubling time (d)	5.92889

5.1.3. Table for Rate of growth and Doubling time at 49 h

Table 12

	LP
Rate of growth (r)	0.156353
Doubling time (d)	4.43321

5.1.4. Table for Rate of growth and Doubling time at 71st h

Table 13

	LF	LP	L07	LA
Rate of growth (r)	0.21282	0.09578	0.14889	0.87786
Doubling time (d)	3.25696	7.23656	4.65543	0.78958

5.2. Discussion for Growth kinetics

- The doubling time for *Lactobacillus acidophilus* is lowest. Hence, it is having the fastest growth to decline phase. However, as peptides are produced from hydrolysis, which is a time-consuming process. Hence, *Lactobacillus acidophilus* might have low uniformity in production of peptides.
- The doubling time for *Lactobacillus plantarum* is the highest. Hence, it is having the slowest growth to decline phase. However, as peptides are produced from hydrolysis, which is a time-consuming process. Hence, *Lactobacillus plantarum* might have high uniformity in production of peptides.
- The growth kinetics was done in order to optimize the peptide production, to understand which hour of the growth curve the peptide was released. This gives us better understanding of how peptide activity is to growth of culture.

- **Formulas in Growth kinetics:**

  Formula for Rate of growth (r):

Rate (r) = ln (OD2/OD1)/(T2-T1)

  Formula for Doubling time (d):

Doubling time (d) = ln 2/ r

  (Martin, Alejandro, 2017);

- *Lactobacillus spp.* stays in stationary phase for 42 h and hasn't declined even at 71 h.

5.3. Results for Antimicrobial Assay

5.3.1. Results for Antimicrobial assay of Lactobacillus spp. in casein medium

- There was no zone of inhibition by *Lactobacillus spp.* in casein medium.

5.3.2. Results from Antimicrobial assay of Lactobacillus spp. in MRS medium

- There were zones of inhibition by *Lactobacillus spp.* in MRS medium under specific pathogens.

5.3.2.1. Table for Zone of inhibition w/o well size at 34 h

Table 14

	LP
E. Coli	-
Salmonella T	0.05 x 0.16 cm
Bacillus S	0.14 x 0.2 cm
Pseudomonas A	0.13x 0.2 cm

5.3.2.2. Table for Zone of inhibition w/o well size at 71 h

Table 15

	LP	LA	L07	LF	Gentamicin Ab
E. Coli	-	-	-	-	1.5 x 1.5 cm
Salmonella T	1.0 x 1.6 cm	0.1 x 0.1 cm	0.05 x 0.05 cm	0.05 x 0.5 cm	2.5 x 2.5 cm
Bacillus S	0.4 x 0.4 cm	0.2 x 0.2 cm	-	-	3.5 x 3.5 cm
Pseudomonas A	0.4 x 0.3 cm	-	0.6 x 1.5 cm	0.5 x 1.5 cm	2.4 x 2.1 cm

5.4. Discussion for Antimicrobial Assay

- There was no zone of inhibition from *Lactobacillus spp.* from casein medium, because it might not have antimicrobial peptides. It could have other bioactive peptides but since the aim was to obtain antimicrobial peptides, the *Lactobacillus spp.* from casein medium is discontinued from the experiment, and only *Lactobacillus spp*. from MRS medium is considered as it has zone of inhibition, indicating presence of antimicrobial peptides.
- Even though, *Lactobacillus plantarum* had higher doubling time which means it had slower growth, it had prominent antimicrobial activity. This suggests that the higher the doubling time, the lower the growth but more uniform the peptide activity.
- The size of the well punched on the agar is 0.5 cm, which is neglected from the well size and in order to understand the actual size of the zone of inhibition.
- The antimicrobial assay for the 34^{th} h was repeated twice due to issues with agar solidification and unstable agar medium.
- The antimicrobial peptides from *Lactobacillus spp*. couldn't inhibit *E. Coli* growth and have antimicrobial activities against only specific pathogens.
- The antimicrobial activity of the peptides was much less effective in comparison to Gentamicin which is an antibiotic drug.

5.5. Results for ACE inhibition Assay

5.5.1. Results of table 1 for ACE inhibition Assay

Table 16

	LF	LP	Captopril - Ctrl
B - A	0.2539	0.2248	0.207
B - C	0.2418	0.1916	0.1822
B-A/B-C	1.050041	1.173278	1.13611416
Inhibition %	105%	117%	113%

5.5.2. Results of table 2 for ACE inhibition Assay

Table 17

	LF	LP	Captopril - Ctrl
B - A	0.156	0.082	0.143
B - C	0.151	0.045	0.085
B-A/B-C	1.033113	1.822222	1.682352941
Inhibition %	103%	182%	168%

5.5.3. Results of table 3 for ACE inhibition Assay

Table 18

	LF	LP	Captopril - Ctrl
B - A	0.08	0.17	0.059
B - C	0.195	0.173	0.147
B-A/B-C	0.410256	0.982659	0.401360544
Inhibition %	41%	98%	40%

5.5.4. Graph for ACE inhibition Assay

- The graph for ACE inhibition is plotted using B-A values from the 3 readings for sample and the control. B-A value is the OD of the supernatant.

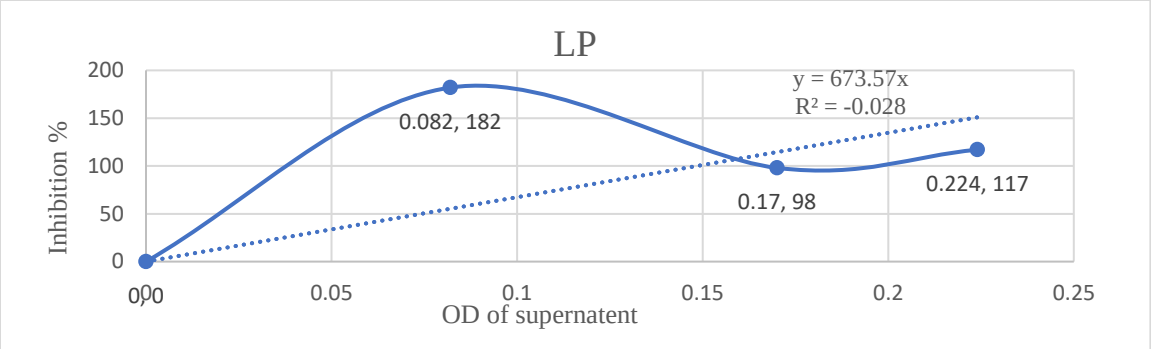

Figure 41: Graph of ACE inhibition assay for *Lactobacillus plantarum*

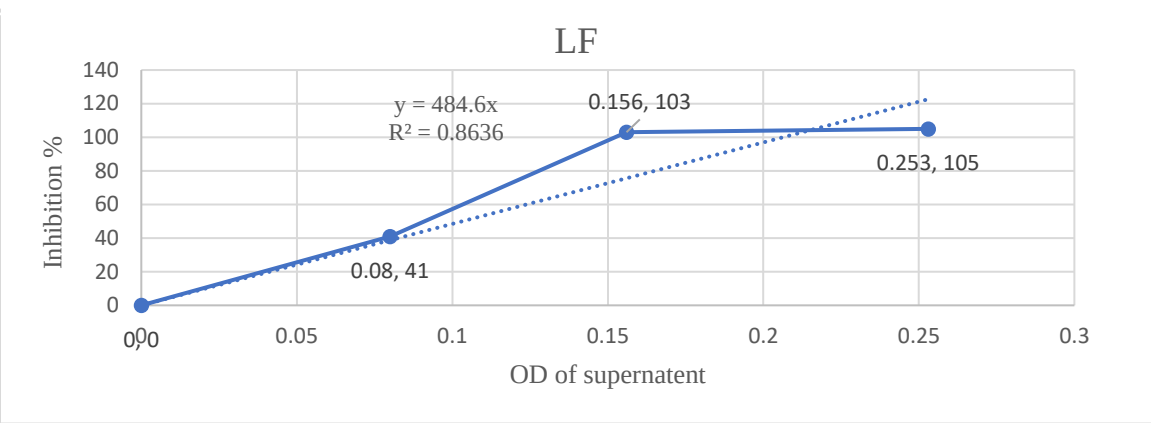

Figure 42: Graph of ACE inhibition assay for *Lactobacillus fermentum*

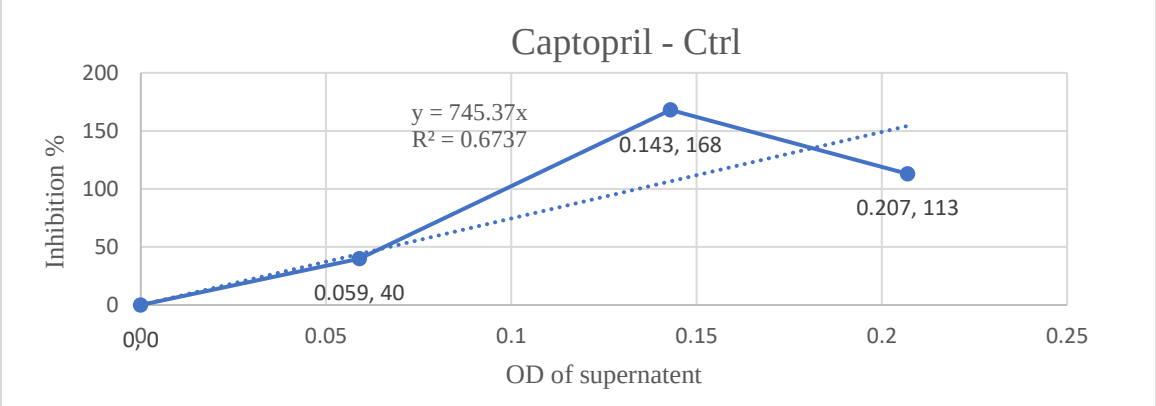

Figure 43: Graph of ACE inhibition assay for Captopril control

5.6. Discussion for ACE inhibition Assay

- The ACE inhibition assay was performed before purification and there was high inhibition %. *Lactobacillus plantarum* had shown the highest inhibition rate throughout all 3 tables.
- ACE inhibition lowers the risk of cardiovascular diseases by reducing angiotensin 2 in the body. The activity and inhibition rate indicate the presence of ACE inhibitory peptides present in *Lactobacillus spp.* in MRS medium (Fan et al., 2019).
- Further purification and characterization of the peptides wasn't performed, so we cannot determine the sequence of ACE inhibitory peptide.
- The readings were taken 3 times due to technical issues. However, due to same proportions of inhibition %, it is understood that Lactobacillus plantarum has ACE inhibitory peptides at higher activity than captopril positive control. This could be due to the dilution of the captopril to increase the volume of the ACE inhibitory drug.

- **Formula for ACE inhibition Assay**

  Formula for Extent of inhibition (%)

Extent of inhibition (%) = (B – A)/ (B – C) x 100

  (Ebrahimi, Ai, Alizadeh and Shariaty, 2017);

  A = Optical density in presence of ACE enzyme and supernatant

  B = Optical density without supernatant [Control]

  C = Optical density without ACE enzyme

- **From the graph for ACE inhibition assay:**
  - LP has max Ace inhibition at OD of 0.082 with 182% inhibition.
  - LF has max Ace inhibition at OD of 0.253 with 105% inhibition.
  - Captopril control has max Ace inhibition at OD of 0.143 with 168% inhibition.

6. CONCLUSION

The species that would be used for further characterization and purification for its bioactive peptides would be *Lactobacillus plantarum* due to its high ACE inhibitory activity and its consistent antimicrobial activity.

- From the study of growth kinetics of *Lactobacillus spp*., it is understood that the release of peptides for *Lactobacillus plantarum* is very late and has high activity at 71st h of growth kinetics. The doubling time for Lactobacillus plantarum has been the highest during the study of growth kinetics. This suggests that its growth rate is much slower than other species, but it has higher activity than other species. Hence, we can assume that slower growth rate isn't a negative attribute, but it might essentially provide consistent peptide activity.
- From Antimicrobial assay from MRS medium, Lactobacillus plantarum had the highest and most consistent inhibitory activity at 71st hour of growth kinetics and the average zone of inhibition for *Lactobacillus plantarum* at the 71st hour was 0.6 x 0.7 cm.
- From the ACE inhibition assay, *Lactobacillus plantarum* had highest ace inhibition at OD of 0.082 with 182% inhibition and *Lactobacillus plantarum* has consistent inhibitory activity in all 3 readings. It had the highest activity even to captopril which is an ACE inhibitor used in treatment for hypertension, diabetes, and Cardiovascular diseases (CVD).
- Lactobacillus plantarum can be further purified for its ACE inhibitory peptides which can be used to create newer, and effective drugs which can treat several medical conditions and several cardiovascular diseases (CVD) (Hamley, 2017).
- After confirming its potential in activity, the growth kinetics can be optimized for a good ratio of production: activity. After that, it can be purified to under 3 kDa for single activity ACE inhibitory peptides. Then the peptide can be characterized and sequenced using bioinformatics tools which can stimulate it under different conditions and to also check its binding to various receptors in the body (Korhonen, 2009).
- If the peptide is viable and has no toxic effects, then it can be manufactured after several clinical trials and tests along with a substrate molecule that maintains the stability of the peptide.

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