



Identification and Molecular Characterization of Bioactive Molecule from Rhizosphere Soil Organism

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INTRODUCTION

The soil environment is very complex and provides diverse microbial habitats which include a number of different kinds of Bacteria, Fungi, Actinomycetes, Algae, Protozoa etc. It varies greatly depending on any change in climate, organisms or land form. Some of the different types of soils are Black soils, Red soils, Laterite soils, Forest and Mountain soils, Arid and Desert soils, Saline and Alkaline soils and so on. Rhizosphere is the zone of soil which surrounds plant roots and most of the rhizosphere soil is believed to have more Bacteria, which is followed by Fungi. Most of the Rhizosphere organism are seen living in a mutual beneficial relation with the plants. Commensalist relationship is also seen with the other relations here. Root cells of plants are under continual attack from microorganisms and it survive by secreting defence proteins and other chemicals which are known as antimicrobial chemicals.

The bacterial domain leads the microbial population in the rhizosphere soil, followed by fungi, actinomycetes and other groups (Mendes et al. 2013; Nunes da Rocha et al. 2013). Among bacteria, the phylum proteobacteria represent the dominant members of the rhizosphere microbiome (Stephane Uroz et al. 2011) followed by other [Source: Mendes et al. (2013)] groups like Firmicutes, Actinobacteria, Acid bacteria, Bacteroidetes, Verrucomicrobia and Planctomycetes (Buée et al. 2009; Turner et al. 2013a). Next to bacteria, fungal communities make up a significant group of the rhizosphere microbiome.

For the treatment of diseases caused by such microorganisms, the inhibitory chemicals employed to kill micro-organisms or prevent their growth, are called antimicrobial agents. Antimicrobial agents are of two types based on their activity. These can act against bacterial infection either by killing the bacterium or by arresting its growth. They do this by targeting bacterial DNA and its associated processes, attacking bacterial metabolic processes including protein synthesis, or interfering with bacterial cell wall synthesis and function. Some of the Antimicrobial compounds that are naturally secreted are essential oils derived from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes obtained from animal sources (e.g., lysozyme, lactoferrin), bacteriocins from microbial sources (nisin, natamycin), organic acids (e.g., sorbic, propionic, citric acid) and naturally occurring polymers (chitosan) (Gutierrez et al., 2008, 2009). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action.

Attainment and maintenance of the native structural state is critical for biological activity in vivo. Polypeptide chains are composed of twenty different amino acids linked to each other via peptide bonds. The chain forms a repeating backbone with alternating side-chains of the amino acid residues (Richardson 1981, Branden and Tooze 1998). The

order of amino acids sequence as well as the length of the chain (number of amino acids) is determined by the genetic code. The sequence of the amino acids in a polypeptide chain defines the primary structure of the protein.

Current estimates for protein therapeutics project a growth rate between 7 and 15% annually for coming years (Walsh 2010). Therapeutic proteins are endogenous (or engineered proteins closely resembling endogenous proteins) and are therefore expected to have better specificity and safety profile as compared to the conventional small molecule drugs (Leader et al. 2008). The major challenges for developing protein drugs include cost, complex manufacturing, relative instability, inadequate pharmacokinetic properties and formulation in conventional dosage forms, such as tablets or capsules (Leader et al. 2008, Swami and Shahiwala 2013).

MATERIALS AND METHODS

Sample Collection

Rhizosphere soil was collected from different parts of the Botanical Garden of Kristu Jayanti College (Lat.-13° 3' 33.2989" N; Long.-77° 38' 29.9958" E) and Prajyoti study house (Lat.-13° 3' 29.2622" N; Long.-77° 38' 33.3229" E) Bangalore, by uprooting the plants and collecting soil at a depth below 30cm in clean sterile ziplock cover bags and preserved at -20°C.

Isolation of organism

One gram of each soil sample was taken and dispersed in 9ml of 0.85% saline water and vortexed for 5 minutes. After that serial dilution was carried out in order to isolate single colonies. 7 dilution blanks each containing 9 ml of sterile distilled water were first set up and numbered 1-7. 1 gm of the soil collected was added to the first tube. The contents of the test tube were gently mixed. This served as the stock solution. From the stock using a sterile micropipette, 1ml of the sample was transferred to the next tube. The same procedure was continued till the desired dilution was reached, that is till 6th dilution tube. From this final dilution tube, 1ml of the liquid was discarded off.(Booth.C.*et.al.*,2006).

Appropriate dilutions (10^3 - 10^6) were plated on nutrient agar plates, by taking 100 µl of suspension after brief vortexing. After that inoculated plates were incubated at 37°C for 24hours. Purity of the cultures were obtained by continuous sub culturing and were checked by staining.

Characterization of Bacteria

The selected colonies were identified based on their morphological, biochemical and molecular characteristics.

Morphological Characteristics-

Partial identification of bacterial colonies was done by using morphological and microscopic observation. Morphological observations were made on the colony size, surface, opacity, colour, motility, cell shape and its growth pattern. Microscopic observations were made on gram staining, endospore staining and motility test.

Gram staining

It divides the bacteria into Gram positive & Gram negative (Gram C. 1884). Bacterial smear was prepared and heat fixed onto a clean glass slide. It was flooded with Crystal violet for 1 minute and then washed off with water. The smear was then flooded with Iodine for another 1 minute and then washed off with water. Then it was washed with Ethanol-Acetone, the decolorizer. The smear was flooded with Safranin for 30 seconds and washed off with water. The slide was air dried and observed under 100x oil immersions.

Endospore staining

Bacterial smear was prepared on a clean grease free slide and heat fixed. It was covered with a square blotting paper that fit the slide and it was saturated using Malachite green stain solution and steamed for 5 minutes. The paper was kept moist by adding more dye as required. Then the slide was washed with water and counterstained with 0.5% safranin for 30 seconds and washed again with water. The slide was air dried and examined under the microscope at 100x oil immersion.

Motility Test (By Hanging Drop Method)

This test was performed to check whether the bacterium is motile or not. For this test a 6-hour old culture of the bacterium in peptone broth was used. A drop of the bacterial culture was placed in the middle of a cover slip. A thin line of petroleum jelly was placed along the edges of the cover slip. The cavity slide was turned upside down and was allowed to gently touch the cover slip. The entire cavity slide-cover slip combination was flipped over and was observed under the microscope (40 x). (Collee *et.al.*, 1996)

Biochemical Characteristics-

Biochemical characterization of the bacterial isolate was performed using tests such as Indole test, Methyl Red test, Voges Proskauer, Citrate, Catalase, Oxidase, Starch hydrolysis, Urea, Nitrate, Motility and Carbohydrate Fermentation Tests were performed. (Macfaddin, 1976).

Molecular Characterization of organism

To carry out molecular identification of the isolate, the isolate was grown overnight and subjected to DNA isolation. The purified genomic DNA of these isolate was amplified using 16S rRNA gene amplifying universal primers. 16S rDNA sequencing was performed using commercial service. These sequences were compared using NCBI BLAST (Basic Local Alignment Search Tool) service.

Protein Analysis

Extraction and Separation of Proteins

The bacteria are inoculated aseptically into Nutrient broth and its kept for incubation at 37°C for 24 hours. Then the broth is subjected to centrifugation at 10,000 rpm for 10 minutes at 4°C to avoid protein degeneration and separate the cells from the broth. The supernatant containing all the crude proteins produced by the organism is separated into a different sterile centrifuge tube. Now the supernatant is treated with saturated Ammonium sulphate solution and its kept overnight at 4°C for protein precipitation. Then this culture broth is centrifuged at 10,000 rpm for 10 minutes at 4°C to extract out all the proteins present in it. The supernatant is discarded and the protein pellet is stored in phosphate buffer saline at 4°C, which is used for further studies. (Ausubel et.al,1991) The proteins were separated using Centrifugal device. They were separated based on their size, two Centrifugal devices were used here, 1K and 3K. It was centrifuged at 10,000 rpm for 10 minutes at 4°C and the retentent as well as the separated protein filtrates were stored separately at 4°C for further studies. (Ausubel et.al,1991)

Protein Sample concentration and Molecular weight determination

Lowry's method was used for the estimation of total amount of proteins obtained in the Protein filtrates extracted from the bacterial culture. (Wilson, K. and Walker,2000) Microsep Advance centrifugal devices with molecular weight cut off 3K and 1 k were used for protein fraction concentration to preserve protein activity and low protein binding. The yield of protein fractions was found to be significantly increased with the centrifugal devices typically centrifuged for 5folds at 7,500 x g with 30 minutes of time period specifically at 20°C. The size and integrity of the respective protein fractions were analyzed using standard Protein Markers on SDS PAGE

TLC separation and Activity fraction studies

The concentrated sample fractions were further analyzed using standard marker using TLC with different types of mixed solvent system (Butanol: acetic acid: water, Ethyl acetate: Methanol and ethanol: water). The concentrated fractions were separated more compatible with ethanol and water solvent mixture.

RESULTS AND DISCUSSION

Isolation of Rhizosphere Soil Microorganism

11 different Bacterial colonies were isolated from the Rhizosphere soil sample collected from the Botanical garden of Kristu Jayanti College and these were sub cultured several times on Nutrient Agar Medium (figure.1). Out of these cultures, the bacteria which produced a different and unique colony morphology pattern was selected for further research



Figure.1- Bacterial Isolates from Rhizospere soil sample

Morphological Characterization

The isolated strain was morphologically characterized according to Bergey's Manual of Determinative Bacteriology (Krieg and Holt 1984). Observations were made on their phenotypic (visual) characters and microscopic analysis. The results

showed that the organism is a Gram positive, motile rod which grows in an opaque, filamentous rhizoid pattern on agar surface (fig. 2 and fig 3.). When the organism is plated on nutrient agar in Drop Inoculation Technique, the border of the colony appears to extend out like tree roots (fig.2). Details

of the colony features of the bacteria are noted down (Table 1).

Table 1. Morphological characterization of the organism

S.No.	Morphological features	
COLONY		
1.	Colour	white
2.	Form	Rhizoid
3.	Elevation	Raised
4.	Surface	Smooth
5.	Texture	Opaque
6.	Size	Large spreading
CELL		
7.	Gram stain	positive
8.	Shape	rods
9.	Spore stain	positive
10.	Motility	positive



Figure 2 - Drop inoculation of bacteria on Nutrient Agar plate



Figure 3- Gram Staining showing Gram Positive Rods

Biochemical characterization

A number of biochemical tests were performed for the identification of bacterial isolates with the help of Bergey's Manual. The tests used for this purpose are Indole test, Methyl Red test, Voges Proskauer, Citrate, Catalase, Oxidase, Starch hydrolysis, Urease, Nitrate, Motility and Carbohydrate Fermentation

test. The organism showed positive results for Methyl Red, Catalase, Oxidase, Starch, Glucose, Mannitol and Motility tests. It showed negative results for Indole, Voges Proskauer, Citrate, Urease and Nitrate tests. These are represented in Table 2 and figures 5-10.

Table 2. Biochemical Characterization of the Bacterial isolate

Biochemical Characterizations		
S. No.	Tests	Results
1.	Indole	Negative
2.	Methyl Red	Positive
3.	Voges Proskauer	Negative

4.	Citrate	Negative
5.	Catalase	Positive
6.	Oxidase,	Positive
7.	Starch hydrolysis	Positive
8.	Urease	Negative
9.	Nitrate	Negative
10.	Motility	Positive
11.	Glucose	Positive
12.	Lactose	Negative
13.	Sucrose	Negative
14.	Maltose	Negative

Molecular Characterization of Bacteria

The Molecular Characterization of the bacterial strain was performed using 16S rRNA gene sequencing. The sequence of the 16S rRNA gene was compared with GenBank entries, using NCBI Tool BLAST and EMBL- EBI FASTA program to understand the phylogenetic relationships. The sequence showed a similarity of 99% with *Bacillus altitudinis*, *Bacillus aerophilus* and *Bacillus pumilus*. From the multiple sequence alignment data and lineage report, it is inferred that the culture is showing affiliation towards *Bacillus altitudinis* and hence the culture under study is conclusively confirmed as *Bacillus altitudinis*.

The sequence of the bacterial isolate received after 16S rRNA sequencing is

P-PRASAD_SER395

LENGTH- 780

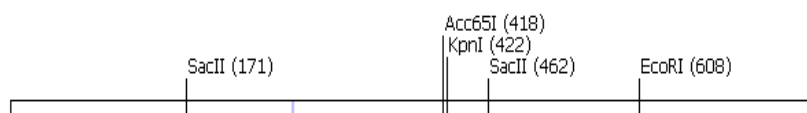
GGCGAGCTTCGCTCCCGGATGTTAGCGGCGGACGGGT
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GAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCT
GTCACCTACAGATGGACCCGCGGCATTAGCTAGTTG

GTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTT
GTTAGGGAAGAACAAGTGCAAGAGTAAGTCTTGAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGG
GAGGGTCATTGGAACTGGGAACTTGAGTGCAGAAAG
AGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAAGTGGCGAAGGCGACTCT
CTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGG
GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AACGATGAGTGCTAAGTGTTAGGGGGTTCCGCC

Restriction Sites

It was observed that the bacterial isolate consisted five Restriction Sites. SacII Restriction enzyme cuts it 2 times. The other Restriction enzymes that can cut this bacterial sequence are Acc651, EcoRI and KpnI. These results were obtained together with 16s rRNA sequence. This is represented in the fig.4.

Restriction map for P-PRASAD_SER395 (780 bases, linear DNA)
Displaying cut positions.



Unique cutters:

Acc65I, EcoRI, KpnI

Enzymes that cut two times:

SacII

Enzymes that cut three times:

None

Enzymes that cut four and more times:

None

Non-cutters:

ApaI, BamHI, Bsp68I, BstXI, EcoRV, HincII, HindIII, NotI, PaeI, PstI, SacI, SalI, SmaI, XbaI, XhoI, XmiI

Figure 4. Restriction map of the isolate sequence

NCBI BLAST Results –

Lineage Report

The lineage report gives a simplified view of the relationships between the organisms, according to their classification in the taxonomy database. This report is 'focused' on the organism which yielded the strongest BLAST hit. According to the generated

Lineage Report it was seen that the query sequence has 29 hits with the *Bacillus altitudinis* complex, in which maximum hits are showed by *Bacillus altitudinis*. Therefore, it can be inferred from the result data that the isolate is *Bacillus altitudinis* (figure 5).

Query ID	Id Query_123091	Database Name	nr
Description	P-PRASAD_SER395	Description	Nucleotide collection (nt)
Molecule type	nucleic acid	Program	BLASTN 2.6.1+ Citation
Query Length	780		

Organism	Blast Name	Score	Number of Hits	Description
root			101	
. Bacteria	bacteria		100	
. . Bacillus	firmicutes		99	
. . . Bacillus altitudinis complex	firmicutes		29	
. . . . Bacillus altitudinis	firmicutes	1395	20	Bacillus altitudinis hits
. . . . Bacillus aerophilus	firmicutes	1395	6	Bacillus aerophilus hits
. . . . Bacillus stratosphericus	firmicutes	1388	3	Bacillus stratosphericus hits
. . . . Bacillus sp. MSSRF N43	firmicutes	1393	1	Bacillus sp. MSSRF N43 hits
. . . . Bacillus pumilus	firmicutes	1393	25	Bacillus pumilus hits
. . . . uncultured Bacillus sp.	firmicutes	1388	3	uncultured Bacillus sp. hits
. . . . Bacillus aerius	firmicutes	1388	7	Bacillus aerius hits
. . . . Bacillus xiamenensis	firmicutes	1388	2	Bacillus xiamenensis hits
. . . . Bacillus sp.	firmicutes	1388	30	Bacillus sp. hits
. . . . Bacillus safensis	firmicutes	1388	1	Bacillus safensis hits
. . . . Bacillus subtilis	firmicutes	1388	1	Bacillus subtilis hits
. . . . uncultured bacterium	bacteria	1388	1	uncultured bacterium hits
. . . . uncultured prokaryote	unclassified	1393	1	uncultured prokaryote hits

Figure 12- Lineage Report showing maximum similarity of isolate sequence with *Bacillus altitudinis*.

Description Report

Description report clearly depicts that the bacterial isolate's sequence showed maximum similarity scores with the *Bacillus altitudinis* strain HQB235 and

Bacillus aerophilus strain HQB230 that is, 1395 and 99 % similarity. With reference to the Lineage report, it can be concluded that the isolate is more similar to *Bacillus altitudinis* than *Bacillus aerophilus*.

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/> Bacillus altitudinis strain HQB235 16S ribosomal RNA gene, partial sequence	1395	1395	99%	0.0	99%	KT758434.1
<input type="checkbox"/> Bacillus aerophilus strain HQB230 16S ribosomal RNA gene, partial sequence	1395	1395	99%	0.0	99%	KT758429.1
<input type="checkbox"/> Bacillus sp. MSSRF N43 16S ribosomal RNA gene, partial sequence	1393	1393	99%	0.0	99%	KU131261.1
<input type="checkbox"/> Uncultured prokaryote clone say K4.30. 16SF 16S ribosomal RNA gene, partial sequence	1393	1393	99%	0.0	99%	KP408433.1
<input type="checkbox"/> Bacillus pumilus strain MNP2 16S ribosomal RNA gene, partial sequence	1393	1393	99%	0.0	99%	JX385492.1
<input type="checkbox"/> Bacillus pumilus strain SC5G40151 16S ribosomal RNA gene, partial sequence	1389	1389	99%	0.0	99%	JX315323.1
<input type="checkbox"/> Bacillus pumilus strain PDD-5b-1 16S ribosomal RNA gene, partial sequence	1389	1389	99%	0.0	99%	DQ512749.1
<input type="checkbox"/> Uncultured Bacillus sp. clone TOPO14 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY911244.1
<input type="checkbox"/> Uncultured Bacillus sp. clone TOPO12 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY911242.1
<input type="checkbox"/> Bacillus pumilus strain J 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY950620.1
<input type="checkbox"/> Bacillus aerophilus strain IHB B 15224 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY933476.1
<input type="checkbox"/> Uncultured Bacillus sp. clone KE-04 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY274849.1
<input type="checkbox"/> Bacillus altitudinis strain BT3 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY820043.1
<input type="checkbox"/> Bacillus altitudinis strain FL65 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY818981.1
<input type="checkbox"/> Bacillus aerius strain FL64 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY818980.1
<input type="checkbox"/> Bacillus aerius strain FL63 16S ribosomal RNA gene, partial sequence	1388	1388	99%	0.0	99%	KY818979.1

Figure 5 – Description Report showing maximum similarity to *Bacillus altitudinis*

BLAST Graphic summary

Graphic summary shows that our query sequence has 99% similarity to *Bacillus altitudinis* (Accession no. KT758434.1), *Bacillus aerophilus* and *Bacillus pumilus*. The alignment result shows that *Bacillus*

altitudinis and *Bacillus aerophilus* have 777/778 (99%) identities with the isolate sequence whereas *Bacillus pumilus* have 776/777(99%). This is represented in figure 6.

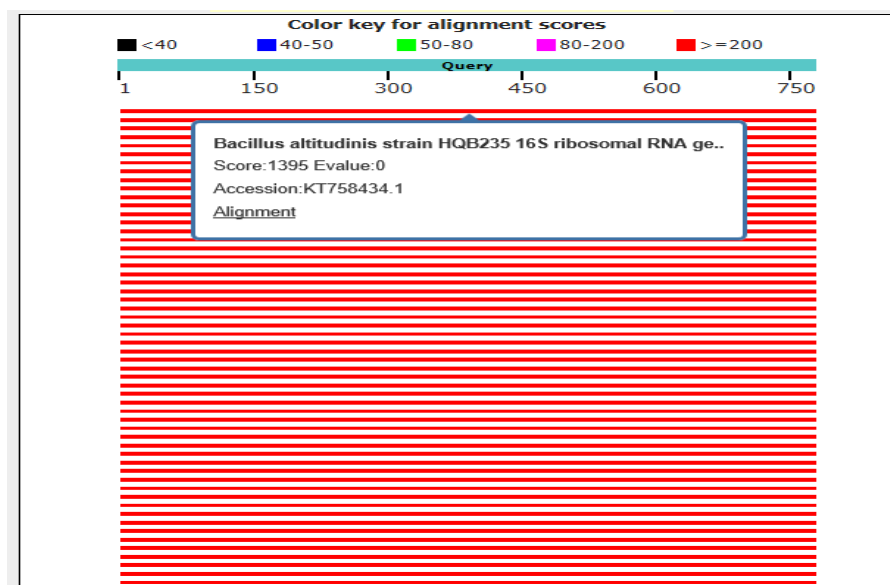


Figure.6 – Graphic summary shows that our query sequence has 99% similarity to *Bacillus altitudinis* (Accession no. KT758434.1)

Bacillus altitudinis strain HQB235 16S ribosomal RNA gene, partial sequence

Sequence ID: KT758434.1 Length: 1462 Number of Matches: 1

Related Information

Range 1: 45 to 822 GenBank Graphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
1395 bits(1546)	0.0()	777/778(99%)	1/778(0%)	Plus/Plus	

Features:

Query 4 GAGCTTCGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT 63

|||||

Sbjct 45 GAGCTTCGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT 104

Query 64 AAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGG 123

|||||

Sbjct 105 AAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGG 164

Query 124 TTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTA 183

|||||

Sbjct 165 TTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTA 224

Query 184 GTTGGTGAGGTAACGGCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG 243

|||||

Sbjct 225 GTTGGTGAGGTAACGGCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG 284

Query 244 CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC 303
 |||
 Sbjct 285 CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC 344

Query 304 GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA 363
 |||
 Sbjct 345 GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA 404

Query 364 AAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGACCTTGACGGTACCT 423
 |||
 Sbjct 405 AAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGACCTTGACGGTACCT 464

Query 424 AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCG 483
 |||
 Sbjct 465 AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCG 524

Query 484 TTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAG 543
 |||
 Sbjct 525 TTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAG 584

Query 544 CCCCCGGCTCAACCGGGGAGGGTCATTGGAACTGGGAACTTGAGTGCAGAAGAGGAGA 603
 |||
 Sbjct 585 CCCCCGGCTCAACCGGGGAGGGTCATTGGAACTGGGAACTTGAGTGCAGAAGAGGAGA 644

Query 604 GTGGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG 663
 |||
 Sbjct 645 GTGGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG 704

Query 664 GCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA 723
 |||
 Sbjct 705 GCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA 764

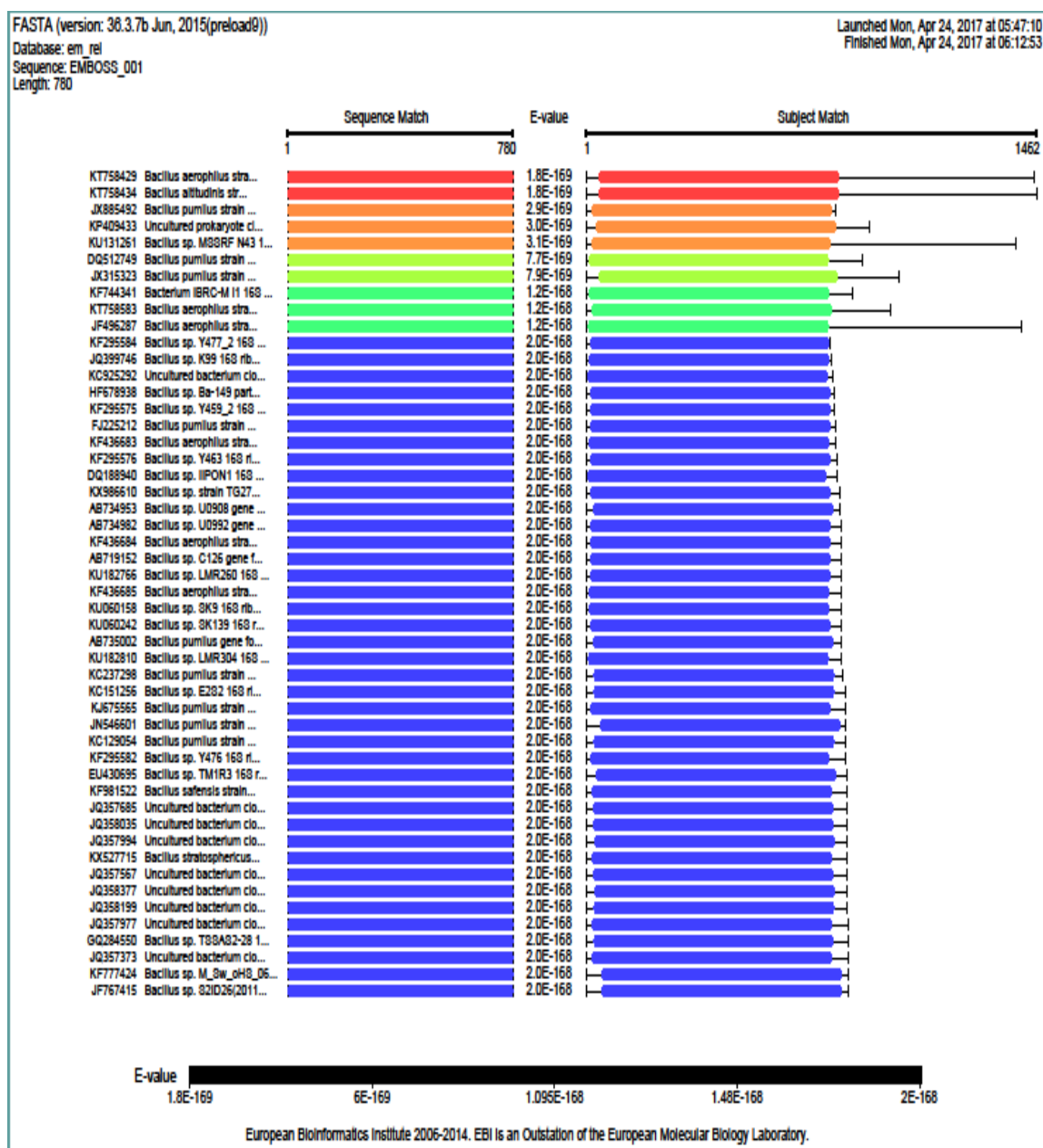
Query 724 GATACCCTGGTAGTCCACGCCGT-AACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCC 780
 |||
 Sbjct 765 GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCC

FASTA Graphic Summary

FASTA similarity sequence search was done in EMBL-EBI which showed same results as that from NCBI

BLAST search, that is, the isolate sequence is 99.7% similar to the *Bacillus aerophilus*, *Bacillus altitudinis* and *Bacillus pumilus* strains (figure 7).

Figure 7. FASTA similarity sequence search graphic summary showing maximum identities against *Bacillus aerophilus* and *Bacillus altitudinis*



Protein sample concentration and molecular weight determination of Rhizosphere soil organism

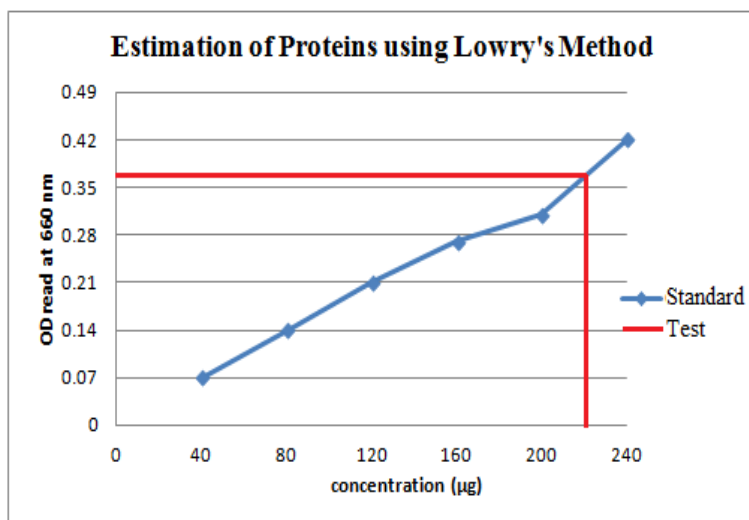


Figure .8- Estimation of total proteins using Lowry's Method.

Lowry's Protein estimation was also performed on different sizes of proteins which were separated using Centrifugal device into 1K,3K and its retentate.

The result showed that retentate contains maximum proteins. And there were more amount proteins in 3K as compared to 1K.

Figure:9 Molecular weight determination of 3K and 1 K Fractions using SDS

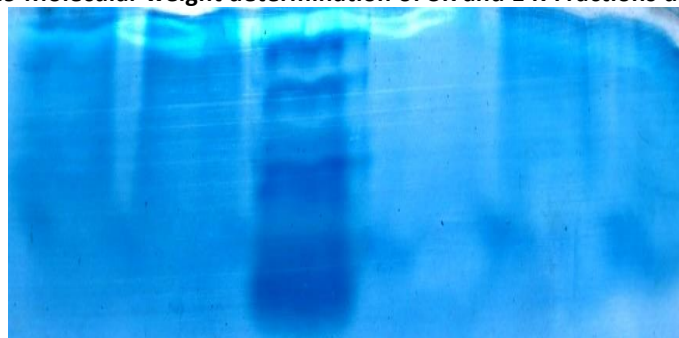
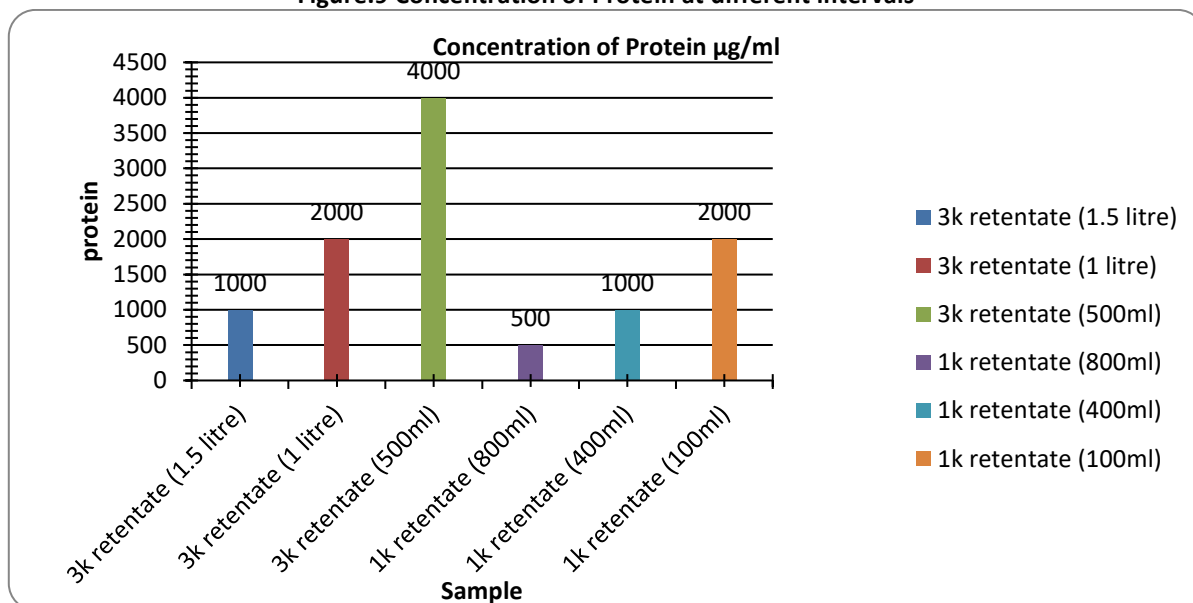
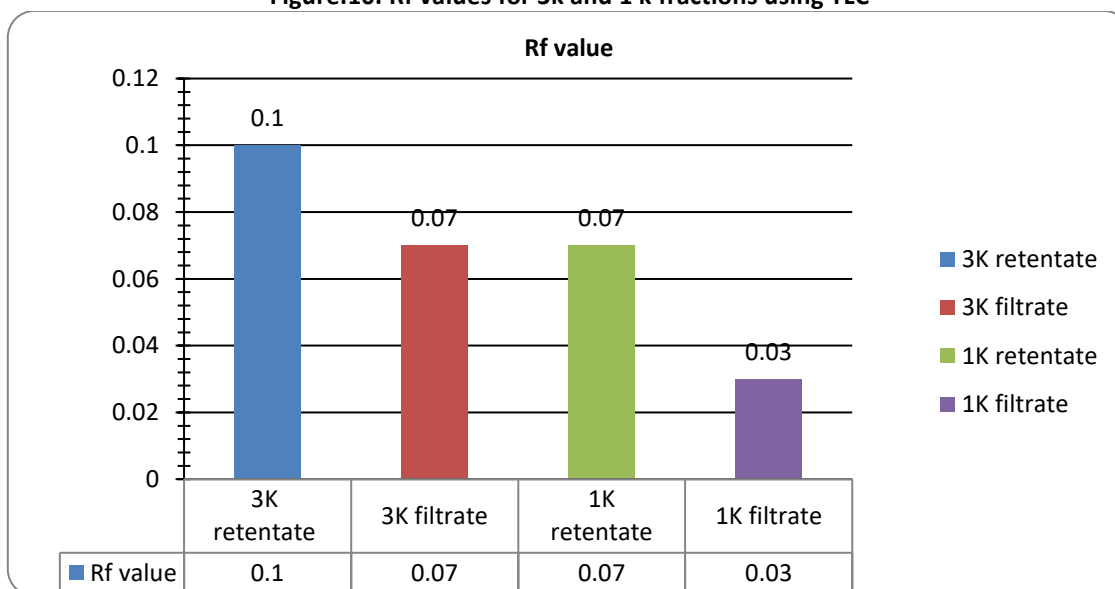


Table:3 Concentration of Protein at different intervals

Sample volume	Concentration of Protein µg/ml
3k retentate (1.5 litre)	1000
3k retentate (1 litre)	2000
3k retentate (500ml)	4000
1k retentate (800ml)	500
1k retentate (400ml)	1000
1k retentate (100ml)	2000

Figure.9 Concentration of Protein at different intervals

Table: 4. Rf values for 3k and 1 k fractions using TLC

Sample Fractions	Rf value
3K retentate	0.10
3K filtrate	0.07
1K retentate	0.07
1K filtrate	0.03

Figure.10. Rf values for 3k and 1 k fractions using TLC


The Protein fractions of 3k and 1 K were separated significantly with increased folds of concentration process. The concentrated protein fractions 3k and 1 k were further quantified periodically using Lowry's method (Table.No.1). The final desired volume of

concentrated sample is 500ml of 3k and 100ml of 1 k fractions were labeled accordingly and found to be stable at respective conditions will be suitable for further work. The Rf value of each separated fractions was calculated (Table No.2)

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