EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 11
266-275
Year: 2018

BIOTECHNOLOGICAL APPROACH OF PHYTOPATHOGENIC ORGANISMS (*LYSTERIA MONOCYTOGENES*) FROM THE LEAF IN *VIGNA RADIATA* (L.)

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Article Received on 25/08/2018 **Article Revised on 15/09/2018** **Article Accepted on 05/10/2018**

ABSTRACT
V. radiata (L.) is a legume cultivated for its edible seeds and sprouts across Asia. The present research work was aimed to identify the dominant autochthonous bacterial flora from the leaf blight disease causative agent on the experimental host plant of *V. radiata* (L.). Totally four different organisms were isolated and the dominant bacterial isolatesc were confirmed as "*Lysteria monocytogenes*" through sequence analysis. Proteins are the major biological factor that plays an important role in the bacterial development and various physiological processes. Consequently, the protein profile of the phytopathogenic bacterial strains especially *L. monocytogenes* has been identified its proteins polypeptides in the form of banding pattern throuha technique of SDS-PAGE. Interestingly PPB3 (*Lysteria monocytogenes*) bacteria expressed the total of six bands, of these six one band was almost prominently thickened appearance than the other five derived bands. In addition these bacteria possessed the highest and lowest molecular weight range between 279 to 162 kDa protein band intensity. From the overall result, interestingly it was revealed that the determined protein SDS banding pattern of disease causative agents. This was the first recordically reported work for assessed the protein profiling of the disease causative organism of *L. monocytogenes* from the legume plant especially leaf of *V. radiata* (L.) plant. This is the first recordically reported work for assessing the protein profile of the disease causative organism from the legume plant especially leaf of *V. radiata* (L.) plant.

KEYWORDS: *V. radiata*, leaf blight disease, sequence analysis, SDS-PAGE.

INTRODUCTION
Legumes are grown agriculturally, primarily for their grain seed called pulse, for livestock forage and silage, and as soil-enhancing green manure. *Vigna radiata* (L.) Wilczek [Synonyms: *Phaseolus radiata* L. (1753), *Phaseolus aureus* Roxb. (1832)], often known as green gram or mung bean, is native to India and Central Asia. It has been grown in these regions since prehistoric times (Vavilov, 1926) and as an important legume crop in India throughout the year. It is an important source of protein and several essential micronutrients. It contains 24.5% protein and 59.9% carbohydrate, 75 mg calcium, 8.5 mg iron and 49 mg B-carotine per 100g of split dual (Bakr *et al*., 2004). Every 100 g of edible portion of green gram seed contains 75 mg calcium, 4.5 mg phosphorus, 24.5 g protein and 348 kilo calories energy (Meena *et al*., 2013).

Plant pathogenic bacteria are heterotrophic organisms, which can develop on host plants as parasites. They are not able to penetrate directly plant tissue. Infection usually occurs through natural openings like stomata, hydatodes, nectaroides as well as injures of various origin. The important places of infection appeared to be leaf scars (Billing, 1987; Agrios, 1997; Sobiczewski, Schollenberger, 2002; Kryczyński *et al*., 2002; Janse, 2005). Necroses, blights, cankers, wilting, soft rots and tumours are the main symptoms of bacterioses. The range of host plants of particular pathogens varies from very wide – consisting of several hundred plant species to very narrow (Billing, 1987; Klement *et al*., 1990; Agrios, 1997).

Mungbean is attacked by different species of insect pests but sucking insect pests (aphid, jassids, white leaf hopper and whitefly) are of the major importance (Islam *et al*., 2008; Swaminathan *et al*., 2012). These insect pests not only reduce the vigor of the plant by sucking the sap but transmit diseases and affect photosynthesis as well (Sachan *et al*., 1994). Under severe cases stem fly may alone cause more than 90 per cent damage with a yield loss of 20 per cent. Whitefly, a potential vector of mungbean yellow mosaic virus (MYMV), can cause losses ranging from 30–70 per cent (Talekar, 1990). In India the girdle beetle, *Oberiopsis brevis* (Swedenbord), a major pest of soybean, sometimes infests mungbean

locally (Talekar, 1990). The field infestation ranges from 7.8–9.9 per cent and 100 per cent destruction of seeds occurred at 9.9 per cent field infestation (Banto and Sanchez, 1972). The annual yield loss due to the insect pests has been estimated to the tune of 30 per cent by Soundararajan and Chitra (2011) in green gram and Justin, *et al.,* (2015) in urd bean and green gram.

MATERIALS AND METHODS

Preparation of sample

The diseased plant *V. radiata* from legume family was collected from in and around of Annikarai (Tamilnadu). The diseased leaf part is separated on the basis of color (brown, yellow and dried green). The brown, yellow and dried green colored leaf samples are grinded separately with distilled water in mortar and pestle. The extract is collected and spread on agar plates. After colonies are grown, they are counted and the number of bacteria in the original sample was calculated. The colonies produced are inoculated in agar slants and used for further studies.

Gram staining

Identification of microorganisms isolated from plant disease parts were done by gram staining. Placed slide with heat fixed smear on staining tray. Gently flooded smear with crystal violet and allowed to stand for 1 minute. Then the slide was gently rinsed with distilled water using a wash bottle. Gently flooded the smear with Gram's iodine solution and allowed to stand for 1 minute. Then the slide was slightly rinsed with distilled water using a wash bottle. The smear appeared as a purple circle on the slide. Decolorized the slide using 95% ethyl alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Immediately rinsed with water. Gently flooded with safranin to counterstain and allowed to stand for 45 seconds. Then the slide was slightly rinsed with distilled water using a wash bottle. Blot dried the slide and viewed the smear using a light microscope.

Sequencing

Plasmid minipreps were prepared from recombinants using the Promega Wizard Plus purification system (Promega UK Ltd.) according to the manufacturer's instructions. Sequencing was performed using the Thermo Sequenase sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Amersham, United Kingdom). The sequencing reactions were set up with 5µl of plasmid DNA, 1 µl of sequencing primer (M13 Universal [fB1/rULWSI – X68376 – 5' GACCCTTCAAAAGGTCTTAG 3' and Reverse [P1/WXint – U54992 5'-GACAGTGTGCTTATAACTTTTA -3'] both labeled with IRD800 dye), 0.7 µl of dimethyl sulfoxide, and 14.3 µl of sterile molecular biology-grade MilliQ-grade water (Millipore). For each clone, 4.5 µl of the sequencing reaction was added to 1.5 µl (each) of A, C, G and T reagent (primer termination mixes for each dideoxynucleotide). A commercial master mix kit was used for PCR amplification under the following cycling conditions: 1x15 min at 95°C, 32x (for the 8f-534r set) or 35x (for the 968f-1401r set) 15 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C, and 1x1 min at 20°C. Optimal amplification conditions were defined for each primer combination by the cycle number before the real time PCR amplification curves entered a plateau with no further increase of total fluorescence. Cycling was performed on a Stratagene MX3000p (Strata gene, Amsterdam, Netherlands). PCR products were removed immediately after the last cycle and stored at -20°C until further analysis (Duan *et al*., 2010).

Sequence analysis

Sequences obtained from the LI-COR image system program were converted to FASTA format and analyzed for chimeric forms using the Chimera-CHECK 2.7 program from the Ribosomal Database Project II. After elimination of chimeric sequences, the partial 16S sequences were then compared with 16S rRNA gene sequences from the public signature sequences.

SDS-PAGE ANALYSIS

Procedure for Electrophoresis Reagents Preparation

Stock solutions

Acrylamide 30% - Bisacrylamide (29.2:0.8) was prepared by mixing acrylamide (29.2 g) and bisacrylamide (0.8 g) in 100 ml distilled water. The solution was filtered through Whatman No.1 filter paper and store at 40°C in a dark bottle. Separating gel buffer (resolving gel) (1.5 M Tris HCl pH 8.8) – 18.17 g Tris was dissolved in approximately 40 ml of distilled water and adjusted the pH to 8.8 with 1N HCl and the final volume was make upto 100 ml. Stacking gel buffer (1 M Tris HCl pH 6.8) - 12.14 g Tris was dissolved in approximately 40 ml of distilled water and adjusted the pH to 6.8 with 1N HCl and the final volume was make upto 100 ml. 10% SDS (Sodium Dodecyl Sulphate) – 1 g of SDS was dissolved in 10 ml of doubled distilled water. The solution was clear and colourless and kept at room temperature. 10% Ammonium per sulphate (APS) – 100 mg of APS was dissolved in 1 ml of distilled water. APS is unstable and decomposes readily at room temperature. TEMED (N, N, N, N – Tetra methyl ethylene diamine) – This reagent was acting as a catalyst for gel formation. Electrode buffers (Reservoir Buffer) – 3.028 g of Tris, 14.45 g of Glycine, 0.5 g of SDS were mixed with 500 ml of distilled water. The solution was stored at 4°C in a dark bottle. Comassive Brilliant Blue stain – 50 ml methanol, 7 ml acetic acid and 200 mg comassive brilliant blue wer mixed with 43 ml distilled water. The solution was blue in color and kept at room temperature. Destaining solution – 30 ml ethanol, 67 ml of distilled water were mixed with 7 ml of acetic acid solution. The solution is colorless and kept at room temperature. This solution once used could be reused. For this purpose, after destaining add a teaspoon of activated charcoal to the solution and allowed to settle the impurities properly. Then the blue color will disappear and the solution was filtered through Whatman

No.1 filter paper and this could be used for destaining again. Sample buffer (3 ml) – It is prepared by mixing 3 ml each of 0.5 M Tris HCl (pH 6.8) and 0.3 ml of 10% SDS, 2.4 ml β-mercaptoethanol, 3 ml glycerol, 3 ml distilled water and 1 pinch of bromophenol blue. The solution is blue in color and stored at 4°C. Resolving gel (15 ml) – It is prepared by mixing 4.9 ml distilled water, 6 ml acrylamide, 3.8 ml Tris (pH 8.8), 0.15 ml of 10% SDS, 0.15 ml of 10% APS, 0.006 ml TEMED and allowed 20-30 minutes for polymerization. Stacking gel (4 ml) – Prepared by mixing 2.7 ml distilled water, 0.067 ml acrylamide, 0.5 ml Tris (pH 6.8), 0.04 ml 10% SDS, 0.04 ml 10% APS and 0.004 ml TEMED. 7% Acetic acid – Mix 7 ml acetic acid and 93 ml distilled water and this solution was used for the preservation of the gel.

Protein sample preparation for electrophoresis

1 ml of the bacterial culture was taken and centrifuged at 6,000 rpm for 10 minutes. The growth media was carefully removed by leaving the bacterial pellet on the bottom of the tube. Add 100 µl of the sample loading buffer to each bacterial pellet and mix by gently pipetting the sample buffer in and out of the pipette tip. Incubate the mixtures at 100°C for 5 minutes in the heating block. The sample is then used for the electrophoresis.

Working Method of Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out using the method of Jeyaraman (1996) with minor modifications. A sandwich was made with two glass plates separated by spacer strips. The spacer strips were coated with Vaseline for adhering mechanisms. The glass plate was kept vertically by placing it on to a stand, which could hold the plates vertically. Few ml of distilled water was poured between the plates to check leakage if any. The resolving gel of 12% (pH 8.8) was poured into the space between the glass plates after removing distilled water. The level should be about 2 cm below from the notch. It was kept undisturbed for polymerization for about 30 minutes and then make a layer of distilled water on the surface of the resolving gel, to avoid contact between the gel surfaces, air and also to make an even surface.

When polymerization was completed the distilled water was removed and the stacking gel of 7.5% (pH 6.8) was poured over the resolving gel and the Teflon comb was inserted into the gel, and allowed to polymerize for 30 minutes. After polymerization, the glass plates were clipped out from the stand and also the bottom spacer was removed. The plate was attached to the electrophoresis apparatus. The electrode buffer was poured to the lower and upper chamber. Then the Teflon comb was carefully removed from the gel, and the sample was added to each well at a volume of 25 µl with the help of micropipette. Marker protein of 14-100 kDa was loaded in one well as standard. Initially a current of 60 V was supplied and when the sample enters into the resolving gel the voltage was continued to 120 V till the marker dye reaches the bottom of the separating gel. At the end of electrophoresis, the gel was removed from the glass plates using a spatula and introduced into the trough containing staining solution for overnight and destaining was done until a clear band could be seen.

RESULTS AND DISCUSSION

Table 1 shows the result of total autochthonous phytopathogenic bacterial population present in the diseased leaf of the legume plant *V. radiata*. Mainly four different coloured colonies are observed, among the four PPB4 shows too numerous too count in nature, followed by other three organisms were countable numbers. While, PPB3 yellow appearance bacterial strain was noticed at 80±5.44 dominant percentage of the pathogenic agent. In addition other two types of colonies white cream nature bacteria showed 4.63±1.05% and 14±2.14% of white leafy appearance colonies also been noticed form the diseased leaf.

Table 1: Autochthonous Pathogenic Bacteria floral population from the host plant of diseased leaf of *V. radiata* (L.).

S. No.	Observed plant pathogenic organism	Color and shape of colonies	Number of observed colonies	Appeared colonies (in %)
1.	PPB1	White cream leafy growth	5±1.24	4.63±1.05
2.	PPB2	White round	21±3.46	14±2.14
3.	PPB3	Yellow round dot growth	120±7.95	80±5.44
4.	PPB4	Yellowish White	TNTC	TNTC

Table 2: Morphological view of plant pathogenic bacteria isolated from the diseased *Vigna radiata* L. leaf extract.

Bacteria No.	Gram Reaction	Shape
PPB1	+ ve	Rod
PPB2	+ ve	Rod in group
PPB3	+ ve	Chained rod
PPB4	- ve	Cocci

The morphological view of the phytopathogenic bacterial strains were observed through the electron microscope (Plate 8). Uniformly the first three bacterial strains were observed to be gram positive and the PPB4 was gram negative. The PPB1, PPB2, PPB3 and PPB4 were rod, grouped rod, chained rod and cocci respectively (Table 2).

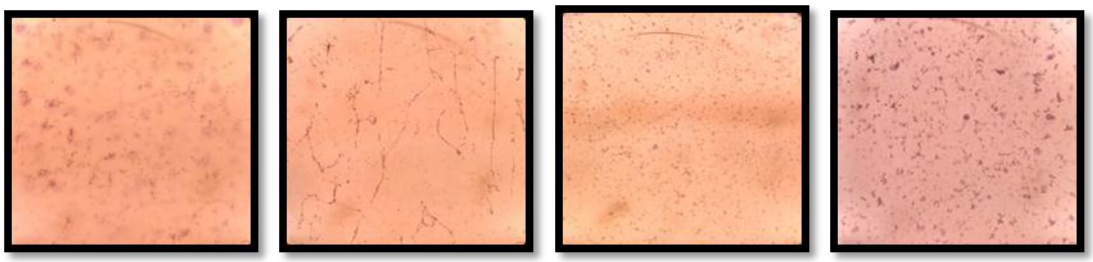

Plate 1.1. PPB 1 **Plate 1.2. PPB 2** **Plate 1.3. PPB 3** **Plate 1.4. PPB 4**

Figure 1: Gram stained microscopic view of isolated pathogenic bacteria from diseased *V. radiata* (L.) leaf extract.

Table 3: Biochemical Characterization of Plant Pathogenic Autochthonous Bacterial flora from *V. radiata* (L.) Plant.

Sl. No.	Characters	PPB1	PPB2	PPB3	PPB4
1.	Cell diameter (mm)	0.8-0.9	0.7-0.8	0.8-0.89	0.8-0.92
2.	Spore	+	+	-	-
3.	Motility test	+	+	+	-
4.	Aerobic growth	+	+	+	+
5.	Urease test	-	-	-	+
6.	Catalase test	+	+	+	+
7.	MR	-	-	-	-
8.	VP	-	-	-	-
9.	Citrate utilization	+	-	-	+
10.	Protease	-	-	-	-
11.	Gelatin hydrolysis	+	+	+	-
12.	Casein hydrolysis	+	+	+	-
13.	Lipase	-	-	-	-
14.	Tributyrin hydrolysis	ND	ND	ND	-
15.	Tween 80 hydrolysis	ND	ND	ND	-
16.	Starch hydrolysis	+	+	+	-
17.	Oxidase production	-	+	-	+
18.	Nitrate reduction	+	-	-	+
19.	Chitinase production	-	-	-	-
20.	Argine dihydrolase	-	-	-	-
21.	Glucose	ND	ND	+	-
22.	Fructose	ND	ND	+	-
23.	Mannose	ND	ND	+	-
24.	Gas production	-	-	-	-
25.	Indole	-	-	-	-
26.	PHB	ND	ND	+	-
27.	Growth pH 5-7	+	+	+	+
28.	Growth 40°C	+	+	+	+

Sequence Analysis
Name of the Identified Organism of your Sample (SID-4): *Lysteria monocytogenes*
Type: Species-specific signature sequences

Figure 6: The PCR primers which target the 16S rRNA Sequence Analysis.

Primer
IA-323--5'-TGTACACACCGCCCGTC-3′
-3'-CTCTGTGTGCCTAGGTA-5 ′

Forward Cycle

ATCAGCAACTAGCCAAGCTACTTCTTCAGCTGCTTCAACTGCAACGAGTGCCAGTGCTACTAGTGCTTCTT
CACAATCAGTTGCTAGCCAAGCTACTAGTTCTGCTACTACTTCTTCAGCTGCTAGCCAAGCTACTTCAA
GTGTTGCAACGAGTGCTGCTTCTTCAGCAAGCTCAACGCAATCAGCTGCTAGCCAAGCTTCAAGTTCTGC
TGCAACCACTAGTCAAGCTTCATCAAGTGCTACTAGCTCAGCTGCATCATCAACAGCTTCAACAACCTCA
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G CATGTTAGGCAGTATGTTGTTGATTGGCCTGATTTTCCAGCTACCACGCTTAACCCAACTCCCATCCTCA
TTAACAACTGGGGTCGAAGAGCTGTTCTTTTTAGCAATTGCACTCATATTAAATCGTGACTGGCTACGGT
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TCACCAAACACTTAGTTTAACCCTATTGCAATTGGGCTTTACGTTCGTACTGGGGCTACTATTATGTTTA
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TAATACGTATGGGAAATAAATTTCATGAATCCCACCAAGAAAATGAATAATAATCGCACTGGGTGCAGA
AGTCCCTTTACCAAACAAGGCAAAGGCTAATAAGACCCCAAGACCTGGACCTGGATCTGGTTCTAGCAA
GAACAGAATTGACTTACCCGCACTAGATGCAGCCTGAATCCCAAGCGGTGTTAAG

Reverse Cycle

GATTGGATGGCAATCCCAAGACCACTAGGCCCTCCTGCTGATACTTCTGATAAAGTTCTTCTAATCCTTT
AAACTGTGGCGCCAGGCCACAGTTGCTAGCCGTATTCACGATTAGCAAGACCTGACCGCGATATTGTGAC
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TTACTTTGTAGATTAGCATGTTTAATTTCAATTTGCAAGAACCATTGTTTTTTAAAAGTTTTTTCAGTAA

Fig- 1a:- Ethidium Bromide stained gel of PCR amplification products from the leaf blight disease caused bacteria of *Lysteria monocytogenes* in *V. radiata* leaf.

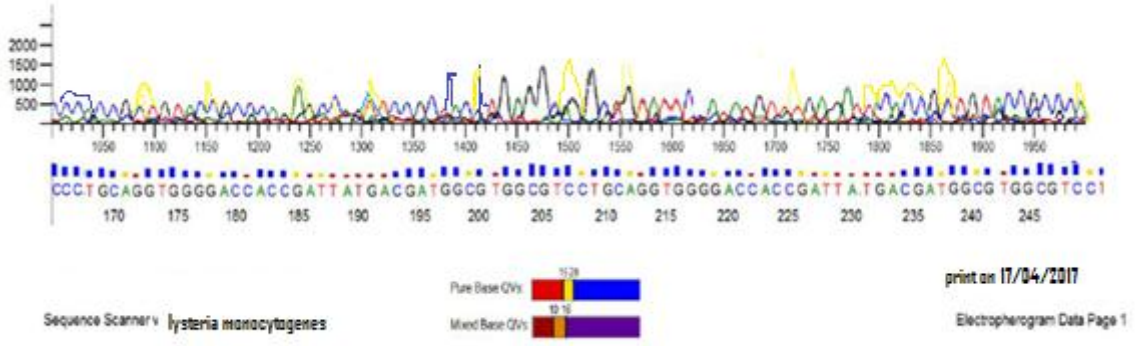

Fig- 2a:- Electropherogram of the experimental Sample of the plant pathogenic organism.

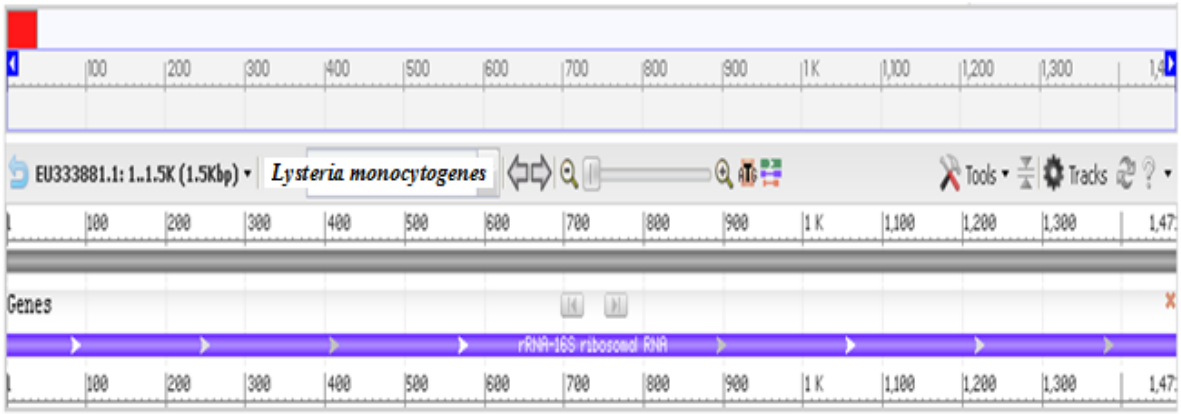

Fig – 2b:- Graphic view from the conformation of Experimental plant pathogenic organisms of *Lysteria monocytogenes.*

Sequence Analysis

The present research work aimed to identify the dominant autochthonous bacterial flora from the leaf blight disease causative agent on the experimental host plant of *V. radiata* (L.). Totally four different organisms were isolated and name was assigned as PPB1, PPB2, PPB3 and PPB4. Among the four isolated organisms PPB4 only dominantly present as well as main agent for disease causing in the host plant. Subsequently, when the experimental plant extracts based AgNP were treated with these four autochthonous bacterial populations significant antibacterial efficiency was noticed against the particular organism. PPB4 was observed to be dominant and hence the reason the present work was designed to know the specific name of the phytopathogenic bacterial isolates. Therefore, after sequence analysis it was confirmed through the amplified PCR product that the748 kilo base pair, such a particular plant dominant PPB4 isolate name was "*Lysteria monocytogenes*"**.** It was represented the sequence analysis confirmatory figures stated on Figure- 1 to 1 a, b and c.

SDS – PAGE Analysis

Proteins are the major biological factor that plays an important role in the bacterial development and various physiological processes. Plate 2 illustrates the SDS-PAGE protein patterns of the total body mass of the experimental phytopathogenic bacterial strains named as PPB1, PPB2 and PPB3 (Lane 2 to Lane 4). Total whole mass of the particular above mentioned phytopathogenic bacterial strains showed one, nine and six polypeptide bands in PPB1, PPB2 and PPB3 respectively. It was maximum number (9) of bands such as mild and thick intensity bands were noticed in PPB2. Furthermore Lane 2 PPB1 bacterial strain consist of one bright protein band and its respective molecular weight range was 187 kDa. While in another bacterial strain PPB2 clearly revealed that totally nine bands and its highest and lowest molecular weight range between 210 to 159 kDa. Among the nine bands three bands were thickened intensity also other six bands showed the faded appearance. Interestingly PPB3 (*Lysteria monocytogenes*) bacteria expressed the total of six bands, of these six one band was almost prominently thickened appearance than the other five derived bands. In addition these bacteria possessed the highest and lowest molecular weight range between 279 to 162 kDa protein band intensity. From the overall result interestingly it was clearly revealed that the determined protein SDS banding pattern of disease causative agents were identified from the *V. radiata* diseased leaf. This was the first recordically reported work for assessed the protein profiling of the disease causative organism of *Listeria monocytogenes* from the legume plant especially leaf of *V. radiata* (L.) plant.

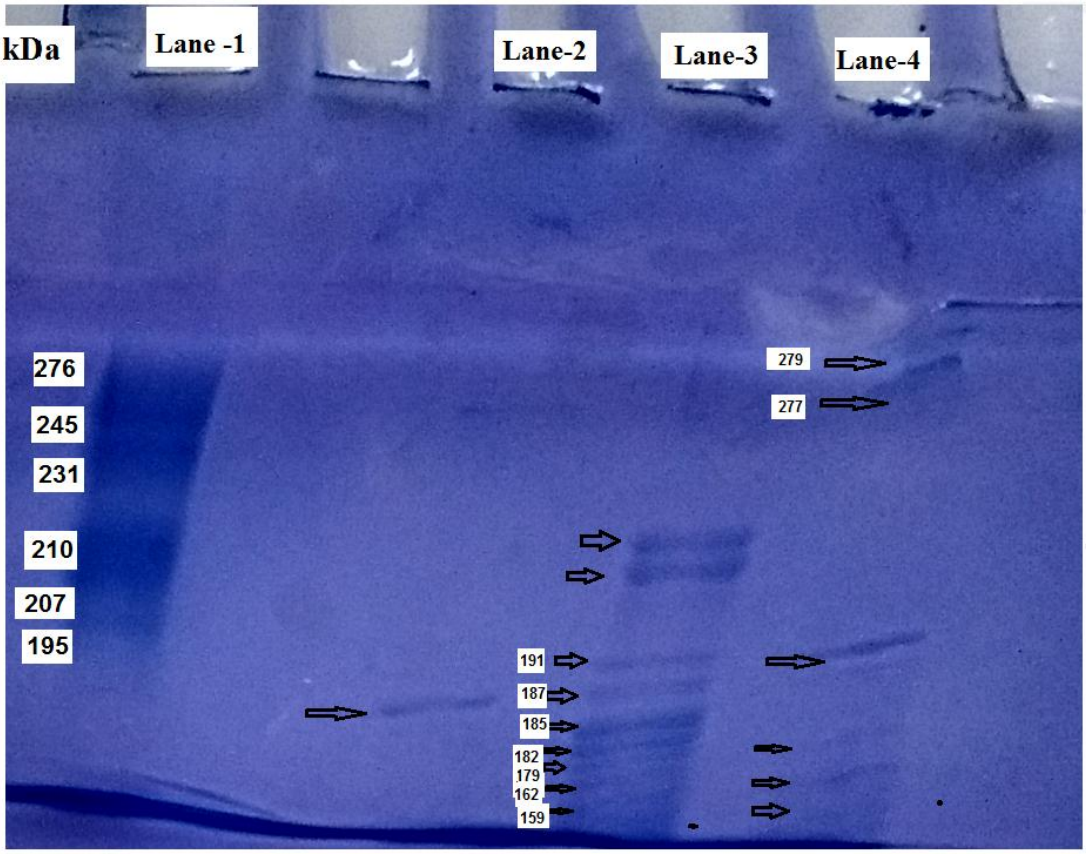

Fig- 3: SDS-PAGE protein pattern of experimental phytopathogenic strains from the diseased leaf in *V. radiate.*

CONCLUSION

The present work was clearly concluded that when the diseased leaf of *V. radiata* (L.) dominantly affected by the phytopathogenic bacterial strain named as *Lysteria monocytogenes.* It was the first report proved with help of recent advanced techniques of sequencing anlysis. Moreover, the total whole mass of the particular above mentioned phytopathogenic bacterial strains showed one, nine and six polypeptide bands in PPB1, PPB2 and PPB3 respectively, inspite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to gain knowledge about the phytopathogenic organisms.

ACKNOWLEDGEMENT

We would like to sincerely express thanks **Rev. Fr. Jose Bright** Secretary and Bursar and deep warm thanks to give our Principal **Dr. J. Thamithanka Kumaran** Malankara Catholic College, Mariagiri for providing the facility and encouragement for publication of this manuscript. We would also like to acknowledge all the staff members and Lab assistant staff in the Biotechnology Department in MCC for providing the necessary facilities for completion of this work.

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