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Isolation and characterization of Gram-positive and Gram-negative Bacterial Membrane Vesicles using Poly Ethylene Glycol Method

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Abstract

Membrane vesicle secretion was predicted earlier as a method of elimination of proteins, lipids, and RNA from the cell. At present vesicles are thought to be a brand-new type of intercellular communication and they are playing a crucial role in numerous physiological and pathological processes. However, in this expanding field, much remains unknown regarding the origin, biogenesis, secretion, target and fate of these vesicles. Vesicle research might become a more established field as a result of a complete understanding of the isolation and analysis techniques currently in use, making it possible to utilize membrane vesicles in therapeutic settings. Thus, standardization of the isolation of vesicles is in great need. This research article deals with the isolation and characterization of two distinct bacterial species-derived membrane vesicles namely, *Lactobacillus acidophilus* a human gut Bacterial MVs and *Pseudomonas syringae,* a model phytopathogen.

Keywords: Membrane vesicles, isolation, characterization, *Lactobacillus acidophilus* and *Pseudomonas syringae.*

Introduction

Communication within the Bacterial MVs community and also between the Bacterial MVs and host was reported to happen through a quorum sensing system. Later, the nano-sized disposed material from the bacterial cells known as membrane vesicles (MVs) which are released to maintain cell homeostasis also shed light on cell-to-cell-communication (1). Further, vesicles are of greater interest due to their emerging appreciation for interspecies as well as interkingdom communication. These are considered extracellular carriers of nucleic acids and proteins. To avoid quick disintegration and circumvent immune surveillance, bacterial cells wrap cargo molecules in MVs resulting in both local and long-distance intercellular communication. Growth stage and stress are two elements that have an impact on the production of outer membrane vesicles (OMVs), with prior studies indicating that production peaks during the late log and early stationary phase (2). Interestingly, both pathogenic and non-pathogenic bacteria are capable of modulating their respective host immunity. Human health and food security are considered the two sides of the same coin with regard to health conservation.

The human gastrointestinal system, also referred to as the “Gut microbiome,” has the largest density of bacteria. Among these *Lactobacillus acidophilus* a well-known probiotic prompts in revamped health benefits.

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
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Membrane vesicles (MVs) of *L. acidophilus* size range fell within 20-400nm and are enriched with bacteriocin (LBA1805), inducer peptide (IP1800), mucus binding protein (Mub), surface protein FmtB and surface layer protein SlpX (3,4).

On the other hand, substantial research underwent in the context of interactions between bacterial vesicles and human hosts while meager evidence was shown to support bacterial vesicles and plant host interactions. Thus, investigation into the interaction between bacterial vesicles and plant hosts may shed light on effective immune modulation, plant health protection and disease control through inhibition of pathogen ingress. The plant-pathogen interaction was the surging area of research interest whereas plant-pathogenic vesicle interaction might provide an in-depth knowledge in immuno-modulation which further supports sustainable agriculture. The model phytopathogenic bacteria *Pseudomonas syringae*, Gram-negative rod-shaped bacteria which causes infections in a wide range of plant species exhibits the property of vesiculation. Vesiculation of *P.syringae* was shown to be greatly influenced by growth stages and stressors (2). Outer membrane vesicles (OMVs) of *P. syringae* are comprised of toxins and other virulence factors that are capable of internalization into host cells. The well-characterized research by McMillan (2) unveiled that the *P.syringae* OMVs modulate plant host immunity and the components that are responsible for modulating the host (*Arabidopsis*) include lipids, carbohydrates, small molecules and other molecules.

This article deals with the isolation and characterization of vesicles from both *L. acidophilus* a probiotic human gut bacteria and *P. syringae* a phytopathogenic bacteria.

Materials and Methods

Bacterial strains and growth conditions

Lactobacillus acidophilus MTCC 10307 was regularly cultured in MRS broth and incubated for 24hrs at 37˚C. Subsequently sub-cultured in MRS broth and incubated for 6hrs, 8hrs and 13hrs to obtain early log, mid-log and late log phase respectively. Similarly, *Pseudomonas syringae* MTCC 11950 was cultured in LB broth and incubated for 24hrs at 30 ± 7˚C. Further, strains are sub-cultured in KB broth and incubated for 9hrs, 11hrs and 13hrs to obtain early log, mid-log and late log phases respectively. All the three above-mentioned phases of both bacteria were subjected to MVs isolation.

Biofilm formation assay

Qualitative assay

MRS and LB broth were dispersed in 6 well plates of about 3mL in each well. Later, 10µL of overnight cultures of *L. acidophilus* and *P. syringae* were inoculated in their respective media. The plates were swirled gently to equilibrate the culture onto broth. Then sterile coverslips were immersed onto each well and the plates were covered gently with lid and then incubated at 37˚C for 24 hrs. After incubation, the coverslips are taken out from the wells carefully and washed with PBS to remove non-adherent cells. Finally, the coverslips containing biofilms were fixed using 2.5% glutaraldehyde for 5 minutes followed by sequential dehydration using a series of 70 to 100% ethanol. Further, the fixed samples are utilized to visualize the biofilm of respective bacteria under Scanning Electron Microscopy (SEM). Samples were placed on a the Samples were then sputtered with Gold palladium . SEM imaging was facilitated by employing a Zeiss EVO/18 (Carl Zeiss) equipped with SmartSEM software under an acceleration voltage of 5 kV.

Quantitative assay

Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well microtitre dishes using a previously reported protocol. 100µl were inoculated from a 1:100 dilution of an overnight *L. acidophilus* and *P. syringae* culture. After inoculation, plates were incubated at 30˚C for 12 h and 24 h. The supernatants were removed and 125µl of a 1%

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.64

solution of Crystal violet(CV) were added to biofilm cells on each well. Then the plates were incubated at room temperature for 15 minutes, rinsed thoroughly and repeatedly with water and scored for the formation of a biofilm. Crystal violet(CV) attached to the biofilm cells were solubilized in 200µl of 95% ethanol, of which 125µl were transferred to a new polystyrene microtitre dish and the absorbance was determined using a plate reader at 600 nm (5, 6).

Isolation and characterization of membrane vesicles

Isolation of membrane vesicles

Poly Ethylene Glycol (PEG) having a molecular weight of about 6000Da were used in isolating vesicles from *L. acidophilus* and *P. syringae*. 16 percent of PEG solution was prepared using 1M NaCl, as this concentration was reported to be optimal for retrieval of vesicles ranging from 20-1000nm. 30 mL of overnight cultures of *L. acidophilus* and *P.syringae* were centrifuged for 45 minutes at 7000 RPM. The supernatant were again centrifuged to remove further cell debris at 7000 RPM for 30 minutes. Further, the unfrosted supernatant of 10mL was transferred to tube containing an equal volume of PEG solution. This mixture was incubated overnight or a minimum of 12 hours at 4 ˚C. Following incubation with PEG solution, the mixture was centrifuged at 5000 RPM for 30 minutes. The centrifugation process was allowed to repeat subsequently until a visible pellet occurs. Finally, the obtained pellet was suspended in filtered PBS, stored at -80 ˚ C and utilized for further analysis(7).

Characterization of isolated membrane vesicles

Estimation of vesicles size through particle size analyzer by dynamic light scattering

Isolated OMVs were diluted 10:990 µL in PBS (pH 7.4) before analysis. Anton Paar Litesizer 500 was used to analyze the particle size of the isolated vesicles (8).

Estimation of vesicles integrity by zeta potential

Isolated OMVs were diluted 10:990 µL in PBS (pH 7.4) before analysis. Anton Paar Litesizer 500 was used to check the integrity of the isolated vesicles (9).

Visualization of vesicles under fe sem

10 µL of isolated vesicles were spotted at the center of the foil, air-dried and were fixed using 2.5% glutaraldehyde for 5 minutes. Following fixation, the samples were dehydrated by applying a series of ethanol ranging from 70 to 100%.

Protein estimation

Vesicles are enriched with proteins thus the protein estimation is necessary for the characterization of vesicles isolated. The protein quantity was obtained by utilizing Nanodrop spectrophotometer, Bradford and *Characterized by SDS-PAGE* assays.

Nanodrop spectrophotometer

Isolated vesicles are subjected to nanodrop spectrophotometer to measure the purity of protein. The A260/280 ratio should be approximately 0.57 for proteins to be considered as pure.

Bradford assay

Further to determine the concentration of the proteins present in vesicles Bradford assay was carried out. Bovine serum albumin (BSA) was used to generate a standard curve from which unknown concentrations of proteins were determined.

SDS-PAGE analysis

12% gel was prepared and the isolated vesicles were loaded after sonication for 1 minute. Low-intensity sonication was done to disarticulate proteins present inside the vesicles. After the gel run the separated proteins were stained overnight using coomassive and later visualized for visible bands after the de-staining process.

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
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Results and Discussion

Biofilm formation Qualitative assay

The quantitative analysis of biofilm formation displayed that comparatively 24h incubation results in higher biofilm formation than 12h incubation. This further ensures the optimal selection of OD for isolating vesicles as biofilm may influence the enrichment of vesiculation. The biofilms are significant for the bacterial community which protects the bacteria from various stressors (10). Considering that, for colonization bacteria must attach themselves preferentially to a surface for instance, *L. acidophilus* as a probiotic has to adhere to the intestinal tracts whereas *P. syringae* would interact and adhere to the plant host. Which in the case of *L. acidophilus* eventually increases the production of mucus extracellular polymeric substances, antimicrobial bacteriocins, and results in enhanced inhibition of pathogens growth by modulating host immune system through the prevention of pathogen adherence and compete for binding sites (11). For *P. syringae* cellulose exopolysaccharide, PsI like polysaccharide was produced as a result of biofilm which enhances the virulence of this phytopathogen (12). The biofilm formation initially starts with reversible attachment and switches to forming irreversible attachment under favorable conditions resulting in macro colony formation. The macro colonies of *L. acidophilus* were visualized using SEM as shown in Fig.1. a and b; the mature biofilm were visualized for *P. syringae* as shown in Fig.1.c. The level of magnification and resolution available with SEM allows for the examination of the overall morphology of the microorganisms making up the biofilm as well as their spatial organization. Moreover, biofilm was a result of quorum sensing mediated cell-to-cell communication. The PsyI/ PsyR (signal synthase/receptor) were the QS system for *P. syringae* and the signal molecule produced was 3-oxo-C6-HSL and C6-HSL(13)

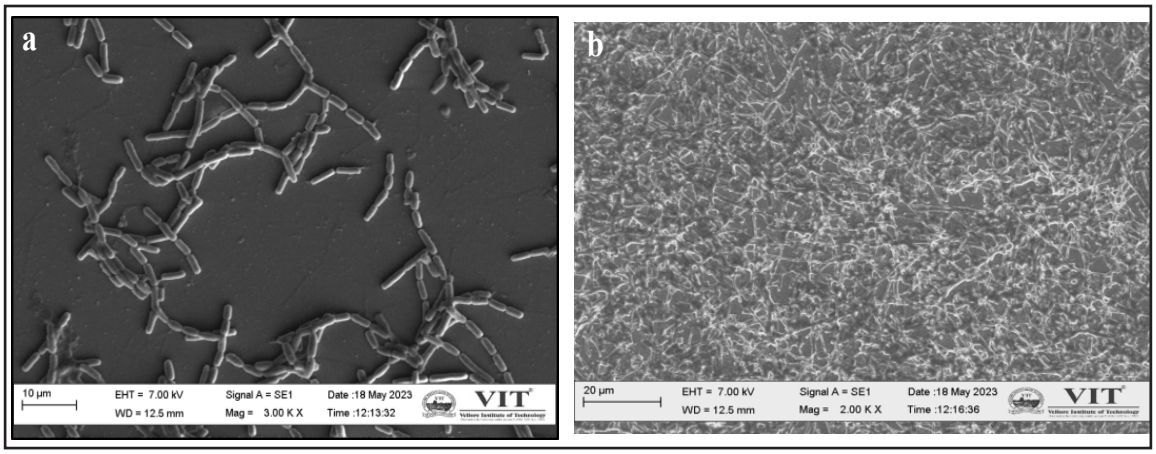

Fig. 1**: V**isualization of biofilm (Qualitative assay) under SEM: a. Biofilm of *L. acidophilus* and *b. P. syringae.*

Biofilm formation quantitative assay:

Biofilm formation was largely dependent on cross talk between the bacterial communities and an increase in biofilm may result in enhanced vesiculation. Research by Zaborowska (14) shows that biofilm-derived vesicles comprise increased drug-binding proteins than vesicles derived from planktonic cells. The qualitative as well as quantitative assay for biofilm states that *L. acidophilus* and *P. syringae* are capable of biofilm formation. Biofilm mass formed is directly proportional to the higher absorbance (15).

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.64

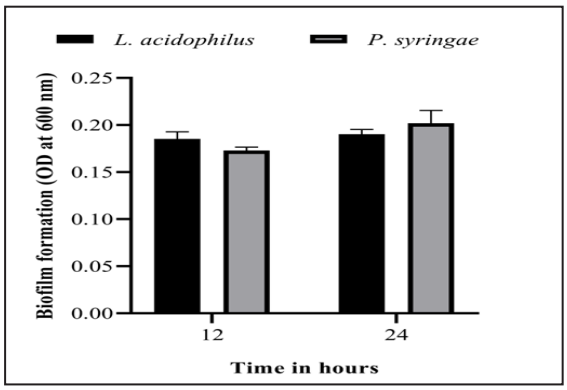

Fig. 2**.** Bacterial biofilm formation-Quantitative assay

Membrane vesicle isolation and characterization

To ascertain the size distribution of the MVs isolated from *L. acidophilus* and OMVs from *P. syringae* cultures, DLS technique were utilized. The sizes were ranging between 200-300 nm in all the three phases of *L. acidophilus* Whereas P.syringae the size distribution ranges between 250-400 nm. Results obtained to support that the isolated vesicles were in line with the pre-existing data.

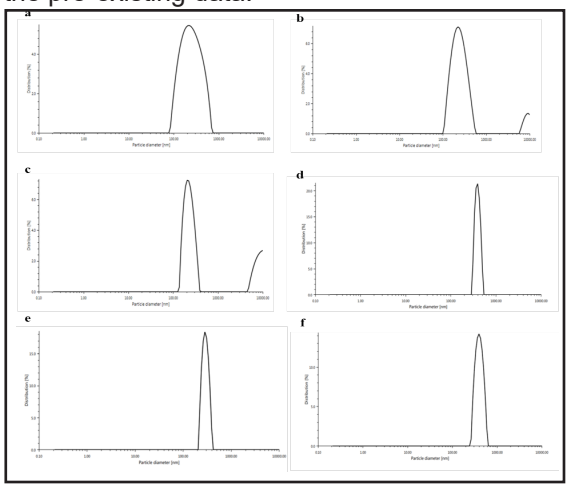

Fig. 3**:** MVs size analysis by DLS a-c - MVs of *L. acidophilus* obtained from early log, mid log and late log phases subjected to DLS analysis showed peak values of 267.8nm, 247.5nm and 228.1nm respectively. d-f – OMVs of *P. syringae* obtained from early log, mid log and late log phases were shown to produce peak value of 391nm, 292.4 nm and 401.6 nm respectively.

The values of zeta potential of *L. acidophilus* and *P. syringae* show that the vesicles isolated were in good integrity in all the selected three phases. As the surface charges are necessary for maintaining integrity of the vesicles, the measurements acquired were negative which further ensures the stability of the isolated vesicles. According to the research by Midekessa 16), reported that zeta potential was lower negative value during acidic pH along with aggregation. Thus, obtained zeta potential records for both *L. acidophilus* MVs and *P. syringae* OMVs were lower negative value which might be due to vesicle aggregation formation.

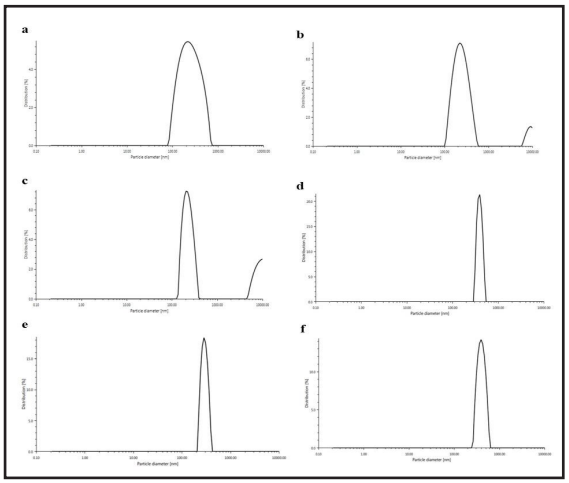

Figure.4: MV's integrity tested by zeta potential analysis a-c –MVs of L. acidophilus obtained from early log, mid log and late log phases results in the mean zeta potential of -14.9 mV, 0.1mV and -1. 2mV respectively. d-f - OMVs of P. syringae from early log, mid log and late log phases showed the mean zeta potential of -11.1 mV, -0.7 mV and -1. 1 mV respectively.

Vesicles were subjected to high resolution Field Emission Scanning Electron Microscopy (FE SEM). FESEM generates sharper, less distorted images with a resolution far clearer than conventional SEM due to the potential

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.64

gradient electron source (17). The report predicts the size of the MVs isolated from early log, mid log and late log phases was 113.1nm, 242.5 nm and 189.2 nm, respectively. Whereas, *P. syringae* size ranges around 101.3 nm, 260.5 and 201.9 nm and which were obtained from early log, mid log and late log phases respectively. FESEM allows visualizing rounded vesicles with diameter ranged from 110-250±50 nm for *L. acidophilus* whereas, *P. syringae* ranged from 100-260±50 nm in dm.

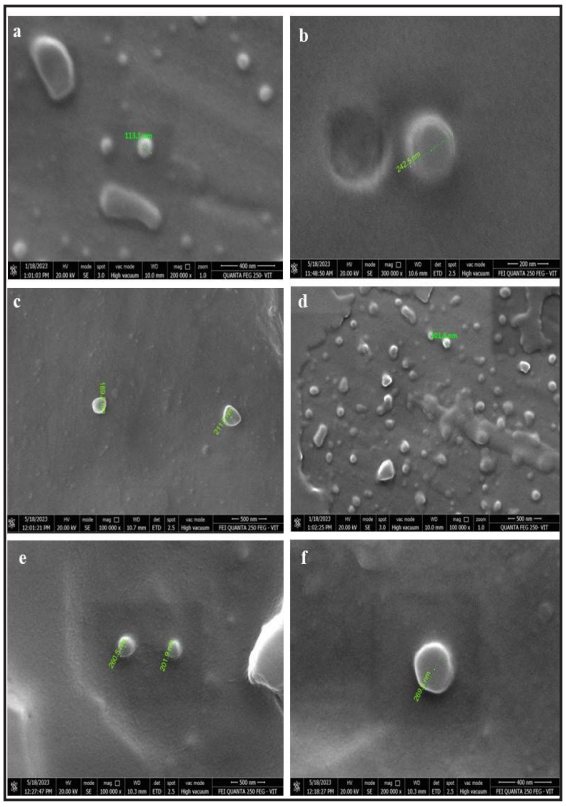

Fig. 5**:** Membrane Vesicle morphology and shape analysis. a-c - MVs from *L. acidophilus* whereas, d-f - OMVs from *P. syringae* under FE SEM

Membrane vesicle protein quantification analysis:

The protein quantification assays confirmed that MVs and OMVs are having a reliable protein concentration and are in consistence with those reported earlier. The protein concentrations were measured using nanodrop and bradford assays. Based on nanodrop analysis our data indicated that the protein concentrations was approximately 0.85, 0.10, 0.73mg/µL for the MVs, while protein concentrations was approximately 0.40, 0.64, 0.73mg/µL for *P. syringae* OMVs obtained from early log, mid log and late log phases respectively. Moreover, the protein concentration was reconfirmed with bradford assay. The values were ranging from 173.61, 312.86, 221.62 µg/mL were obtained for *L. acidophilus* MVs. whereas, for *P. syringae* OMVs the protein concentration was about 214.72, 268.18, 252.19 µg/mL. In the graph LB1, LB2, LB3 represents protein concentrations of MVs isolated from early log, mid log and late log phases respectively whereas PS1, PS2, PS3 represents OMVs isolated from early log, mid log and late log phases respectively. Though protein quantification can be observed by nanodrop reconfirmation is essential and could be efficiently done by good old bradford assay. We could observe slight variations in the protein concentrations obtained by nanodrop and bradford assays. Nanodrop would be reliable for nucleic acids and thus reconfirmed with bradford assay.

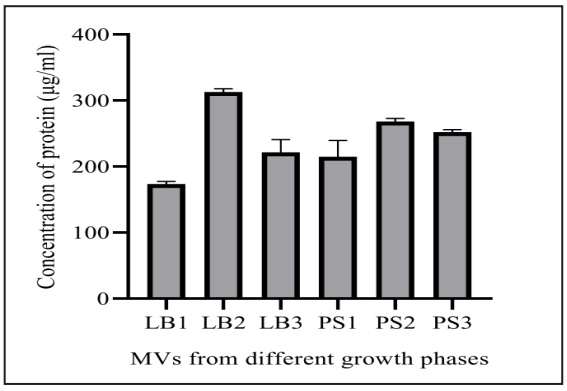

Fig. 6: Protein estimation of isolated vesicles by Bradford assay: LB1, LB2, LB3 represents MVs isolated from early log, mid log and late log phases respectively whereas PS1, PS2, PS3 represents OMVs isolated from early log, mid log and late log phases respectively.

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.64

Protein size analysis by SDS PAGE

P. syringae OMVs composed of bacterioferritin with 17.9 kDa of molecular weight, which is an iron storage protein with ferroxidase activity. OmpA of 30 kDa are present in the outer membrane and has porin and monoatomic ion transport activity. Further, Outer membrane lipoprotein OprI of 46.1 kDa, lipoprotein of 7.7 kDa, outer membrane porin OprD having 48.4 kDa, were also present in OMVs of *P. syringae* as reported by Kulkarni et al,.

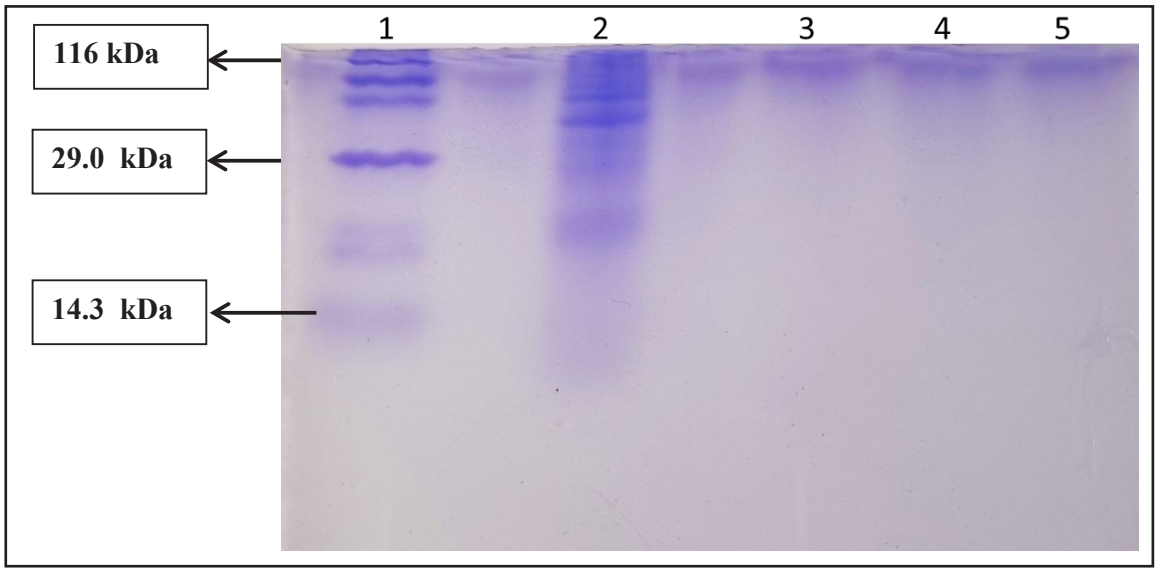

Fig. 7: Protein size analysis by SDS PAGE: Lane 1 represents protein medium range ladder and lane 2 represents whole cells of *P. syringae* and Lane 3-5 represents P. syringae OMVs isolated from early log, mid log and late log phases respectively.

In this study, the proteins obtained were under the molecular weight ranging of 50 to 100 kDa, thus P. syringae OMVs may have the components of OmpA, OprI and OprD regard to existing reports ranging around 50 kDa. As the bands were not prominent further optimal confirmation was required. Further, as an interpretation of this experiment, the protein concentration might be low in the isolated OMVs. Thus, the Mass spectrometry method would be the possible way to further quantifying low-abundance proteins present in the isolated vesicles.

The whole bacterial cell possesses a vast number of proteins in comparison with nanosized MVs which have less number of proteins due to size constrain. Eventually, bands obtained are satisfactory as whole cells have more proteins and OMVs show fewer bands. Further, as regards to aforementioned lack of a prominent band could be due to low protein concentration which could be rectified by standardization. Thus, PEG method-based vesicle isolation is exquisite for both Gram-positive and Gram-negative bacteria.

Conclusion

Membrane vesicles in the best of its position to engross researchers and are considered to be safe to utilize. Vesicles are made of distinct cargo with reference to various organisms and even differ within organisms from the same genus. This diversified cargo can modulate various hosts immune system and the exact mechanism of how the vesicles activate immune response in their Host's cell is undiscovered till now. Researchers are attracted to existing scanty reports on vesicle mediated immune ac-

Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.64

tivation, while lodestone will be laid in the near future by distinctive applications of vesicles. The vesicle-host interaction would be crucial in engaging research for developing new technologies for disease management. A study by Garcia-Romero [19] recorded that PEG renders a higher harvest of vesicles in comparison with the ultra-centrifugation and the kits used (Exo-Quick and PureExo). This provides additional confirmation of utilization of PEG will result in good yield of vesicles and are more reproducible [20]. PEG-based vesicle isolation prompts that the *L. acidophilus* membrane vesicles and *P. syringae* outer membrane vesicles are released efficiently on all three selected phases. The isolated MVs and OMVs could possibly be explored in future applications. For instance, MVs of Gram-positive probiotic strains of the genera Lactobacillus have demonstrated the ability to treat skin infections and inflammatory conditions in the intestine, namely Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD), by altering the host's immune system. The mobilization of inflammatory cells was inhibited by treatment with a mixture of the Lactobacillus-derived MVs, which also decreased myeloperoxidase (MPO) activity. The fact that MVs generated from Lactobacillus can interact with pathogens and stop HIV-1 replication provides yet another piece of evidence. Additionally, MVs secreted from *L. acidophilus* were found to have the ability to destroy the opportunistic gut pathogen *L. delbrueckii*. So, at present probiotics are thus being replaced by bacterial MVs [21, 22, 23]. On the other hand, OMVs from *P. syringae* were able to protect bacteria from chemical stress induced by the growth-inhibitory effects of membrane-active antibiotics. Further, research showed that OMVs from P. syringae activate plant immune responses that protect against bacterial and oomycete pathogens [2]. *P. syringae* OMVs can be explored as a tool to uncouple plant growth and defense activation which may further signify immuno-modulatory properties. Furthermore, omics-based studies including proteomics and transcriptomics will be done for the isolated vesicles which can be utilized for future *in vivo and in silico* studies. Using *in silico* studies predictions can be made to understand the exact mechanisms and the molecular pathways involved in the interactions of vesicles-hosts interaction.

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Vol. 17(Supplementary issue - 3B) 1293-1302,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
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