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Screening and extraction of biofilm inhibitors from *Lactobacillus* isolated from fruit extracts against *Vibrio cholerae*

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Abstract
Bacteria being resistant to various antimicrobial treatments, due to its ability to form biofilm, it can survive harsh conditions and withstand the host's immune system. This has drawn considerable interest from researchers to identify new therapy options to treat biofilm-associated infections. The need for development of antibiofilm agents that are nontoxic is increasing rapidly. The new antibiofilm agents have the potential to disperse bacterial biofilms *in vivo* and could positively impact human medicine in the future. The current study deals with the effect of *Lactobacillus* isolate which inhibit the biofilm formation of *Vibrio Cholerae* by producing a potent inhibitor. The organism was identified and screened for the production of bioactive compounds. Our results revealed the inhibition of biofilms up to 80%. Antibiofilm agents that can both disseminate and destroy biofilm bacteria could have some useful applications but remain rare.

Keywords: Biofilm, *Vibrio cholerae, Lactobacillus*, quorum sensing

Introduction
Microbial biofilms develop when bacteria adhere to the substratum and grow inside a secreted extracellular matrix. The formation of biofilm follows defined stages like reversible and irreversible attachment; surface motility, and initiation of micro colony formation, maturation, ageing and differentiation of micro colonies and finally, biofilm dissolution and generation of specialized dispersal cells. The properties of the individual strains of bacteria present, i.e. rate of extracellular polymer production, are an important influence on these biofilms. Biofilms have the ability to form on living or non-living surfaces, and represent a prevalent mode of life in natural, industrial and hospital settings [1]. Cholera is a devastating diarrheal disease that affects millions of people in the world each year. It is an infection of the small intestine caused by the bacterium *Vibrio cholerae* causing profuse watery diarrhea and vomiting. Transmission is primarily through the consumption of contaminated drinking water or food. Primary treatment is with oral rehydration solution and if these are not tolerated intravenous fluids, antibiotics are beneficial in severe cases.

Vibrio cholerae, a curved motile Gram negative halophilic facultative pathogen with a polar flagellum. It belongs to the family Vibrionaceae. *V. cholerae* live in marine, estuarine and freshwater environments in association with zooplankton, phytoplankton, crustaceans, insects and plants. *Vibrio* adapt to environmental stress through biofilm formation. The biofilm formation begins with attachment of free floating microorganisms to a surface, initially adhered through weak reversible Vander Waal forces and then anchor permanently using cell adhesion structures such as pili. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. During their colonization the cells communicate with each other *via* quorum sensing using products such as Acyl Homoserine Lactones (AHL). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. Dispersal of cells from the biofilm colony enables the biofilms to spread and colonize new surfaces.

Biofilm cells show a remarkable tolerance to a variety of antimicrobial measures as compared to their planktonic growing counterparts. The emergence of multiple drug resistance is a serious clinical problem in treatment and containment of disease. The large volumes of contaminated waste released into the environment during disease and the remarkable ability of *V. cholerae* to thrive not only in the marine but also in the fresh water environments enabling the spread of epidemic and pandemic disease. In the new millennium we are encountering an alarming increase in multiple-resistant bacteria, and as a result infection that were once treatable have become non-treatable.

This calls for the development of alternative treatment strategies which relay on identification of a suitable drug target and development of cognate pharmaceutically applicable drugs. Quorum sensing systems playa a key role in orchestrating the expression of exo-proteases, siderophores, exotoxins and several secondary metabolites, and participate in the development of biofilms [2, 3, 4, 5]. Environmental changes generally lead to a wide variety of morphological and physiological responses in bacteria. Quorum sensing is a regulatory mechanism which enables bacteria to make collective decisions with respect to the expression of a specific set of genes. This mechanism contains an element of co-ordination and to achieve this, bacteria communicate by means of small, diffusible signal molecules. QS Bacteria communicate with one another using chemical signal molecules. These bacteria produce and release chemical signals molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. *Lactobacilli* belong to the group of *lactic acid bacteria (LAB)*, that have several distinguished abilities such as production of lactic acid, enzymes such as β-Galactosidase organic acids, free fatty acids, ammonia, reuterin, diacetyl, hydrogen peroxide and bacteriocin, which have the capacity to inhibit the growth of food spoilage and pathogenic organisms. Lactic acid bacteria are traditionally used as starters for food fermentations. Since they have the ability to inhibit spoilage and pathogenic bacteria, they are important in food preservation and intestinal prophylaxis. Lactic acid bacteria are the most important groups for industrials purposes, since their fermentative activity involves a notable preservative capacity as a result of the drop in the pH and the antimicrobial activity of their metabolites. Many lactic acid bacteria, including members of great genera *Lactococcus, Lactobacillus, Carnobacterium Enterococcus and Pediococcus,* are known to secrete bacteriocin. Bacteriocins are in general cationic, amphipathic molecules as they contain an excess of lysyl and arginyl residues. Bacteriocins are among the most promising preservatives in the food industry, and are a family of microbial defense system, which meant they may prohibit the invasion of other strains or the change of the environment, both biotic and abiotic.

The discovery of QSI (quorum-sensing inhibitors) compounds requires live screening systems which can identify the presence of QSI activities of pure as well as mixture of compounds. There are basically three different targets in gram negative QS systems -the signals generator, the signal molecules and the signals receptor. An obvious strategy is to screen for compounds that prevent the signal molecules from being synthesized by the LuxI-encoded AHL synthesis. If no N-acyl homo serine lactones (AHL) are produced, the bacteria will be unable to sense when a quorum is reached hence; QS controlled gene will not activate. If the signal production cannot be prevented, the signals themselves can be targeted with degradation in mind [6]. Examination of completed *V. cholerae* genome sequence showed that AI-2 quorum sensing circuit was present. Genetic analysis was used to demonstrate that, in addition to this AI-2 system, *V. cholerae* possesses two other parallel sensory circuits. Information from the three systems is channeled to the two component Quorum sensing controls biofilm formation in *V. cholerae*. Multiple quorum-sensing circuits regulate diverse function in *V. cholerae* including virulence, protease activity and biofilm formation, all of which are modulated by the activity of the LuxO response regulator and hapR DNA binding protein [7]. Surprisingly, in contrast to other bacterial species in which quorum sensing activates virulence gene expression at high cell densities, in *V. cholerae*, quorum sensing appears to repress ToxR-regulated virulent genes [7, 8].

At low cell density *V. cholerae* activates the expression of virulence factor and forms biofilms. At high cell density the accumulation of two quorum-sensing autoinducer represses these traits. These two autoinducers, cholerae autoinducer-1(CAI-1) and autoinducer-2 (AI-2), function synergistically to control gene regulation although CAI-1 is the stronger of the two signals *V. cholerae* AI-2 is furanosyl borate diester (2S-4S)-2-methyl-2, 3, 3, 4-tetra hydroxyl tetra hydrofuran borite. Autoinducer AHL's which is produced by *V. cholerae* consist of a lactone ring covalently linked to 4- to 14-carbon acyl side chain through an amide bond. AHL-degrading enzymes hydrolyze the AHL's in to inactive molecules, there by blocking the QS systems that are closely linked to virulence factor production and bio film formation [9].

Materials and Methods

Sample Collection

The marine water sample and fruit wastes were collected for isolation of *Vibrio cholerae* and *Lactobacilli*, respectively.

Total viable bacterial count

Vibrio cholerae were isolated from marine water sample, they were serially diluted using 9ml sterile saline. The scrapping from plate samples were serially diluted using 9ml sterile saline. Total viable bacterial counts were enumerated by pour plate method, using nutrient agar medium (Hi-Media, Mumbai, India). 1ml aliquot of appropriate dilution was pipetted out into the sterile petriplates and 20ml of nutrient agar was added into each petriplate. The sample was mixed thoroughly by rotating the plate clockwise and anticlockwise direction and allowed to solidify. Then the inoculated plates were incubated at 37°C, duplicate plates were also maintained. Petriplates with 30-300 colonies were selected after 24-48 h incubation and the total viable bacterial counts were enumerated. The bacterial population was expressed as number of colony forming units CFU mg^{-1} of soil sample. Diluted samples were streaked on selection plates (MMB) containing 0.1% L -tyrosine and glucose, black colonies suspected of melanin production were isolated and grown again on L -tyrosine-containing plates and on similar plates without tyrosine (negative control).

Bacterial Identification

The bacterial strains isolated from the samples were identified up to generic level by employing the standard morphological and biochemical characteristics described in Bergey's manual of systematic bacteriology.

DNA isolation

Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA; pH 8) buffer and lysozyme (10 mg/ml) were added in the pelleted cells of the dominant isolate and incubated for 30 min at room temperature. SDS and Proteinase K (10 U/µl) were added and incubated at 55°C for 2 h. DNA was extracted with phenol, chloroform and iso-amyl alcohol, and was precipitated with ethanol and dissolved in TE buffer.

PCR amplification, cloning and sequencing of 16S rRNA genes

16S rRNA genes of the bacterial isolates were amplified with genomic DNA isolates as template and 8F and 1490R primers

in the following composition and amplification cycle. Each reaction mixture contained 2 µl of template DNA (100 ng), 0.5 µM of two primers, and 25 µl of Enzyme Master Mix (Bioron). The PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 92°C for 30 sec, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min was carried out in Thermal Cycler (Thermo Hybaid). After the last cycle, a final extension at 72°C for 20 min was added. The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected and isolated plasmids with insert were sequenced with M13 Sequencing Primers using ABI Bio systems automated sequencer. Nucleotide database was searched with the sequences obtained with NCBI BLAST (Blastn) tool (http://www.ncbi.nlm.nih.gov/BLAST).

Biofilm Inhibition Assay

The biofilm assay was done by the method previously described by the Pratt *et al*., (1998). Briefly, 10µl of overnight culture of *V. cholerae* was inoculated in 96-well microtitre plates (polystyrene) containing LB broth and different concentrations of *Lactobacillus* extract (5, 10, 15 and 20% v/v) followed by incubation for 48 hrs at room temperature. The biofilms were stained by 210µl of 0.1% crystal violet solution (w/v for 10 min, after which the dye was discarded and the wells were rinsed twice with distilled water. The wells were allowed to air dry before solubilization of the crystal violet with 210µl of dimethyl sulfoxide (DMSO). The optical density was determined at 595 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad).

Extraction of bioactive compound

The potential isolate *Lactobacillus* was inoculated into 100ml of MRS broth and incubated at 37°C on a rotary shaker for two days. After maximum growth was appeared the culture broth was centrifuged at 10,000 rpm for five minutes at 15°C. Bioactive compound containing supernatant was extracted using equal volume of different solvents such as hexane diethyl ether, ethyl acetate and butanol. The solvents were concentrated by flash evaporator at 40°C. The crude fraction was dissolved in DMSO so as to prepare a stock of 100mg/mL. Biofilm inhibition assay is also tested using the different concentration of this crude extract. Crude extract was subjected to FTIR analysis.

Results

Isolation and identification

Characterization of *Lactobacillus* and *Vibrio cholerae* was performed using various tests (Table 1). *Lactobacillus* was found to be positive for methyl red, starch hydrolysis, acid and gas production tests. *Vibrio cholerae* was positive for motility, indole, Voges Proskauer, Citrate, Gelatin hydrolysis, Catalase, Oxidase and acid production (Table 1).

Table 1: Characterization of *Lactobacillus* and *Vibrio cholerae*

S.No	Tests	Lactobacillus	Vibrio Cholerae
	Performed	Observation	Observation
1	Morphology	Rod	Curved Rods
2	Gram staining	Positive	Negative
3	Motility	Negative	Positive
4	Indole	Negative	Positive
5	Methyl red	Positive	Negative
6	Voges Proskauer	Negative	Positive
7	Citrate	Negative	Positive
8	Starch hydrolysis	Positive	Negative
9	Gelatin hydrolysis	Negative	Positive
10	Lipid hydrolysis	Negative	Negative
11	Catalase	Negative	Positive
12	Oxidase	Negative	Positive
13	Acid Production	Positive	Positive
14	Gas Production	Positive	Negative

Biofilm Assay

All isolates were tested for biofilm inhibition activity against *Vibrio cholerae.* Among them five strains showed biofilm inhibition activity. Biofilm assay using crystal violet stain revealed that 20% of cell extracts inhibited biofilm above 80% (Figure 1,2). The MIC was recorded as the lowest concentration that produced complete suppression of visible growth.

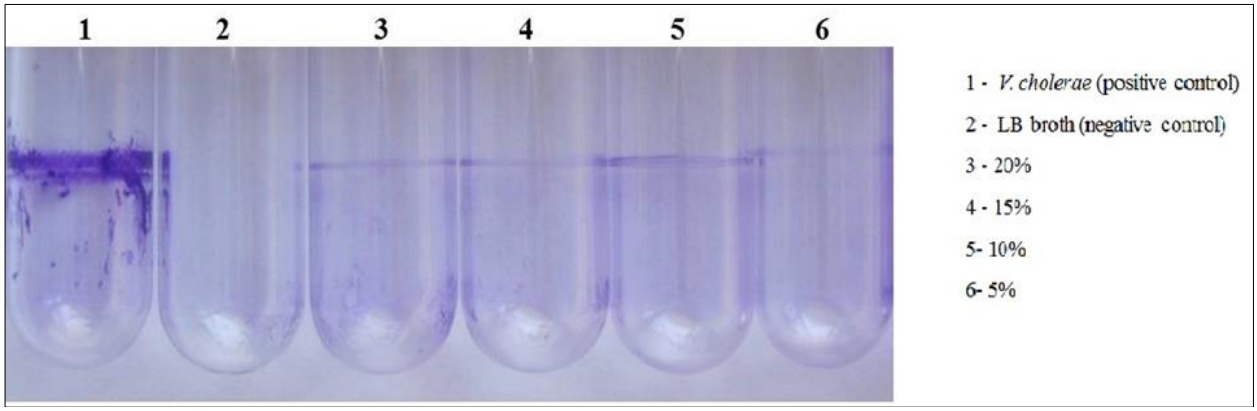

Fig 1: Biofilm Assay

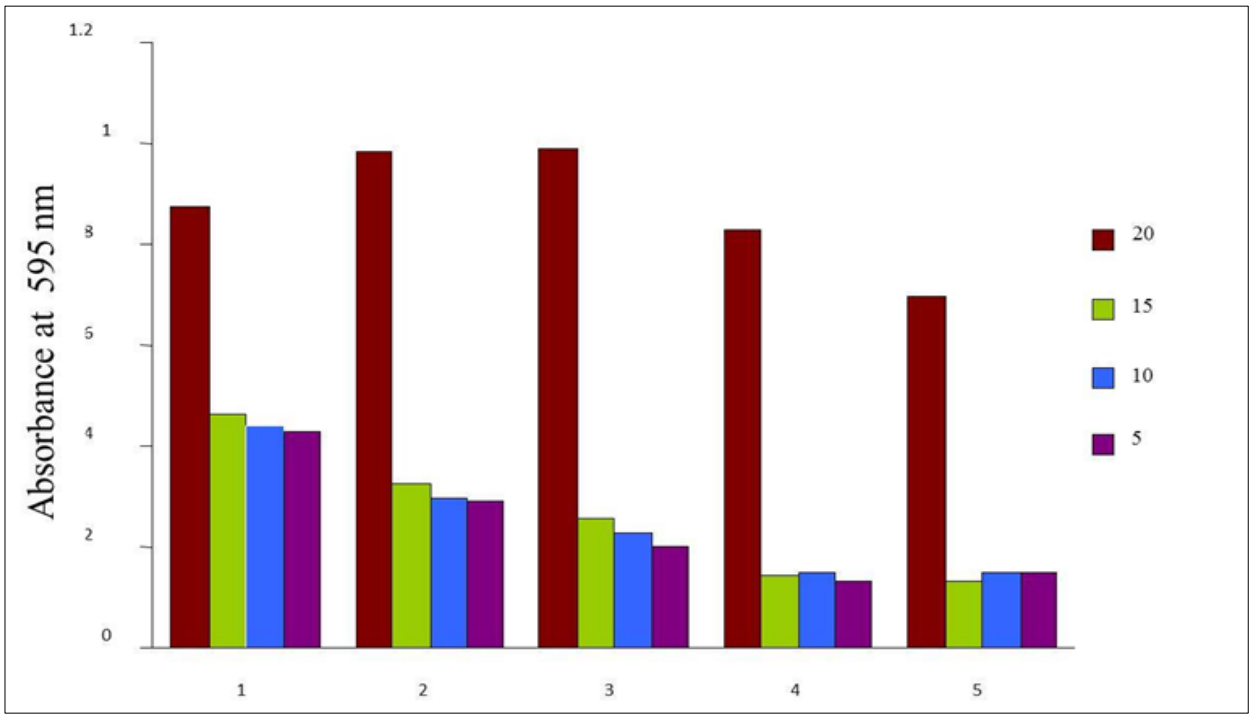

Fig 2: Decrease in biofilm formation of V. cholerae with varying percentage of *Lactobacillus*

Extraction of bioactive compound

Lactobacillus isolates diethyl ether fraction showed biofilm inhibition at a concentration of 300µg/ml. The fractions of hexane, ethyl acetate and dichloromethane did not show any biofilm inhibiting property.

Air-liquid coverslip assay

Biofilm inhibition was again confirmed by Air-liquid Interphase coverslip assay (Figure 3). Based on the MIC results, the test strain was shown to be the most promising isolate to be studied in detail.

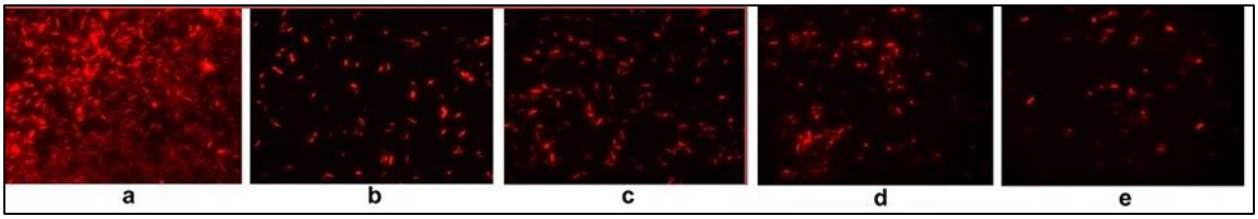

Fig 3: Air-liquid coverslip assay

Discussion

V. cholerae can form biofilms in association with animate and inanimate surfaces. Bacteria attach to surfaces and aggregate in a hydrated polymeric matrix to form biofilms. Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections [10]. *P. aeruginosa* has the ability to convert to an antibiotic resistant phenotype after being exposed to an antibiotic with enhanced biofilm forming ability [11].

Bacteria being recognized as highly interactive organisms with complex survival nature and plays a critical role to cause disease. In particular many species inhabit dense, surface-bound communities termed biofilms, within which they communicate and respond to local cell density through a process known as quorum sensing [14]. Quorum sensing is

known to control biofilm formation in several bacterial species such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Aeromonas hydrophilla*. EPS secretion is under quorum sensing control in a numbers of bacterial model systems. Many species, including the pathogen, *P. aeruginosa*, activate EPS production at high cell density [2, 12]. Diverse array of physiological activities are being regulated using quorum sensing communication circuits by Gram-positive and Gram-negative bacteria. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilms formation. In general gram-negative bacteria use acyl homo serine lactones as autoinducers and gram-positive bacteria use processed oligopeptides to communicate. A family of autoinducer known as autoinducer-2(AI-2) is involved in both gram - negative and gram-positive bacteria [13]. Recent advantages in the field indicate that cell-cell communication via. autoinducers occurs both within and between bacterial species.

Jaine *et al*. [14], proved that development of surface attached biofilm bacterial communities is considered as an important source of nosocomial infections. Recently, bacterial interference via signaling molecules and surface-active compounds was shown to antagonize biofilm formation, suggesting that non-antibiotic molecules produced during competitive interactions between bacteria could be used for biofilm reduction.

Different kinds of bacteriocins have been found from different bacteria in the recent years, earlier in 1969, WHO announced that nisin was a kind of food preservative with high efficiency and safety, and later in 1983, FDA declared that nisin was generally recognized as a safe food preservative. Since LAB and their metabolites have been consumed in high quantities by countless generations of people in cultured foods with no adverse effects, the LAB continue as the preferred source for food-use bacteriocins, either in the form of purified compounds or growth extracts. The low molecular weight bacteriocins of Gram-positive bacteria demonstrate bactericidal activity which is directed principally against certain other Gram-positive bacteria. For example, the lantibiotic nisin has been shown to be effective against many strains of Gram-positive bacteria, including S*taphylococci, Streptococci, Bacilli, Clostridia*, and *Mycobacteria*.

Mounting data suggests that bacterial auto inducers elicit specific responses from host organism. Although the nature of chemical signals, the signal relay mechanisms and the target genes controlled by bacterial quorum sensing systems differ, in every case the ability to communicate with one another allows bacteria to co-ordinate the gene expression, and therefore the behavior, of the entire community [15]. The phenomenon of quorum sensing, or cell- to- cell communication, relies on the principle that when a single bacterium releases autoinducers into the environment, their concentration is too low to be detected. However, when sufficient bacteria are present, autoinducer concentrations reaches threshold level that allows the bacteria to sense a critical cell mass and, in response, to activate or repress target genes [16]. The emergence of multiple-drug-resistant strains of bacteria has let the focus towards finding novel strategies for treating bacterial infections. The discovery, that a wide spectrum of organisms uses quorum sensing to control virulence factor production makes it an attractive target for anti-microbial therapy. Through blocking this cell to cell signaling mechanism, pathogenic organisms that use quorum sensing to control virulence could potentially be rendered a virulent.

From this study, it can be concluded that the *Lactobacillus* isolated from the fruit extracts have the biofilm inhibition activity against *Vibrio cholerae*; they inhibit the biofilm formation of *V. cholerae* by producing a potent inhibitor [17]. Here, we selected *Lactobacillus* because it is heavily exploited as a source of useful enzymes and therapeutically useful bioactive molecules. Biofilm formation is important for the life cycle of *V. cholerae*, facilitating environmental persistence within natural aquatic habitat during inter epidemic periods [18, 19]. As multidrug resistance is rapidly emerging in *V. cholerae* and related pathogens, there is an urgent need for novel compounds which will interfere with quorum sensing [23]. Hence, the bioactive compound present in the cell extract of selected *Lactobacillus* may be a good candidate to prevent infections caused by *V. cholerae*.

Conclusion

The diethyl ether fractions obtained from the *Lactobacillus* was found to inhibit the biofilm formation. This was further confirmed using Air-liquid coverslip assay. However, further have to be carried out to determine the biofilm inhibiting compounds present in it

Conflicts of interest: The authors declare no conflict of interest

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