

# Critical Evaluation of Biofilm Formation among Food Borne Bacterial Pathogens Isolated from Grape Peel and Methods to Tackle Biofilm

Suganthi Ramasamy, Jagadeshwari Akalu Srinath

Department of Biotechnology, Dr. G. R. Damodaran College of Science, Coimbatore Tamil Nadu, INDIA.

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## ABSTRACT

Biofilm forming food borne pathogens are a serious threat to food products and food industries as they have the capacity to attach to the substratum or to each other and covered by an exopolysaccharide matrix. Spoilage and pathogenic bacteria may form biofilms which increases post-processing contamination and risk to public health. The present study comprised of isolating the biofilm producing food borne pathogens from the peel of grape. The antibiotic resistance test was carried out and the isolates showing multiple antibiotic resistances were taken for the further study. For the selected isolates, the biofilm forming assays like Tube test method, Modified Congo Red Agar (MCRA) method and Micro Titre Plate (MTP) method was carried out. All the isolates showed moderate biofilm activity. Based on the morphological, physiological tests and 16s rRNA sequencing, the isolates were identified as *Kocuria kristinae*, *Staphylococcus haemolyticus*, *Burkholderia cenocepacia* and *Enterobacter cloacae*. The sequences were deposited in NCBI, Genbank and the accession numbers were assigned as MK615917, MK615918, MK615919 and MK615920 respectively. Different natural preservatives such as vinegar, lemon, tamarind, citric acid, sodium azide, sodium bicarbonate and Nisin were used for the biofilm eradication. Nisin in the concentration greater than 6.25mg/ml is suitable to inhibit the biofilm formation in *Staphylococcus haemolyticus* and 0.0487mg/ml for the remaining isolates. Vinegar in 1:2 dilution, Lemon in 1:4 dilution, Citric acid in 31.25mg/ml and Sodium azide in 12.5mg/ml will be optimal for inhibition of all the isolates. Sodium bicarbonate in 250mg/ml is optimal for inhibition of *Staphylococcus haemolyticus* and *Enterobacter cloacae*.

**Key words:** Biofilm formation, Food Pathogen, Food preservatives, Nisin, Micro titre Plate Assay, Biofilm eradication.

## Correspondence:

**Dr. Suganthi R.**, Associate Professor and Head of the Department, School of Biotechnology, Dr. G. R. Damodaran College of Science, Avinashi Road, Coimbatore-641014, Tamil Nadu, INDIA. Phone no: +91 9843134681

Email: sugantham2000@gmail.com

## INTRODUCTION

Food contaminated by bacteria and/or their toxins, parasites, viruses, chemicals, or other agents cause Food borne illness (commonly known as food poisoning). Food borne illness occurs when people eat or drink food or beverages contaminated with pathogens, chemicals, or toxins.<sup>[1]</sup> Biofilm can be defined as an association of micro-organisms in which microbial cells adhere to

each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance (EPS).<sup>[2]</sup> The production of chemotactic particles within a biofilm enhances the bacteria to communicate with each other and they are intrinsically more resistant to antimicrobial agents. In the food industry, biofilms have the ability to cause food-borne disease outbreaks. Furthermore, inefficient cleaning regimes may be a contributing factor in the spread of resistance in hospital environments.<sup>[3]</sup> Such biofilms could be continuous sources of contamination of foods in contact with them and may also lead to spoilage of foods or transmission of foodborne diseases.<sup>[4]</sup> The increased resistance of biofilm cells to antibacterial agents and sanitizers has also been observed.<sup>[5,6]</sup> Different species of microor-

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ganisms may possess diverse ability to attach or form biofilm on different surfaces. For example, biofilms can exist on all types of surfaces in food plants and medical devices ranging from plastic, wood, glass, metals and food products.<sup>[7]</sup> There have been many studies reporting the biofilm formation by foodborne pathogens such as *L. monocytogenes*, *S. typhimurium*, *S. aureus*, *E. coli* O157:H7, *Pseudomonas* spp. on food contact surfaces.<sup>[8,9]</sup> Most studies have focused on the biofilm-forming abilities (adhesion and colonization) of foodborne pathogens with regard to the environmental factors, cell surface characteristics and substratum properties. However, relatively fewer studies have been reported on the detachment (dispersal) process of biofilm.<sup>[10,11]</sup> Food preservatives have to be economical and should not have an effect on taste and aroma of the original food, or any substance in food. Most preservatives are inhibitory at acceptable levels.<sup>[12]</sup> Chemicals that have been used in food preservation include sodium chloride, sodium benzoate, citric acid, vinegar, sodium bicarbonate. These chemical agents are employed to prevent microbial growth in food.<sup>[13]</sup> The main objectives of the present study are screening for food preservatives that inhibit the growth of the multidrug resistant bacterial pathogens and elucidation of MIC of the food preservatives that eradicates the biofilm forming activity of the multidrug resistant bacteria.

## MATERIALS AND METHODS

### Sampling

Grape peel was selected as a source for isolating food borne pathogens. It was collected from the fruit vendor under sterile conditions. Grape peel was separated from the fruit was soaked in 0.8% saline. 1 ml of the sample is added to 10 ml of sterile nutrient broth and kept for incubation at 37°C for 24 h in a shaking incubator. After incubation, 100 µl of the inoculum was spread plated on different selective medium such as Mannitol Salt agar (MSA), Eosin Methylene Blue agar (EMB), *Pseudomonas* isolating agar (PIA), PALCAM agar, *Salmonella Shigella* agar (SSA) by spread plate method. The plates were incubated for 24 h at 37°C. The different isolates from each culture were selected and sub cultured on their EMB Agar and MSA selective medium and maintained. Morphological characterization of the isolates was carried out by studying the colony characteristics and by Gram staining.

### Molecular identification of the isolates

DNA was extracted from the bacterial cultures grown in Brain heart infusion agar for overnight culture. DNA was harvested from one loop of bacterial colony inoculated in 200µl of Lysis buffer (10mM Tris

HCl-100 µl, 1 % Triton -100µl, 0.5% Tween 20-50 µl, 1mM EDTA-20 µl make up to 1 L) and the mixture was kept for incubation at 70°C for 10 min. The whole lysate was centrifuged at 3000 rpm for 6 min. After centrifugation the supernatant of extracted DNA is used for PCR amplification. Full-length bacterial 16S rRNA genes were quantified using PCR (Applied Biosystems). The following primer set was used 16S27F Forward - 5- AGA GTT TGA TCC TGG CTC AG -3' and 16S1492R Universal Reverse - 5'- TAC CTT GTT ACG ACT T -3'. The reaction mix is prepared by adding 12µl Amplicon master mix, 2µl Forward primer, 2µl reverse primer, 5µl genomic DNA and 4µl Nuclease free water to make up the volume to 25µl. The PCR cycling conditions consisted of denaturation at 95°C for 2 min and subsequent 24 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 1 min and extension at 72°C for 2 min, final extension at 72°C for 10 min. The PCR products obtained were sequenced. The 16S rRNA sequences determined in this study were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI Basic Local Alignment Search Tools, nucleotide (BLASTn) program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Compared the Query (input) sequence against the sequences available in the database results with the list of hits. The sequence submitted to gen bank and to attain the accession ID.

### Antibiotic resistance test

The isolates were inoculated overnight in LB broth. Muller Hinton Agar plates were prepared and the swab of the respective isolates was performed with the help of sterile cotton swabs. The HimediaDodeca G-VI plus multiple antibiotic discs consisting of 12 different antibiotics (Ab-Antibiotics, LZ-Linezolid, RO-Roxithromycin, P-Penicillin, AMX-Amoxicillin, VA-Vancomycin, CB-Carbenicillin, MET-Methicillin, TEI-Teicoplanin, CD-Clindamycin, L-Lincomycin, AZM-Azithromycin, RIF-Rifampicin) in different concentrations was placed on the swabbed plate. The plate was kept for incubation at 37°C for overnight. The formation of zone shows that they are sensitive and the absence of zone shows that they are resistive against that specific antibiotic. The diameter of the zone was measured.

### Assay for biofilm formation

Three biofilm assays were performed such as Tube test method, Modified Congo Red Agar (MCRA) method; Microtiter Plate (MTP) method.

### Tube test method

Brain heart infusion (BHI) broth with 2% sucrose (10 ml) was inoculated with a loopful of bacteria from

overnight culture plates and incubated for 24 h at 37°C. The tubes were then decanted and washed with PBS (pH 7.3) and dried. Dried tubes were then stained with crystal violet (0.1%) for half an hour. Excess stain was removed; tubes were then dried and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as absent, moderate or strong.

#### **Modified Congo red agar method (MCRA)**

In the MCRA (Blood agar - 37 g/L, Glucose - 50 g/L, Congo red - 0.8 g/L) the CRA is modified by replacing sucrose with glucose, replacement of BHI Agar by an alternative agar, that is, Blood Base Agar. The MCRA plate was inoculated with bacteria from a fresh plate with overnight growth and then it was incubated for 24 h at 37°C and subsequently 2 to 4 days at room temperature. Positive result was indicated by black or red colonies with a dry crystalline consistency.

#### **Microtitre Plate (MTP) Method**

Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18–24 h at 37°C in a stationary condition. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the bacterial inoculums and only broth served as a control to check sterility and non-specific binding of the medium. These plates were incubated for 24 h at 37°C. After incubation, the content of the well was gently removed and then were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating “planktonic” bacteria. They were stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro Enzyme-Linked Immunosorbent Assay (ELISA) auto reader at wavelength of 570 nm (OD 570 nm). These OD values were considered as an index of bacteria adhering to the surface and forming biofilms.

#### **Selection of food preservatives**

The inhibition of growth of the different isolates was carried out by the use of food preservatives such as lime, vinegar, sodium chloride, nisin, sodium benzoate, sodium bicarbonate and citric acid. Muller Hinton Agar plates were prepared and the swab of the respective isolates was performed with the help of sterile cotton swabs. Wells were punctured and each preservative was

serially diluted in two fold dilution for seven different concentrations in sterile LB broth and 50 µl of each concentration was added to different wells. The plates were kept for overnight incubation at 37°C. The zone of inhibition was observed at different concentration for each type of food preservative.

#### **Minimum inhibitory concentration**

The lowest concentration for each preservative from the zone of inhibition was taken as the starting concentration for minimum inhibitory concentration which was serially diluted in two fold dilution for eight dilutions. The isolates from overnight LB broth was added to each well at 100µl concentration and left for overnight incubation. The contents in the well were discarded and the crystal violet assay was performed and the OD values were taken at 570nm using ELISA reader.

#### **Effect of preservation methods on biofilm formation**

Biofilm formation by the isolated bacteria was analyzed under various preservation conditions. The tests were conducted by assessing biofilm formation by each strain using the microplate assay under different preservatives like vinegar, citric acid, lemon, nisin, tamarind, sodium azide, sodium bicarbonate in nutrient broth individually.

## **RESULTS**

#### **Isolation of food borne bacterial pathogens**

Bacterial cultures isolated from Grape peel were streaked two selective medium (MSA and EMB agar) and the two isolates that showed good morphology in EMB agar was identified to be Gram negative and two isolates that were streaked on MSA plates were confirmed to be Gram Positives. On determining the 16S rRNA sequence of each bacterial culture, it was identified as *Kocuria kristinae*, *Staphylococcus haemolyticus*, *Burkholderia cenocepacia* and *Enterobacter cloacae*. The sequences were submitted in NCBI, GenBank and the accession numbers were assigned as MK615917, MK615918, MK615919 and MK615920 respectively.

#### **Antibiotic resistance patterns**

Antibiotic resistance study revealed that the isolated strains showed resistance towards many antibiotics at varying magnitudes. *Kocuria kristinae* showed resistance against the antibiotics amoxicillin and teicoplanin. *Staphylococcus haemolyticus* showed resistance against the antibiotics penicillin, amoxicillin, carbenicillin and teicoplanin. *Burkholderia cenocepacia* showed resistance against the antibiotics linezolid and teicoplanin. *Enterobacter cloacae* is a multidrug resistant organism

showing resistance against most of the antibiotics except roxithromycin and rifampicin. Among the antibiotics, all the isolates showed resistance against teicoplanin. Linezolid was found to be more effective among the antibiotics against the tested isolates with 30 mm inhibition for *Kocuria kristinae*, *Staphylococcus haemolyticus* and *Burkholderia cenocepacia* against the antibiotic Clindamycin (Table 1).

#### Biofilm formation by food borne pathogens

The lineformation was observed in *Burkholderiacenocepacia* and *Kocuria kristinae* after crystal violet staining. *Staphylococcus haemolyticus* and *Enterobacter cloacae* showed-stained violet indicating a positive result for biofilm formation by tube test method. Theblack crystalline colonies produced by *Kocuria kristinae*, *Burkholderia cenocepacia* and *Enterobacter cloacae* showed strong biofilm formation and the red colonies produced by *Staphylococcus haemolyticus* indicated weak biofilm production (Figure 1).

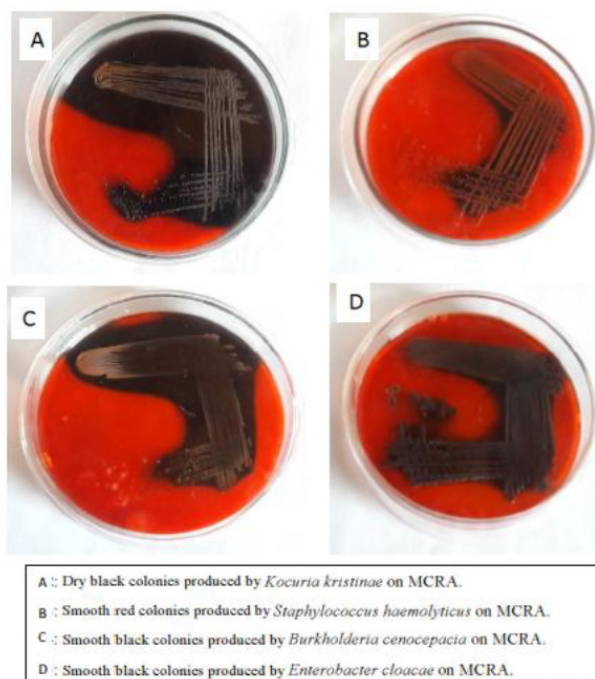
#### Microtitre Plate (MTP) Method

The OD values weredetermined with a micro Enzyme Linked Immuno Sorbent Assay reader and the values between 0.120-0.240 were considered moderate biofilm formation (Table 2).

**Table 1: Antibiotic resistance assay of *Kocuriakristinae*, *Staphylococcus haemolyticus*, *Burkholderiacenocepacia* and *Enterobacter cloacae*.**

Ab	Disc conc in mg	K. kristinae	S. haemolyticus	B. cenocepacia	E. cloacae
LZ	30	S	S	R	R
RO	15	S	S	S	S
P	10	S	R	S	R
AMX	30	R	R	S	R
VA	30	S	S	S	R
CB	100	S	R	S	R
MET	5	S	S	S	R
TEI	30	R	R	R	R
CD	2	S	S	S	R
L	2	S	S	S	R
AZM	15	S	S	S	S
RIF	5	S	S	S	R

R- Resistance, S- sensitive, Ab-Antibiotics, LZ-Linezolid, RO-Roxithromycin, P-Penicillin, AMX-Amoxicillin, VA-Vancomycin, CB-Carbenicillin, MET-Methicillin, TEI-Teicoplanin, CD-Clindamycin, L-Lincomycin, AZM-Azithromycin, RIF-Rifampicin



**Figure 1: Food borne bacteria streaked on Modified Congo Red Agar (MCRA).**

**Table 2: OD values of MTP assay and the biofilm formation.**

Isolates	OD at 570nm	Biofilm formation
<i>Kocuriakristinae</i>	0.171	Moderate
<i>Staphylococcus haemolyticus</i>	0.159	Moderate
<i>Burkholderia cenocepacia</i>	0.121	Moderate
<i>Enterobacter cloacae</i>	0.136	Moderate

#### Minimum inhibitory concentration for different preservatives

Different concentrations of various chemical food preservatives were used to inactivate selected food borne pathogenic bacteria by agar diffusion method. The zone of inhibition was observed around the wells which indicated the antibacterial activities of the chemical preservatives. Different isolates demonstrated different rankings for the inhibiting effects of chemical preservatives. The least dilution of zone formation was taken as the initial concentration for the minimum inhibitory concentration assay (Table 3).

#### Nisin

Nisin was taken at 100mg/ml concentration and was serially diluted in two fold dilutions for seven concentrations and well diffusion method was performed to obtain the zone of inhibition. Only *Staphylococcus*

**Table 3: MIC for food preservatives at different Concentrations/dilutions for inhibiting the growth of Food borne bacteria.**

Food borne bacteria	MIC for Food Preservatives at different Concentrations/dilutions					
	Nisin (100 mg/ml)	Vinegar (undiluted)	Lemon (undiluted)	Citric acid (250 mg/ml)	Sodium azide (100 mg/ml)	Sodium bicarbonate (250 mg/ml)
<i>Kocuria kristinae</i>	No zone	No zone	1:8 dilution	15.625 mg/ml	6.25 mg/ml	No zone
<i>Staphylococcus haemolyticus</i>	1.56 mg/ml	1:2 dilution	1:8 dilution	15.625 mg/ml	12.5 mg/ml	250 mg/ml
<i>Burkholderiacenocepacia</i>	No zone	1:1 dilution	1:4 dilution	31.25 mg/ml	1.56 mg/ml	No zone
<i>Enterobacter cloacae</i>	No zone	1:4 dilution	1:8 dilution	31.25 mg/ml	1.56 mg/ml	250 mg/ml

**Table 4: Zone of inhibition for different concentrations of nisin.**

Conc in mg/ml	100	50	25	12.5	6.25	3.125	1.56
Zone in cm	2	1.8	2.2	1.6	1.5	1.2	1

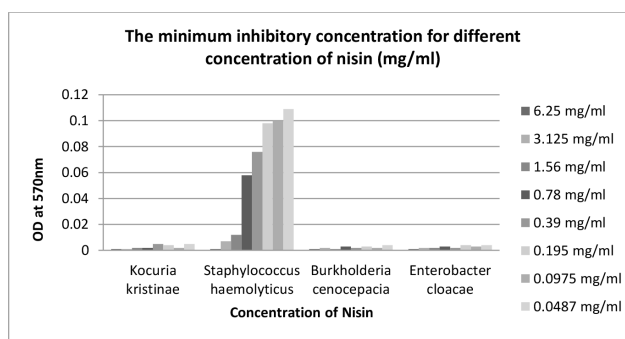
*haemolyticus* showed positive result for all the dilutions. 6.25mg/ml of nisin which is the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 4).

The MIC was observed in *Staphylococcus haemolyticus* which exhibited increased biofilm activity from 6.25mg/ml minimum inhibitory concentration to 0.00487 mg/ml concentration. Therefore concentration greater than 6.25mg/ml of Nisin is suitable to inhibit the biofilm formation in *Staphylococcus haemolyticus* where as 0.0487mg/ml of Nisin was sufficient to inhibit the biofilm formation in *Kocuria kristinae*, *Burkholderia cenocepacia* and *Enterobacter cloacae*. This shows that Nisin in very low concentration can be used to eradicate the biofilm formation (Figure 2).

### Vinegar

Undiluted vinegar was serially diluted in two fold dilutions for seven concentrations and well diffusion method was performed to obtain the zone of inhibition. Isolates *Staphylococcus haemolyticus*, *Burkholderia cenocepacia* and *Enterobacter cloacae* showed positive results at different dilutions. 1:2 dilutions of vinegar and sterile distilled water which is the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 5).

*Staphylococcus haemolyticus* exhibited zone formation for the dilutions 1:1 and 1:2. *Burkholderia cenocepacia* exhibited zone formation the dilution 1:1 and *Enterobacter cloacae* for the dilutions 1:1, 1:2 and 1:4 (Figure 3). The MIC was observed in *Staphylococcus haemolyticus*, *Burkhold-*

**Figure 2: MIC for different concentration of nisin (mg/ml).****Table 5: Zone of inhibition for different concentrations of vinegar.**

Concentration	1:1	1:2	1:4
Zone in cm			
<i>Staphylococcus haemolyticus</i>	1.5	1.1	-
<i>Burkholderia cenocepacia</i>	1.6	-	-
<i>Enterobacter cloacae</i>	2	1.5	1.3

*eriacecepacia* and *Enterobacter cloacae* which exhibited increased biofilm activity from 1:2 dilution. Hence vinegar used in 1:2 dilutions will be optimal for biofilm inhibition.

### Lemon

Undiluted lemon was serially diluted in two fold dilutions for seven concentrations and well diffusion method was performed to obtain the zone of inhibition. All the isolates showed positive results at different dilutions. 1:4 dilutions of lemon and sterile distilled water which is the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 6).

*Kocuria kristinae*, *Staphylococcus haemolyticus* and *Enterobacter cloacae* exhibited zone formation for first four dilutions and, *Burkholderia cenocepacia* and produced exhibited zone formation for first three dilutions (Figure 4). The MIC was observed in all the food borne pathogenic bacterial

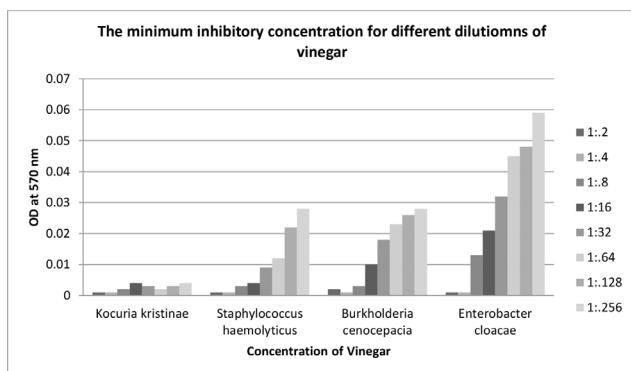


Figure 3: MIC for different concentration of Vinegar.

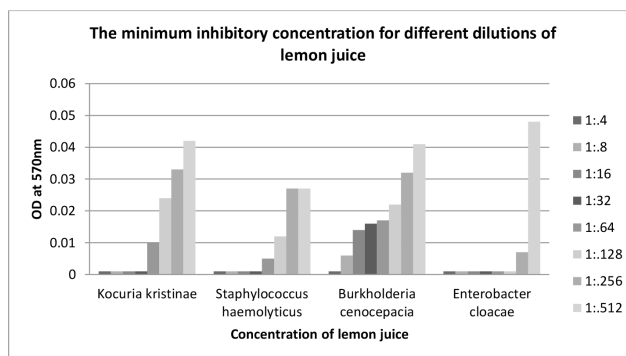


Figure 4: MIC for different concentration of lemon juice.

Table 6: Zone of inhibition for different concentrations of lemon.				
Concentration	1:1	1:2	1:4	1:8
Zone in cm				
<i>Kocuriakristinae</i>	1.5	1.3	1.1	1
<i>Staphylococcus haemolyticus</i>	1.7	1.5	1.3	1.1
<i>Burkholderia cenocepacia</i>	1.4	1.1	1	-
<i>Enterobacter cloacae</i>	1.2	1.2	1	1

Table 7: Zone of inhibition for different concentrations of citric acid.					
Concentration in mg/ml	250	125	62.5	31.25	15.625
Zone in cm					
<i>K. kristinae</i>	3.5	3	2.5	2.1	1.5
<i>S. haemolyticus</i>	2.5	2	2	2	1.5
<i>B. cenocepacia</i>	2	1.7	1.5	1.3	-
<i>E. cloacae</i>	1.7	1.5	1.3	1.2	-

isolates which exhibited increased biofilm activity from 1:4 dilution. Hence lemon used in 1:4 dilutions will be optimal for biofilm inhibition.

**Citric acid**

250mg/ml of citric acid was serially diluted in two fold dilutions for seven concentrations and well diffusion method was performed to obtain the zone of inhibition. All the isolates showed positive results at different dilutions. 31.25mg/ml of citric acid which is the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 7).

*Kocuria kristinae* and *Staphylococcus haemolyticus* exhibited zone formation for first five dilutions and *Burkholderia cenocepacia* and *Enterobacter cloacae* exhibited zone formation for first four dilutions (Figure 5). The MIC was observed in the food borne pathogenic bacterial isolates which exhibited increased biofilm activity from 31.25mg/ml. Hence citric acid in the concentration of 31.25mg/ml will be optimal for biofilm inhibition.

**Sodium azide**

100mg/ml of sodium azide was serially diluted in two fold dilutions for seven concentrations and well diffusion method was performed to obtain the zone of inhibition. All the isolates showed positive results at different dilutions. 12.5mg/ml of sodium azide which is

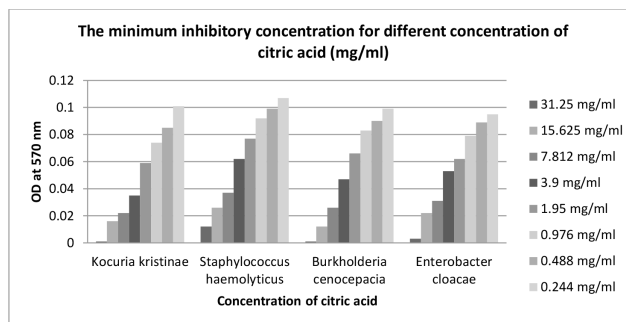


Figure 5: MIC for different concentration of citric acid.

the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 8).

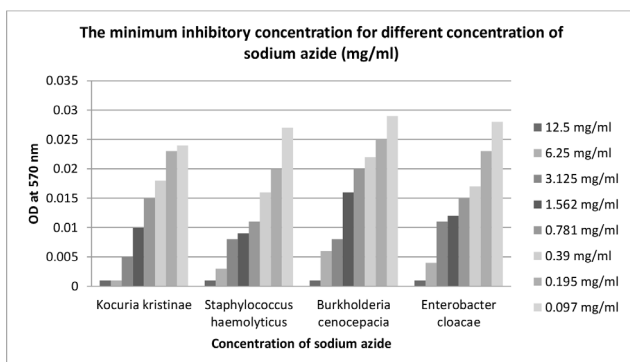
*Kocuria kristinae* exhibited zone formation for first five dilutions. *Staphylococcus haemolyticus* exhibited zone formation for first four dilutions. *Burkholderia cenocepacia* and *Enterobacter cloacae* exhibited zone formation for all dilutions (Figure 6). The MIC was observed in all the food borne pathogenic bacteria isolates which exhibited increased biofilm activity from 12.5mg/ml. Hence sodium azide in the concentration of 12.5mg/ml will be optimal for biofilm inhibition.

**Sodium azide**

250mg/ml of sodium bicarbonate was serially diluted in two fold dilutions for seven concentrations and well

**Table 8: Zone of inhibition for different concentrations of sodium azide.**

Concentration in mg/ml	100	50	25	12.5	6.25	3.125	1.56
<b>Zone in cm</b>							
<i>K. kristinae</i>	3	2.6	2	1.5	1.1	-	-
<i>S. haemolyticus</i>	3	2.5	2	1.5	-	-	-
<i>B. cenocepacia</i>	4	3.5	3.4	3.2	2.8	2.4	2
<i>E. cloacae</i>	4.5	4	3.6	3.2	2.8	2.4	2

**Figure 6: MIC for different concentration of sodium azide.**

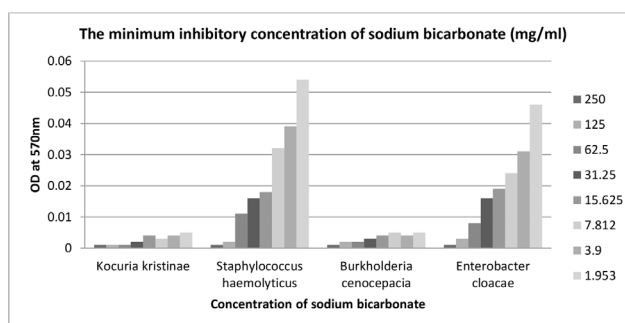
Concentration in mg/ml	250
<b>Zone in cm</b>	
<i>Staphylococcus haemolyticus</i>	2.3
<i>Enterobacter cloacae</i>	2.3

diffusion method was performed to obtain the zone of inhibition. Isolates *Staphylococcus haemolyticus* and *Enterobacter cloacae* showed positive results at one dilution. 250mg/ml of sodium bicarbonate which is the minimal inhibitory concentration was serially diluted in two fold dilutions for eight concentrations and the MTP assay was performed (Table 9). *Staphylococcus haemolyticus* and *Enterobacter cloacae* exhibited zone formation for first dilution only (Figure 7).

The MIC was observed in *Staphylococcus haemolyticus* and *Enterobacter cloacae* which exhibited increased biofilm activity from 250mg/ml. Hence sodium bicarbonate in the concentration of 250mg/ml will be optimal for biofilm inhibition.

## DISCUSSION

In many parts of the world, including India, there is an increase in consumption of raw fresh produce like vegetables, fruits and sprouts. Vending of cut salad vegetables, fruits and sprouts is a common practice in

**Figure 7: MIC for different concentration of sodium bicarbonate.**

India. It has become increasingly popular to eat healthy raw salad vegetables, fruits and sprouts preferentially to any other fast food.<sup>[14]</sup> WHO street-vended food survey revealed that fruits and vegetables form 86% of the total market. Several outbreaks of human gastro-enteritis have been linked to the consumption of contaminated fresh vegetables, fruits and sprouts.<sup>[15,16]</sup> The low sanitation standards during postharvest handling and an increased consumption of raw produce and related products have generated heightened concerns for food safety in developing countries.<sup>[17]</sup> Fresh produce, including apples, grapes, lettuce, peaches, peppers, spinach, sprouts and tomatoes, are known to harbor large bacterial populations. Since fresh produce is often consumed raw, such pathogens can cause widespread disease outbreaks. The consumption of raw produce may represent an important means by which new lineages of commensal bacteria are introduced into the human gastrointestinal system. More generally, produce-associated microbes can have important effects on the rates of food spoilage.<sup>[18]</sup>

Biofilm is attached to substrates as well as express virulence phenotypes and the biofilm formation is one of the important traits of pathogens. In the present study, *Kocuria kristinae*, *Staphylococcus haemolyticus*, *Burkholderia cenocepacia* and *Enterobacter cloacae* were moderate producers of biofilm. In the study conducted by Amrutha *et al.*<sup>[19]</sup> the biofilm forming ability of *E. coli* and *Salmonella* spp., isolated from fresh fruits and vegetables were reported. Biofilm formation is a serious concern in food industry, as pathogenic micro-organisms are capable of attaching and growing on food surfaces and surfaces of other processing equipments. Earlier investigations have shown that the presence of pathogens with ability to form biofilms is one of the leading causes of food contamination and precipitation of disease. In the present study, *Staphylococcus haemolyticus* was a moderate producer of biofilm. There are no reports on the

biofilm formation for *Kocuria kristinae*, *Burkholderia cenocepacia* and *Enterobacter cloacae*.

A study conducted by Nadaraja *et al.*<sup>[20]</sup> included the preservation studies to tackle the biofilm forming food pathogens under different temperature, salt concentrations and pH. The antibiotic resistant assay and the hydrolytic enzyme activity were also studied.

In the present study, antibiotic resistant assay was carried out with a multiple antibiotic disc consisting of 12 antibiotics and the multidrug resistance was observed for different isolates. *Enterobacter cloacae* is a multidrug resistant organism showing resistance against most of the antibiotics except roxithromycin and rifampicin. Among the antibiotics, all the isolates showed resistance against teicoplanin. Linezolid was found to be more effective among the antibiotics against the tested isolates with 30 mm inhibition for *Kocuria kristinae*, *Staphylococcus haemolyticus* and *Burkholderia cenocepacia* against the antibiotic Clindamycin.

Preservatives retard degradation caused by microorganisms and therefore maintain the color, texture, flavor of the food item and extend the shelf life of certain food products.<sup>[21]</sup> They included preservatives like vinegar, citric acid, salt sugar, Sorbic acid. Thus in the present study, food preservatives/food products such as vinegar, lemon, citric acid, sodium azide and sodium bicarbonate were used in the biofilm eradication. The use of these preservatives/food products for biofilm inhibition is not being reported yet. The above were used as they are the most commonly used in day to day activities and are easily accessible. Each of the preservative in different concentration showed different activities against the bacterial isolates. These concentrations were obtained by the Minimum Inhibitory Concentration (MIC) assay performed for each preservative in different concentrations. The least concentration of the preservative which inhibited the growth of the bacterial pathogen was taken as the initial concentration for MIC assay. In the study conducted by Mahdavi *et al.*<sup>[22]</sup> bactericidal effect of different concentrations of nisin on bacterial biofilms had no significance. Also, the effects of nisin on various bacterial biofilms were equal. But in the present study, it is evident that nisin in very low concentration can be used to eradicate the biofilm formation which is in contradiction to the existing study. Vinegar is now mainly used as a cooking ingredient, or in pickling. Vinegar is used in cooking not only for its flavor qualities but also its chemical properties. Because it is highly acidic, vinegar is naturally resistant to bacterial growth and spoilage. The present study showed that Vinegar used in 1:2 dilution will be optimal

for biofilm inhibition for *Staphylococcus haemolyticus*, *Burkholderia cenocepacia* and *Enterobacter cloacae*.

According to the study conducted by Aruoma *et al.*<sup>[23]</sup>; Karimi *et al.*<sup>[11,6]</sup>, citrus juices are consumed majorly because of their nutritional value and special flavor. Citrus fruits are also known to contain bioactive compounds such as phenolics, flavonoids, vitamins and essential oils which are believed to be responsible for a range of protective health benefits including antioxidative, anti-inflammatory, antitumor and antimicrobial activities. The present study showed that lemon used in 1:4 dilutions will be optimal for biofilm inhibition for all the bacterial pathogens.

Citric acid is most concentrated in lemons and limes and exists in a variety of fruits and vegetables. Because it is one of the stronger edible acids, the citric acid is used as a flavoring and preservative agent in food and beverages. The present study showed that citric acid in the concentration of 31.25mg/ml will be optimal for biofilm inhibition for all the bacterial pathogens.

Sodium bicarbonate is widely used in foods at levels up to 2% for leavening, pH-control, taste and texture development. Low cost and lack of toxicity would favor its use as a preservative (Corral *et al.* 1988).<sup>[24]</sup> The present study showed that sodium bicarbonate in the concentration of 250mg/ml will be optimal for biofilm inhibition for all the bacterial pathogens.

## CONCLUSION

In the present study four food borne pathogens were isolated from grape peel and the 16s rRNA sequence were determined and submitted to NCBI gene bank. The antibiotic resistance test showed multidrug resistance. The biofilm forming assays were carried out in three different methods. Different natural preservatives such as vinegar, lemon, tamarind, citric acid, sodium azide, sodium bicarbonate and Nisin were used for the biofilm eradication. Nisin is suitable preservative to inhibit the biofilm formation. Vinegar in 1:2 dilution, Lemon in 1:4 dilution, Citric acid in 31.25mg/ml and Sodium azide in 12.5mg/ml will be optimal for biofilm inhibition of all the isolates. Sodium bicarbonate in 250mg/ml is optimal for inhibition of *Staphylococcus haemolyticus* and *Enterobacter cloacae*. This study recommends the use of food preservatives in washing the fruits before consumption.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**MCRA:** Modified Congo Red Agar; **MTP:** Micro Titre Plate; **EPS:** Extracellular polymeric substance; **SSA:** Salmonella Shigella agar; **MSA:** Mannitol Salt agar; **EMB:** Eosin Methylene Blue agar; **PIA:** Pseudomonas isolating agar; **BLASTn:** Basic Local Alignment Search Tools, nucleotide; **MIC:** Minimum inhibitory Concentration; **PCR:** Polymerase Chain Reaction; **BHI:** Brain heart infusion; **Ab:** Antibiotics; **LZ:** Linezolid; **RO:** Roxithromycin; **P:** Penicillin; **AMX:** Amoxicillin; **VA:** Vancomycin; **CB:** Carbenicillin; **MET:** Methicillin; **TEI:** Teicoplanin; **CD:** Clindamycin; **L:** Lincomycin; **AZM:** Azithromycin; **RIF:** Rifampicin.

## SUMMARY

The biofilm eradication of the food borne bacterial pathogen isolated from grape peel using food preservatives/food products was carried out and showed that lower concentrations of these preservatives were sufficient for the inhibition of biofilm formation. The preservatives were chosen in such a way that they could be easily accessible and available in our daily routine. *Burkholderia cenocepacia* and *Enterobacter cloacae* exhibited biofilm formation only at pH 8 which makes its preservation much simple. *Burkholderia cenocepacia* and *Enterobacter cloacae* exhibited no biofilm formation at any concentration of NaCl. This shows that it can be preserved easily under extreme salt concentrations. *Kocuria kristinae* exhibited a drastic decline in biofilm formation which shows its preservation at high salt concentration. *Kocuria kristinae* and *Staphylococcus haemolyticus* could be preserved at lower temperatures whereas *Burkholderia cenocepacia* and *Enterobacter cloacae* could be preserved at higher temperatures.

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