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Effect of organic acids on biofilm formation and quorum signaling of pathogens from fresh fruits and vegetables

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ABSTRACT

Organic acids are known to be used as food preservatives due to their antimicrobial potential. This study evaluated the ability of three organic acids, namely, acetic acid, citric acid and lactic acid to manage *E. coli* and *Salmonella* sp. from fresh fruits and vegetables. Effect of these organic acids on biofilm forming ability and anti-quorum potential was also investigated. The effect of organic acids on inactivation of *E. coli* and *Salmonella* sp. on the surface of a selected vegetable (cucumber) was determined. The minimum inhibitory concentration of the organic acids were found to be 1.5, 2 and 0.2% in *E. coli* while it was observed to be 1, 1.5 and 1% in *Salmonella* sp. for acetic, citric and lactic acids respectively. Maximum inhibition of biofilm formation was recorded at 39.13% with lactic acid in *E. coli* and a minimum of 22.53% with citric acid in *Salmonella* sp. EPS production was affected in *E. coli* with lactic acid showing reduction by 13.42% while citric acid and acetic acid exhibited only 6.25% and 10.89% respectively. Swimming and swarming patterns in *E. coli* was notably affected by both acetic and lactic acids. Lactic and acetic acids showed higher anti-quorum sensing (QS) potential when compared to citric acid. 2% lactic acid showed a maximum inhibition of violacein production by 37.7%. Organic acids can therefore be used as potential quorum quenching agents in food industry. 2% lactic acid treatment on cucumber demonstrated that it was effective in inactivating *E. coli* and *Salmonella* sp. There was 1 log reduction in microbial count over a period of 6 days after the lactic acid treatment. Thus, organic acids can act as effective potential sanitizers in reducing the microbial load associated with fresh fruits and vegetables.

1. Introduction

Food borne pathogens associated with fresh produce are one of the major concerns in food borne outbreaks recorded worldwide. These pathogens are more hazardous when they are in the form of biofilms. In the food industry, spoilage or pathogenic bacteria are able to attach themselves to processing surfaces and form biofilms [20]. Quorum signaling plays a vital role in the biofilm formation [3]. reported that bacterial virulence is in many cases controlled by quorum sensing. Quorum sensing controls the process of biofilm formation, which gives the bacterial population in biofilm an edge over other non-biofilm producers by providing them with superior access to nutrients [21]. There has been much evidence to support the association of pathogens, biofilm formation and foodborne illness. The adhesion of different pathogenic bacteria including *Bacillus, Salmonella, Listeria, Staphylococcus*, and *Escherichia* with subsequent formation of biofilms in food processing surfaces can cause food spoilage or transmission of diseases [4,22]. Fresh fruits and salads in raw form put the consumers at an increased risk of acquiring foodborne illnesses. The proportion of food-borne outbreak-associated illnesses associated with raw produce increased from 1% in the 1970s to 12% in the 1990s [30]. The chemicals such as chlorine and hydrogen peroxide, though used in food industries on a regular basis have not helped in reducing the number of outbreaks. With the increase in occurrence of food borne outbreaks associated with fresh produce, there is mounting interest in the use of novel biocide applications to prevent or reduce microbial contamination in food industries.

Organic acids are approved as generally recognized as safe (GRAS) by the Federal Drug Administration (FDA). Organic acids have been documented to possess antimicrobial activities against different pathogens such as *E. coli*, *Salmonella* sp. and *Listeria*

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monocytogenes. Undissociated organic acid molecules freely permeate into microbial cell membrane and lower the intracellular pH by dissociating inside the cell and become inhibitory to microorganisms [5,31]. The antimicrobial mechanism of action of organic acids has been reported to be due to decreased gastric pH resulting in unfavourable conditions for pathogenic bacteria [9]. Secondly, organic acids in undissociated form are lipophilic and can therefore diffuse across bacterial cell membranes to reach the interior of the cell to disrupt cell function [12,24]. Given the weak acid nature of most of these compounds, pH is considered a primary determinant of effectiveness because it affects the concentration of undissociated acid formed [11]. Several studies have proved the antimicrobial activity of organic acids [19]. reported that citric acid was an antimicrobial chemical which significantly affected the biofilm elasticity of pre-formed *Pseudomonas* biofilms. Another study demonstrated that gallic acid has the potential to inhibit bacterial motility and thus prevent and control biofilms of these pathogenic bacteria [7]. Malic acid was found to be effective for inhibition of *S. enterica* serovar *typhimurium* biofilm in carrot and other food contact surfaces [29] [23]. showed that citric acid, malic acid, and tartaric acid exhibited strong antibacterial activity at 75.0 mM against pathogens such as *E. coli* O157: H7, *Listeria monocytogenes* and *Salmonella typhimurium*. Though research has been carried out to control pathogens in food industry, chemical methods have not been efficiently applied to alleviate microbial load associated with fresh produce. Studies have been limited to laboratory alone and it has to be taken to the industry level for practical implementation so that foodborne outbreaks can be managed. Pathogens in the biofilm are more resistant to antimicrobial agents and thus, the present study aims on quorum quenching based agents for alleviation of biofilms on fresh fruits and vegetables. The effect of organic acids on quorum signaling and their role in *E. coli* and *Salmonella* spp. biofilms from fresh fruits and vegetables was analyzed. Further research and proper validation of organic acid sanitizing in fresh produce industries can be of great significance in food safety.

2. Materials and methods

2.1. Bacterial strains and conditions

A total of 12 strains including 8 *E. coli* and 4 *Salmonella* sp. isolated from fresh fruits and vegetables were taken for the study. Reference strains of *E. coli* (MTCC 727), *S. typhimurium* (ATCC 14028) and *C. violaceum*/CV 026 (CECT5999) were also included.

2.2. Determination of minimum inhibitory concentration (MIC) of organic acids

The organic acids, namely Acetic acid, Citric acid and Lactic acid were prepared in different concentrations ranging from 0.2 to 2%. Different concentrations (0.2–2%) of organic acids were added to LB broth inoculated with *E. coli* and *S. enterica* and incubated at 37 °C for 24 h. Growth inhibition at lowest concentration of nanoemulsion was noted as MIC. The MIC was recorded as the lowest concentration, which showed complete inhibition of visible growth. All further experiments in the present study were performed at sub-MIC concentrations of each acid.

2.3. Effect of organic acids on biofilm formation of pathogens

2.3.1. Anti-biofilm assay

The effect of organic acid on biofilm formation of test isolates was determined by quantifying the biofilm biomass through MTP assay [32]. Briefly, 1% of overnight cultures of test isolates were added into 1 ml of fresh LB medium and cultivated in the presence and absence of organic acids 30 °C. After incubation, the planktonic cells in MTPs were removed by rinsing the wells twice with sterile water. The surface-adhered cells in the MTP wells were stained with 200 μL of Crystal violet (CV) solution (Hi Media, India). After 5 min, excess CV solution was removed and CV in the stained cells was solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of CV at 650 nm using UV–visible spectrophotometer.

2.3.2. Exopolysaccharide (EPS) production

Test isolates were grown in 100 ml LB medium with 1 ml of different organic acids and incubated at their optimum temperature of 30°c. The biofilms adhered to walls were harvested during late-log-phase by centrifugation at 8000 rpm for 30 min at 20 °C. The supernatant was filtered and three volumes of chilled 100% ethanol was added to it followed by incubating overnight at −20 °C to precipitate EPS [16]. The precipitated EPS was collected by centrifuging the overnight filtered supernatant at 7000 rpm for 30 min at 5°c. The pellet was then dissolved in 1 ml of MilliQ water. EPS was quantified by adding 1 ml of 5%phenol and 5 ml of conc. H2SO4 to 1 ml of the dissolved EPS against different concentrations of glucose [13].

2.3.3. Swimming and swarming motility

For swimming assay, overnight cultures of the test bacteria were point inoculated at the center of the medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with different concentrations of organic acids (200 μL). For swarming assay, test bacteria were point inoculated at the center of the medium consisting of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter sterilized D-glucose with various concentrations of organic acids (200 μL). The plates were incubated at 30 °C in upright position for 16 h. Following incubation, swimming and swarming migration were observed.

2.4. Anti-quorum efficacy of organic acids

2.4.1. Well diffusion assay

To detect the anti-QS activity of organic acids, well diffusion assay was carried out. Briefly, 100 μl of exogenous HHL (*N*-hexanoyl-DL-homoserine lactone) was added to 100 ml of sterilized LB agar, gently mixed and poured into petriplates. Overnight culture of *C. violaceum* CV026 isolates was then swabbed uniformly onto the solidified agar surface. Wells were punched into the agar and different concentrations of organic acids were loaded into them. The plates were then incubated at 37 °C for 24 h. Anti quorum activity was scored as an obscure, colorless, but doable halo around the discs. Sonicated Tween80 and water were used as control.

2.4.2. Flask incubation assay

For the quantitative determination of violacein inhibition by organic acids, flask incubation assay was carried out. CV 2656 inoculated LB broth supplemented with HHL (5 mM) and organic acids at different concentrations were incubated for 24 h at 30 °C. Violacein extraction was carried out as described by Ref. [10]. The experiment was repeated for triplicate values and the percentage of inhibition was calculated by the formula as follows:

$$\%\text{Inhibition} = \frac{\text{control OD}_{585\text{ nm}} - \text{test OD}_{585\text{ nm}}}{\text{control OD}_{585\text{ nm}}} \times 100$$