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Effect of Flavonoid Application on Biofilm Forming Ability of *B. cereus*

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Abstract: ***The well known human pathogen Bacillus cerus found ubiquitously in a wide of environments including soil and raw food surfaces. It is also known as a potential food poisoning agent and can form biofilm, which is one of the significant public health concerns. The present study was aimed to understand the ability of the Bacillus cereus strain to form biofilm on different packaged fresh produces including spinach, cabbage and cucumber surfaces. Crystal violet assay results revealed significant difference in biofilm formation abilities on target vegetable surfaces. Next, the inhibitory effect of combined formulations of flavones was undertaken. The biofilm inhibitory effect of formulations 1 was observed highest (almost 88 %.) on surface of cucumber produce. Our study demonstrated ability of flavnoids in food safety. An environmental friendly application on raw vegetable surfaces with minimal treatment approach prior to packaging may prevent the contamination of such food products and help in maintaining the safety as well as improving the quality of the packaged fresh produces.***

Keywords: Bacillus cerus; Biofilm Formation; Flavones formulations

1. Introduction

All microorganisms grow as single cells (planktonic) in suspension medium which provides them all the required growth factors for their survival and maintenance, after many studies on the growth pattern and interaction of microorganisms with other surfaces, it has been came to the conclusion that these microorganismsshow surface association that leads to the different interaction that eventually form dynamic structure on the attached surface which is called as "biofilm". [1] Such surface attached phenomenon of forming biofilm was first discovered by van Leeuwenhoek, who first describes and observed microbial biofilm. [2]

Biofilms arestable architecture formed by the microbial consortia in which microorganisms reside in the matrix secreted by them only, which act as shelter and give them many benefits such as protection from the tough environmental conditions, includingosmotic imbalance,desiccation, high temperature, high pH etc. [3] and resistance to antimicrobial agents. [4] Apart from the benefits being biofilm could be the fatal in many cases, biofilms can be the major cause life threatening disorders[5] their impact on different industries leads to the many economic problems among the major industries impacted by biofilm food industry is one of them which is facing economic loss as well as the spoilage of the food (end product) by biofilms, this is because of the properties of the food and related products that act as media for attachment of many microbes which eventually forms biofilm on food surfaces[6],among the different foods, present study focuses on the fresh vegetable produce which are the major part of the human dietsuch as vegetables, fruits which are the source of active phytoingreadients, micro and macro nutrients and poses properties like antioxidant, anti-inflammatory as well as anticancer [7] major problem with the direct consumption of such raw produce is that they undergoes minimal treatment after harvest before their packaging and thus they are highly susceptible to the post-harvest contamination and become the carrier of many microorganisms that form biofilms on the fresh produce surfaces and make them unfit for the consumption; the pathogenic (food poisoning) microbes also present in soil and eventually on these surfaces and transmit life threatening diseases to the consumers. [7],[8],[9]

Among all the significant food poisoning pathogens *Bacillus cereus* is one persist in the soil and environment. With its resistive spores quite resistant to the different antimicrobials and temperatures. [10] Different outbreaks have been reported because of the consumption of the food contaminated with *Bacillus cereus* due to the persistence of the spores in the food and production of food poisoning toxins that leads to the emetic syndrome and diarrheal syndrome. [11], [12], [13] These issues highlight the importance of searching for alternative agents to prevent biofilm formation. Diverse biofilm formation abilities of many microorganisms were demonstrated in quite a few research studies [14], [15], [16] depending on their source of isolation. Surface diversities may result in variation in total biomass production, ability of spore formation, and the EPS composition etc. In this study, our focus was to perform quantification of biofilm formation by *B. cerus* and evaluate the biofilm inhibitory effect of the two different formulations of flavonoids on the surface of spinach, cabbage and cucumber respectively.

2. Materials and Methods

Chemicals Reagents and Bacterial Strain

All the solvents and media were obtained from the Merck Pvt.Ltd, Mumbai and Hi media Pvt.Ltd, Mumbai respectively. The test samples of flavonoids including rutin, quercetin and morin were obtained from the local supplier alpha chemicals, Mumbai, India.

The microbial strain *Bacillus cereus* was isolated from the contaminated vegetables in our prior studies and identified based on biochemical characterisation. The bacterial culture was maintained on slant as stocks and sub-cultured on regular interval on newly prepare fresh nutrient agar medium prior to use in experiment. The packaged food material including fresh produce cabbage, spinach and cucumber

have been collected from the local organic packaged food market in Mumbai, India.

Biofilm Formation Assay

Static biofilms were grown as per previously described [17] protocol with slight modification. Disc-shaped pieces of vegetables were cut using a sterilized 2-cm diameter cork-borer and stored in 100 mm x 15 mm petri dishes with distilled water to preserve humidity. Cabbage and cucumber pieces were stored at 4^0C until the time of inoculation and used within 48 h of their preparation. Sterile pieces of each cut vegetable was inoculated along with sterile nutrient broth medium per tube followed by aseptic transfer of isolated strain of *B. cereus* with 1% (v/v) of overnight culture. Negative control tubes filled with same medium and without bacterial inoculation were used as a control. All tubes were kept at 37°c for 24 h respectively for the biofilm formation and assay was performed in triplicates.

Biofilm Quantification

Crystal violet (CV) assay was used based on earlier protocol described [15] to measure biofilm formation. Each set of experimental tubes after incubation subjected for assay. The medium was removed and the tubes were washed three times with phosphate-buffered saline (PBS) (Life Technologies, USA). Vegetable cuts were stained with 0.1% (w/v) CV for 30 min. After incubation, extra CV which did not bind to biofilms was discarded. The vegetable cuts were washed again three times with PBS. Subsequently, 70% (v/v) ethanol was added to each tube with vegetable cuts and incubated for 30 min to release biofilm bound by CV. The solubilised CV was quantified by measuring the absorbance value at a wavelength of 595 nm.

Preparation Of Flavnoid Formulations

Two formulations were prepared using different falvnoids in combination as, one with mixture of Rutin and Quercetin and another with mixture of Rutin and Morin. The formulation prepared was labelled as 1 and 2 respectively. The final concentrations of each of these formulations was diluted as 80, 100, 120, 160, 200, 240ug/ml using 0.1%DMSO (Dimethyl sulfoxide) as diluents and kept in aliquots ready to use.[18]

Determination of Minimum Inhibitory Concentration of Formulations

The Minimum inhibitory concentrations of each of the formulations 1 and 2 were determined based on the earlier studies with slight modification.[19] Different concentrations of formulation 1 and 2 were prepared (40, 80, 100, 120, 160, 200, 240µg/ml and DMSO) incubated with the bacterial inoculums in broth dilution assay at 37^0C for 24h. After incubation period the growth turbidity was measured along with viable plate count and results were expressed with concentration where no growth was observed. The assay was performed in triplicate with each of the formulation and concentration.

Effect of Flavnoid Formulation on *B. Cereus* Biofilm Formation

Antibiofilm Assay was performed on cut pieces of vegetables surfaces of each fresh produce in the sterile petriplates were treated with 0.1ml of formulation 1 and 2 respectively and was allowed to settle for 30 mins.After 30 mins. Cut vegetable pieces were inoculated with the 1ml of the overnight culture of *Bacillus cereus* and incubated in nutrient broth at 37^0C for 24 h. After an overnight incubation, the quantitative estimation of biofilm was performed. The medium was removed and the tubes were washed three times with phosphate-buffered saline (PBS). Vegetable cut pieces were stained with 0.1% (w/v) CV for 30 min. After incubation, extra CV was discarded. The vegetable cuts were washed again three times with PBS. Subsequently, 70% (v/v) ethanol was added to each tube with vegetable cuts and incubated for 30 min to release biofilm bound by CV. The solubilised CV was quantified by measuring the absorbance value at a wavelength of 595 nm. The observed readings were subjected for calculation of % inhibition using formula:

$$\% \text{ Inhibition} = \frac{\text{O.D. of Control - O.D. of Test}}{\text{O.D. of Control}} \text{ X100}$$

3. Results and Discussion

Biofilm Formation Assay (Tube Assay)

The biofilm formation tube assay was performed to evaluate maximum biofilm forming ability of the isolated bacterial strain on different cut vegetable surfaces including Spinach, Cabbage and Cucumber.

Quantitative assay was performed to determine how much biomass was present on the surface of each fresh produce. Theassay was performed which involves visualization of the stained and fresh produce surface followed by crystal violet assay The results obtained from the biofilm formation assay were taken as a average of the three readings observed in each of the experiment set. Normal visual inspection confirmed that spinach leaf was totally spoiled after incubation of 24h. The soft and fragile nature of leaves that made them unsuitable for long incubation hours and it also results in imparting greenish tinge after crystal violet assay. The obtained optical density (O.D.) values were significantly observed higher than that of cabbage and cucumber after 24h, of incubation. For further studies spinach leaves were excluded.

Minimum Inhibitory Concentration of Formulations on *B. Cereus*

The lowest concentration of the formulation 1 and 2 found inhibitory against *B. ceros* isolate was found to be 200µg/ml and same was used for further experiment which was found correlated with earlier studies with other food born pathogen. [19]

Effect of Flavnoid Formulations on *B. Cereus* Biofilm Formation

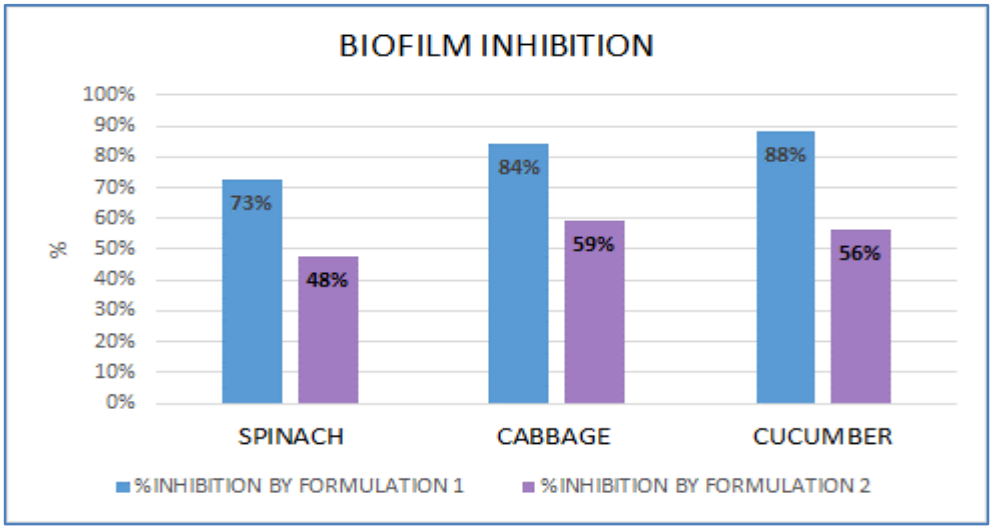

Figure 1: Comparative inhibition in the % of biofilm on the fresh produce with both formulations

The biofilm inhibition assay was performed using the formulation 1 (Rutin + Quercetin) and 2 (Rutin + Morin) with the concentration of the 200µg/ml. The results of the biofilm inhibition assay showed significant % inhibition with formulation 1 (Spinach 73%, Cabbage84% and Cucumber88%) and formulation 2 (Spinach 48%, Cabbage 59% and Cucumber 56%) respectively. Both formulations possess the biofilm inhibition activity however, the formulation 1 found to be more potent than that of formulation 2 as represented in Fig.1. The formulation 1 consists of Quercetin which is proven to be versatile flavonoid that helps in inhibiting the biofilm formation. [20] Quercetin alone has potency to inhibit the biofilm but its effect found to be enhanced in its synergy with flavonoid Rutin, where Rutin act as enhancer for their biofilm inhibition. [15]

The underlying mode of antibiofilm action against *Bacillus cereus* by formulation 1 may be correlated with the earlier research studies, [21] which confirmed that Quercetin act as quorum sensing quencher in biofilm associated infections. In-silico studies were evidenced that Quercetin bind more rigidly with the receptor protein than the signalling molecule which proves that quercetin may act as a potential competitive inhibitor of signalling molecules. In addition, flavonoids are phenolic compounds and phenolic compounds could inhibit specific biological mechanisms of biofilm formation such as adhesins synthesis and EPS production during biofilm development and maturation.[22] Therefore, the biofilm inhibitory effect of Rutin and Quercetin observed in the present study could be through similar mechanisms, avoiding any selective pressure over bacterial growth.

4. Conclusion

The fresh cut produce is any fresh cut fruit or vegetable or any combination thereof that has been physically altered (either cut, sliced, or diced etc.) but remains in the fresh state. Fresh cut produce has become increasingly popular. Rapid growth of this industry is witness by its ever increasing consumers who perceived the product as being attractive due to its uniform piece size, and convenient due to reduced preparation time. Fresh cut produce may represent a food safety concern because fresh-cut processing does not preserve the produce as it does not include any effective microbial elimination step. *Bacillus cerus* common pathogen found associated with fresh-cut products. This pathogen of particular concern for fresh cut produce safety as it may cause severe illness and is able to grow at refrigeration temperature and also able to form biofilm.

The results of the present study demonstrate the comparative biofilm formation ability of Bacillus *cereus* on various cut vegetables. Study also confirmed biofilm inhibitory potential of flavonoids in different formulations. The present study attempt to demonstrate the effective way to prevent the contamination of minimally processed fresh produce by food borne pathogens and thus helps in preventing the further associated illness and ensures the food safety.

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