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Functionalized polycaprolactam as an active food package for antibiofilm activity and extended shelf life

Veluchamy Prabhawathi, Thulasinathan Boobalan, Ponnurengam Malliappan Sivakumar, Mukesh Doble*

Department of Biotechnology, Bhupat & Jyoti Mehta School of BioSciences Building, IIT Madras, Chennai 600036, India

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ABSTRACT

Papain is covalently crosslinked on polycaprolactam and tested as a wrapper for packaging cottage cheese, against *E. coli* biofilm. The bacterial count on neat polycaprolactam (NP) was 50×10^6/ml on the 5th day which dramatically increased to 300×10^6 colony forming units (CFU)/ml by the end of 30th day. The corresponding CFU/ml on papain functionalized polycaprolactam (FP) was 10×10^2 on 5th day and 20×10^2 by the end of 30th day. Fourier transform infrared spectroscopic (FTIR) analysis of biofilm on NP showed the presence of polysaccharide, protein, lipid and metabolites which was three times reduced on FP. FT Raman spectroscopy showed the effect of papain on functional groups such as hydroxyl, amino, carbonyl, phosphoryl and aliphatic, leading to the inhibition of the biofilm. Motility, hydrophobicity and zeta potential of *E. coli* on NP and FP were 10.67 and 5.65 μm/s/V/cm; 88 and 20%; 8.93 ± 2.09 and 2.65 ± 0.52 mV respectively, thereby decreasing the biofilm forming ability of *E. coli*.

1. Introduction

E. coli is a Gram negative food pathogen causing life threatening infections in foetuses, newborns and immuno-compromised people. From 1987 to 2010, there were at least 133 outbreaks associated with infections from raw milk and its products which caused a large number of illness, hospitalization and deaths.[1] One of the outbreak victims, a toddler had been hospitalized with haemolytic uraemic syndrome (HUS), a serious condition that developed after an *E. coli* infection (see footnote 1). Food pathogen also leads to enormous wastage of packed foods due to contamination.

Small inoculums at the time of packaging due to its fast doubling time can develop into a significant burden of organisms by the time it reaches the consumer. As the number of days of storage increases, the contamination rate also increases. *E. coli* successfully contaminates packed foods because it persists on surfaces in the form of biofilm [1]. The process of biofilm formation occurs in five stages namely attachment of the bacteria, formation of exopolysaccharide matrix, biofilm maturation, its development into three dimensional structures and finally the dispersion of the cells to other place. It can tolerate stress, antibiotics and host immunological defences, which is the reason for its resilience in most medical and industrial settings. Biofilm formation on food and food contact surfaces also enhances the risk of transfer of virulence factor genes between the strains, thereby increasing the risk of spreading such bacteria to the consumer. During the last decade, the negative impact of *E. coli* biofilm in human health has stimulated research aimed at providing solutions for combating it [2]. Initial microbial attachment on a surface is the crucial step which should be addressed to prevent contamination and food spoilage.

Biofilm coated surfaces are particularly difficult to decontaminate since they are 500–5000 times more resistant to antimicrobials than the planktonic counterparts [3]. *E. coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract, where it thrives in an environment with structural characteristics of a multispecies biofilm [3]. With over 250 serotypes, it is a highly versatile bacterium ranging from harmless gut commensal to intra- or extra intestinal pathogens, including common colonizers of medical devices and the primary causes of recurrent urogenital infections [3].

Common antimicrobials used in food packages include silver nanoparticle, zeolite, triclosan, allylisothio cyanate and EDTA [4,5]. Chitinase, glucanase, lysozyme and glucose oxidase are few nonoxic antimicrobials available in the market [6]. Currently there are no commercial non-toxic, antimicrobial food package towards milk

* Corresponding author. Tel.: +91 044 22574107.
E-mail addresses: mukeshd@iitm.ac.in, mukesh.doble0@gmail.com (M. Doble).
URL: http://www.biotech.iitm.ac.in/doble_research (M. Doble).

[1] http://foodpoisoningbulletin.com/2013/where-did-mo-e-coli-and-hus-victims-get-the-raw-milk-products/ (19.04.13).

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pathogens. In this study we have used papain as an antibiofilm agent because it is non toxic, environmentally friendly and used in the production of milk product including cheese ripening [7]. Lysozyme, chitinase, B-glucanase, glucose oxidase, subtilisin, lactoperoxidase and oxidoreductase are few of the enzymes which are commonly used in the food industry [8].

Need for sustainable development has given rise to more emphasis on biodegradable polymer wrappings for food packages. So, in the present study, polycaprolactam, a biodegradable polymer is covalently linked with papain as a means of imparting antimicrobial property to the former. This work focuses on preventing the attachment of *E. coli* and its long term effect on the biofilm formation and proliferation. Motility plays an important role in the formation of biofilm and the role of papain on this phenomenon is also investigated.

2. Materials and methods

Papain, a protease from papaya, and polycaprolactam were purchased from Super Religare Laboratories and marine industrial polymers, Chennai, India respectively. All the chemicals and solvents used in the experiments were obtained from Sigma (St. Louis, MO), Super Religare Laboratories (SRL), and HiMedia (Mumbai, India). *E. coli* NCIM 293 was purchased from National Chemical Laboratory (NCL), Pune, India, and was stored in glycerol stock at 20 °C.

One gram of papain was dissolved in 25 mM of Tris–HCl (pH 8.0) to reach a final volume of 500 ml. It was then dialyzed against 5 mM of Tris–HCl buffer (pH 8.0), centrifuged at 10,000 × *g* for 30 min at 4 °C and then lyophilized. It was dissolved in 5 mM of Tris–HCl buffer such that 100 μL of the papain exhibited 100 international units (IU) of protease activity, which was used for all experiments. Polycaprolactam was dissolved in tetrahydrofuran and casted as thin film which was used for the current study.

2.1. Determination of papain activity

Protease activity was determined at 30 °C [9]. To 50 μl of papain solution diluted in 350 μl of buffer (50 mM phosphate buffer, pH 7.5, containing 38 mM EDTA and 34 mM cysteine), 400 μl of 1% (w/v) aqueous solution of casein was added and the reaction mixture was incubated at room temperature for 10 min. The reaction was stopped by adding 800 μl of 10% of trichloroacetic acid (TCA) solution. Then the mixture was incubated at 30 °C for 30 min, centrifuged at 12,000 × *g* for 10 min and the absorbance of the supernatant was measured at 280 nm (Perkin-Elmer, Lambda 35, Shelton, CT). One unit of protease activity is defined as the amount of papain that hydrolyzes casein to produce 1 μmol equivalent absorbance of tyrosine/min with tyrosine as standard.

2.2. Immobilization of papain onto preactivated polycaprolactam

Polycaprolactam (1 × 1 cm) was pre-treated for an hour with a solution containing anhydrous $CaCl_2$ 20% (w/v), 20% (v/v) $D.H_2O$ and 60% (v/v) methanol. Then it was removed, rinsed with distilled water and incubated for 30 min with 6 M of HCl solution. This partially hydrolyzed polycaprolactam was repeatedly washed with $D.H_2O$, dried and stored at 4 °C for later use. The hydrolyzed polycaprolactam was suspended in 0.025% of glutaraldehyde in 25 mM of Tris–HCl buffer (pH 8.0), under mild stirring for 12 h at 25 °C. The polycaprolactam was removed, washed with buffer solution and used for further experiments.

A modified methodology was adapted to immobilize papain on the preactivated polycaprolactam [10]. The latter was incubated with 0.025% of glutaraldehyde solution along with 1% of papain in 25 mM of Tris–HCl at a pH of 8 and 25 °C for 12 h under mild stirring. The polymer was then rinsed with 25 mM of Tris–HCl buffer at a pH 8 to remove the excess glutaraldehyde, then incubated for an additional period of 12 h at the same temperature to achieve an intense cross-linking and stored at 4 °C. Periodically, the polymer and supernatant were withdrawn, and the papain activity was determined.

2.3. Characterization of biofilm

E. coli was grown on NP (Neat Polycaprolactam) and functionalized polycaprolactam (FP) (of size 1 × 1 cm) in nutrient broth. The viable colonies in the biofilm were estimated according to a reported procedure [11]. Protein and the extracellular polymeric substances (EPS) in the biofilm formed on these surfaces were estimated as per Lowry's method [12], using crystalline bovine serum albumin as the reference standard and phenol sulphuric acid method using glucose as the standard respectively [11].

The FTIR spectrum of the ultra thin polymer substrate as well as the biofilm formed on the polymer surfaces were recorded in the frequency range of 500–4000 cm^{-1} using a Perkin-Elmer PE 1600 FTIR spectrometer. This was repeated on three different samples to verify the repeatability of the experiments. Infrared spectra of polymer surfaces are usually collected in transmission mode, that is, the IR beam is passed through the sample and the transmitted IR intensity is measured. However, for the studies of biofilm on polymer surface, attenuated total reflectance (ATR) mode is used mainly because of the surface sensitivity of this technique. The contact angle was measured using the sessile drop technique with a Goniometer (Kruss, Germany) [13]. FT-RAMAN spectrometer is used to characterize the biofilm. Spectral range is 3000–0 cm^{-1}, resolution is 2 cm^{-1} and laser source is Nd: YAG 1064 nm. Model number is BRUKER RFS 27: Stand alone mode. No baseline correction or normalization of the spectra was performed for better comparison of the data.

1×10^7 number of microbes along with 1 × 1 cm of NP or FP was cultured in a 100 ml flask for 24 h. The film was removed, sonicated in 1 ml of fresh nutrient broth and its optical density (OD) value was adjusted to 0.1 at 600 nm (Perkin Elmer, Lambda 35, Shelton, USA). Motility of the microbe attached on these two surfaces was measured using a Microtrac Inc. Nanotrac particle analyzer (Model: Zetatrac; serial number MW12031907-U2839Z, USA). Simultaneously, motility assay was also performed in nutrient agar medium [14]. Here, *E. coli* was grown on NP and FP (of size 1 × 1 cm) surfaces in nutrient broth, until the growth reached mid-log-phase. 10 μl of this culture was inoculated in the centre of a petri plate containing 0.3% (w/v) of nutrient agar. The diameters of the swimming zones were measured after incubation for 15 h at 37 °C.

The biofilm on the surface was fixed with glutaraldehyde (in 0.1% of phosphate buffer at a pH 7.0) for an hour, washed twice with 25 mM of phosphate buffer and once with distilled water, dried overnight in a dessicator, coated with gold and viewed under a Scanning Electron Microscope (Jeol JSM 5600 LSV model).

The live and dead cells present in the biofilm after 24 h of incubation were observed using a mixture of two nucleic acid staining dyes namely, SYTO9 and propidium iodide (PI) (Baclight®, Invitrogen, USA) [11]. The former stains all cells green whereas the latter dye enters only the dead cells (i.e. membrane damaged cells) and fluoresces red. The biofilm was grown on NP and FP, and washed with distilled water. 20 μl of the dye mixture was placed on them and incubated in the dark for 10 min. Excess dye was washed and the surfaces were viewed under a fluorescence microscope (Leica DM5000, Germany) with a blue filter at an excitation of 475 nm.