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Immobilization of Subtilisin on Polycaprolactam for Antimicrobial Food Packaging Applications

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ABSTRACT: Subtilisin was immobilized on polycaprolactam and used for food packaging applications to reduce the transference of microorganisms from the packaging material to the packaged food material. The optimized conditions for subtilisin immobilization was as follows: pH, 8; temperature, 4 °C; glutaraldehyde, 0.5%; incubation time, 25 h; and subtilisin concentration, 600 μL. The formation of −CH═N− at 1576 cm^{-1} in the Fourier transform infrared (FTIR) spectrum confirmed the immobilization. Subtilisin-immobilized polycaprolactam (SIP) exhibited the highest residual activity of 106.67 ± 4.41% and 104.67 ± 0.88% at 40 °C and pH 8 and retained residual activity of 94% at the end of 56 days when compared to 21.33 ± 4.10% in the case of free subtilisin. SIP significantly ($p < 0.05$) lowered the colony forming units (CFU), dry weight, and protein and carbohydrate contents in bacterial and fungal biofilm. Practical application of the SIP on ham steaks at 4 and 20 °C showed a 2−3 times reduction of *Staphylococcus aureus* as well as *Escherichia coli* cells in the range of $p < 0.05$.

KEYWORDS: Polycaprolactam, adhesion, subtilisin, immobilization

INTRODUCTION

There is an increased demand on easily prepared, minimally processed fresh produce without any foodborne microbial contamination.[1] This has intensified the research on antimicrobial packaging technologies.[2]

Microbial contamination of food occurs primarily at the surface, because of post-processing handling, especially during food packing. The microbes that are adhered to these packages become transferred to the packed food. At first, these microbes are bound to the substratum using flagella.[3] After a while, they secrete sticky extracellular polymeric substances (EPS), forming a biofilm matrix embedded by cells.[4] EPS are predominantly composed of polysaccharides, proteins,[5,6] nucleic acids, uronic acids, and humic substances.[7] Polysaccharides are partly responsible for bacterial adhesion and the formation of the biofilm on the surface.[8] EPS serve some of the important functions, including forming and maintaining the microcolony,[9] enabling the bacteria to capture nutrients,[10] facilitating cell−cell communication,[11] and functioning as a stabilizer of the biofilm structure and as a barrier against hostile environments.[12,13]

Therefore, any substance that disturbs the integrity of EPS will be ideal for preventing biofilm. Effects of coating various surfaces, including cloth and polymer, with antibacterial agents that disturb the biofilm and slime have been reported.[14,15] In the food industry, antibacterial sprays or dips have been reported. However, direct surface application of antibacterial substances onto foods have limited benefits because the active substances are neutralized on contact or diffuse rapidly from the surface into the food mass.[16] Antimicrobial food packaging materials have to extend the lag phase and reduce the growth rate of microorganisms, which will extend the shelf life and maintain product quality and safety.[17] This solution appears to lead to the lowest risk to the consumer.[18] Currently, there is a strong interest in the use of renewable and nontoxic supports for immobilization to make the process more ecofriendly.[19]

Protease exhibits both antibacterial and antifungal properties. Protease from *Bacillus proteolyticus* CFR3001 exhibits antibacterial activity against *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, and *Yersinia enterocolytica* by lysing the cell walls.[20] Protease produced by *Pseudomonas aeruginosa* M-1001 exhibited antifungal activity against pathogenic fungi, *Fusarium solani*.[21]

The protease subtilisin is widely used in commercial products, for example, in laundry and dishwashing detergents, skin-care ointments, contact-lens cleansers, cosmetics, food processing, and pharmaceuticals.[22] In the food industry, they are used for various applications, including meat tenderization, cheese ripening as a digestive and an animal feed supplement, flavor development, and milk coagulation.[23] These applications are because of its peptidase and esterase activities. It also exhibits antimicrobial, antifungal, and antibiofilm characteristics.

Polycaprolactam belongs to a class of polymers called polyamine, which contains amine and carbonyl groups and comes in the trade name, Nylon 6. It is better suited than polyethylene and polypropylene when immobilized with protease.[24] In this study, subtilisin has been immobilized on polycaprolactam and used for antimicrobial food packaging applications. The main drawback of the enzyme in such applications is the loss of its activity and stability during prolonged use. Therefore, an attempt has been made to improve its long-term stability and then test its suitability as an ideal active food wrapper.

MATERIALS AND METHODS

Materials. Subtilisin, a protease from *Bacillus subtilis* (EC 3.4.21.62), was purchased from Sigma (St. Louis, MO). Polycaprolactam was

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purchased from marine industrial polymers, Chennai, India. All of the chemicals and solvents used in the experiments were obtained from Sigma (St. Louis, MO), Super Religare Laboratories (SRL), and HiMedia (Mumbai, India). The strains used in this study, namely, *Staphylococcus aureus* National Collection of Industrial Microorganisms (NCIM) 5021, *E. coli* NCIM 293, *Salmonella typhimurium* NCIM 2501, *B. subtilis* NCIM 2718, *Aspergillus niger* NCIM 596, *Candida albicans* NCIM 3471, and *Fusarium proliferatum* NCIM 1105 were purchased from the National Chemical Laboratory (NCL), Pune, India. They were stored in glycerol stock at −20 °C and used when required.

Preparation of Subtilisin. A total of 1 mL of the subtilisin was diluted with 4 mL of 25 mM phosphate buffer (pH 7) and dialyzed against 5 mM of the same buffer (pH 7). It was then centrifuged at 15000*g* for 30 min at 4 °C, lyophilized, and dissolved in 5 mM phosphate buffer, such that 50 μL of the subtilisin exhibited 50 international units (IU) of protease activity. This sample is used for all of the future experiments.

Determination of Subtilisin Activity. The subtilisin assay was performed according to the work by Kunitz,[25] with slight modifications. A total of 1.9 mL of 1% (w/v) Hammersten casein prepared in 25 mM phosphate buffer (pH 7) was taken in test tubes and preincubated at 40 °C for 10 min in a water bath. Then, 0.1 mL of subtilisin was added to the substrate solution, and the tubes were incubated in a hot water bath (Human Engineering Co., Korea) for 10 min at 40 °C. The reaction was stopped by the addition of 3 mL of 5% (w/v) trichloroacetic acid, and the test tubes were kept at ambient temperature (30 ± 2 °C) for 15 min. The precipitate was then removed using Whatman no. 1 filter paper, and the absorbance of the filtrate at 280 nm was determined in an ultraviolet (UV) spectrophotometer (Perkin-Elmer, Lambda 35, Shelton, CT). A total of 1 IU of protease activity is the amount of subtilisin that liberates 1 μM tyrosine per minute.

Zone of Inhibition. Subtilisin was tested for antimicrobial activity by the agar-well diffusion assay.[26] A clear zone of inhibition around the well was taken as an indication of possible antimicrobial activity. The inhibition zone (in millimeters) was measured from the ridge of the well to the end of the halo around the well, using an antibiotic zone scale (Himedia, Mumbai, India).

Preparation of Preactivated Polycaprolactam. Glutaraldehyde-preactivated polycaprolactam was prepared by suspending polycaprolactam pieces (1 × 1 cm) in 0.5% glutaraldehyde in 25 mM phosphate buffer (pH 7.0). The polycaprolactam was kept under mild stirring for 15 h at 25 °C.[27] Then, the polymer pieces were taken out and washed thoroughly with 25 mM phosphate buffer and then with Milli-Q water. This support was used immediately because of the low stability of the aldehyde groups.

Immobilization of Subtilisin onto Preactivated Polycaprolactam. The methodology followed by Lopez-Gallego et al.[28] was adapted with slight modifications. The polycaprolactam prepared as previously described was incubated with 0.5% glutaraldehyde solution (unless otherwise specified) along with 1% subtilisin in 25 mM phosphate buffer at a pH of 7 and 25 °C for 1 h under mild stirring. This treatment permitted the full modification of the primary amino groups in the subtilisin and the support with just one glutaraldehyde molecule.[29] The polymer was then removed and washed with 25 mM phosphate buffer at a pH of 7 to remove the excess glutaraldehyde. It was incubated for an additional period of 20 h at 25 °C for achieving a more intense cross-linking between the subtilisin and the polycaprolactam. Periodically, the polymer and supernatant were withdrawn, and the subtilisin activity recovery was determined, as described below. Subtilisin-immobilized polycaprolactam (SIP) was stored at 4 °C for further experiments.

Optimization of Immobilization Parameters. The different parameters studied and their corresponding values are given in Table 1. They were chosen on the basis of a literature reference.[29]

Table 1. Immobilization Parameters and Their Corresponding Values

parameters studied	values chosen
glutaraldehyde (%)	0.2−1.0
pH	5−10
temperature (°C)	4−50
time of incubation (h)	5−30
subtilisin (μL)	200−1200

The activity recovery was calculated as follows:

$$\text{activity recovery (\%)} = \text{total activity of SIP/total activity of subtilisin} \times 100$$

where total activity of SIP = total activity of soluble subtilisin − total activity of unbound subtilisin.

Residual activity is the subtilisin activity that retains in the SIP with respect to the control under standard assay conditions. SIP was also tested for its stability and activity during prolonged use.

Fourier Transform Infrared (FTIR) Spectroscopic Analysis. The FTIR spectrum of the samples were recorded in the frequency range of 500−4000 cm^{-1} using a Perkin-Elmer PE 1600 FTIR spectrometer. The analysis was performed 3 times with three different samples to verify the repeatability of the experiment.

Contact Angle Analysis. The contact angle was measured on the basis of the sessile drop technique[30] using a contact angle measuring apparatus (Kruss, Germany). The polymer film was centered on a glass slide, and a drop of distilled water (Millipore grade) was placed on it using a syringe. The image of the drop was processed by Digital Scrapbook Artist 2 Software (DSA2) software [determination of static and dynamic contact angle (SW4001)], which calculated both the left and right angles that it made with the polymer surface to an accuracy of ±0.1°. It was measured on five different locations on the polymer, and the average values were reported here.

Biological Evaluation of SIP and Unmodified Polycaprolactam (UP). The microbial growth on SIP and UP were ascertained by measuring the live bacterial cell, fungal biomass, protein, and carbohydrate contents. Microbial cell count on the polymer surfaces was measured on the basis of an earlier reported methodology.[14] Microorganisms from the stock culture were grown on a nutrient agar plate at 37 °C, and a single colony from that was inoculated into 20 mL of nutrient broth and incubated at 180 revolutions per minute (rpm) and 37 °C for 10 h. A total of 500 μL of the above culture was inoculated into 50 mL of nutrient broth and cultured under the above conditions. After 10 h, the cultural broth was centrifuged at 10000*g* and 4 °C for 10 min. The supernatant was discarded, and the bacterial pellet was resuspended in 0.9% saline and adjusted to an optical density of 0.1 at 660 nm (approximately 1 × 10^9 cells/mL) using an UV spectrophotometer (Perkin-Elmer, Lambda 35, Shelton, CT). The bacterial suspension was later used for adhesion experiments.

A. niger and *F. proliferatum* were maintained in Czapek Dox (CD) agar medium, and *C. albicans* was maintained in yeast peptone dextrose (YPD) agar medium. Fungal strains were subcultured in CD broth, and *C. albicans* was subcultured in YPD broth at 35 °C and a pH of 7 for at least 4 days before use. The culture was then taken in the test tube and diluted such that the final concentration was 1.0 × 10^6 cells, which was counted using a hemocytometer (Neubauer Chamber, Merck, S.A.).

The SIP and UP of size 1 × 1 cm were immersed into separate conical flasks containing 25 mL of nutrient broth. A total of 1 mL of bacterial suspension was inoculated into these flasks and incubated under static conditions at 37 °C for 24 h. Samples were removed after 24 h using sterile forceps and washed twice with sterile water to remove unbound cells. The strongly bound bacteria were then removed from the surfaces

by water-bath ultrasonication (Thosan Pvt., Ltd., Ajmer, India) (total of 10 min, with 1 min intervals), and the viable colonies were counted visually in nutrient agar plates. For fungi and yeast, the above experiment was performed in CD and YPD broths, respectively, for 3 days, after which the dry weight was measured using a four-digit balance (Sartorious CP64, Germany).

Protein and carbohydrate in the biofilm formed on the polymer surface were estimated as per Lowry's method using crystalline bovine serum albumin as the reference standard[31] and the phenol sulfuric acid method,[32] respectively.

Scanning Electron Microscopic (SEM) Analysis of the Biofilm. The polymer surface, after exposure to the bacteria and fungi, was washed with distilled water and then fixed using 3% glutaraldehyde (in 0.1% phosphate buffer at a pH of 7.2) for 1 h.[14] Later, it was washed twice with phosphate buffer and once with distilled water. Then, it was dried overnight in a desiccator, coated with gold at 30 mA for 1 min, and viewed under a SEM (JEOL JSM 5600 LSV model, supplied by JEOL, Tokyo, Japan).

Confocal Laser Scanning Microscopic Analysis of the Biofilm. The live and dead *S. aureus* cells present on the polymer surface were determined using the Live/Dead Baclight Bacterial Viability Kit (Invitrogen, Germany). This kit consists of SYTO9 and propidium iodide dyes.[33] These two dyes differ in their ability to stain the bacterial cells. SYTO9 (green color) stains the live as well as dead cells, and propidium iodide (red color) stains only the dead cells. When the later is added, it reduces the fluorescence of the former by penetrating into the dead cells. Hence, live cells fluoresce green, and dead cells fluoresce red.[34] Polymer film after the bacterial adhesion experiments was stained with Baclight dye and incubated for 10−15 min in the dark. Then, it was covered with a coverslip, and the images were captured using a confocal laser scanning microscope (CLSM) (LSM 710 Carl Zeiss, MicroImaging GmbH, Germany). The thickness of the biofilm formed on the surface was also measured using the same instrument. Zen 2009 software (Carl Zeiss MicroImaging GmbH, Germany) was used for data analysis. All images were obtained through a plan-ApoChromate 40×/0.0 Korrm75 objective using 594 (HeNe 594 nm) and 488 (argon 488) lasers, ch1-493-551 and ch2-598-712 filters, and MBS-MBS 488/594 beamsplitter.

Food Packaging Applications. A slight modification to the methodology reported by Besse et al.[35] was followed here. Freshly

Table 2. Zone of Inhibition (mm), Exhibited by 50 μL of Subtilisin Possessing 50 IU of Protease Activity against Various Microorganisms

number	microorganism	zone of inhibition (mm)
1	*B. subtilis*	10.80 ± 0.45
2	*S. aureus*	10.94 ± 0.24
3	*S. typhimurium*	9.51 ± 0.23
4	*E. coli*	8.37 ± 0.12
5	*A. niger*	11.3 ± 0.84
6	*F. proliferatum*	9.84 ± 0.85
7	*C. albicans*	12.45 ± 0.54

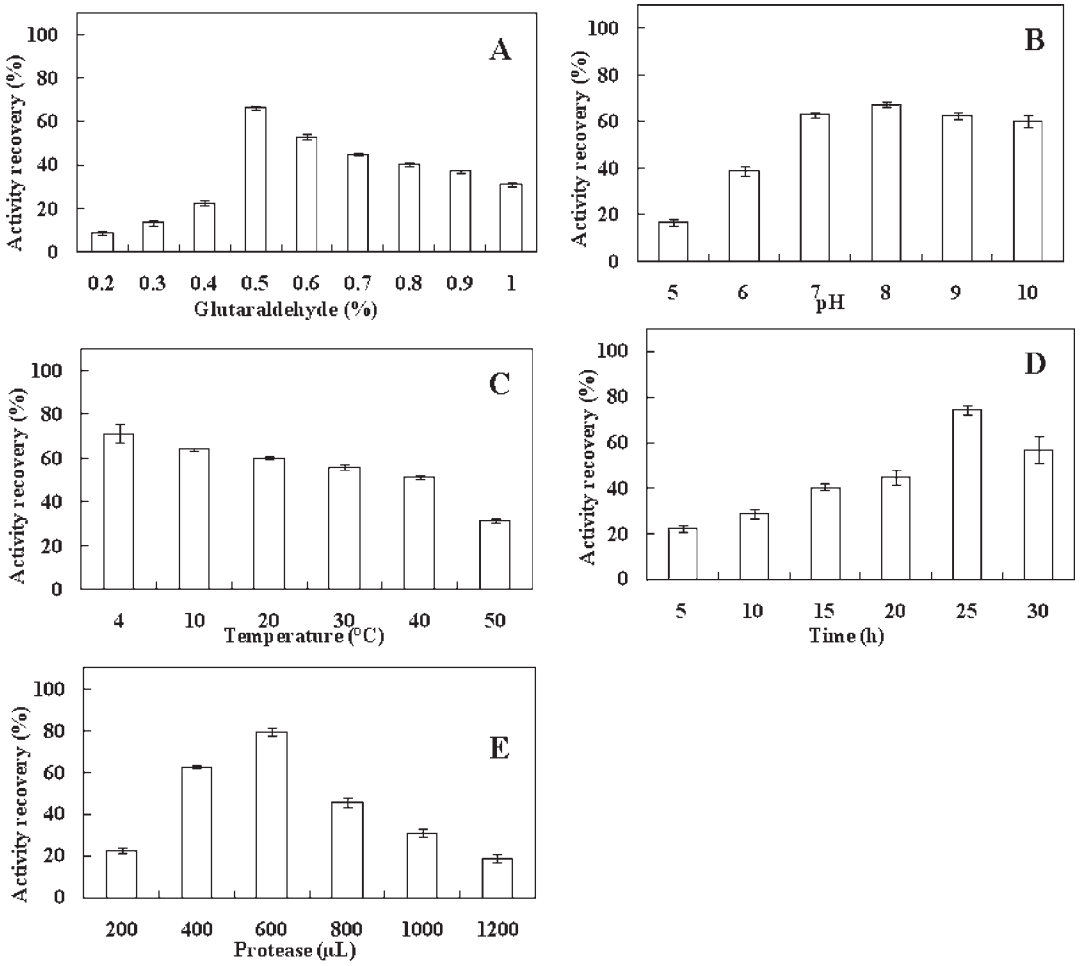

Figure 1. Effect of different parameters on the percentage activity recovery of immobilized subtilisin: (A) glutaraldehyde, (B) pH, (C) temperature, (D) incubation time, and (E) subtilisin concentration.

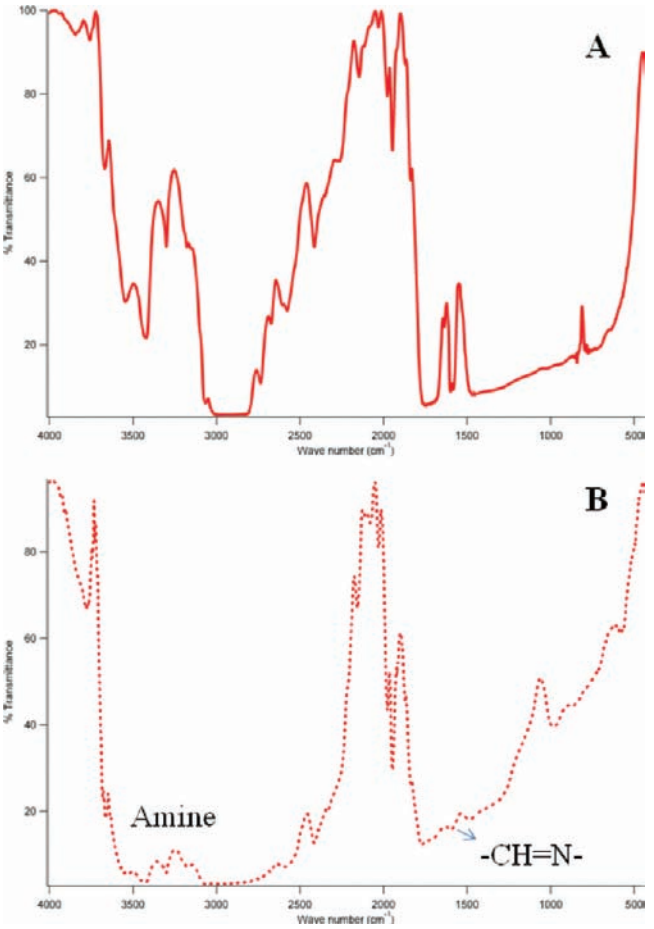

Figure 2. FTIR spectrum of polycaprolactam (A) before (red —) and (B) after (red · · ·) the immobilization of subtilisin. The peak at 3300 cm^{-1} is attributed to secondary amide. The appearance of the amine link (−CH═N−) is confirmend by the presence of peaks at 1298 and 1576 cm^{-1}.

processed ham steak samples were purchased and kept frozen at −20 °C and thawed at 2 °C for 1 day immediately before use. Ham steak was cut into small pieces, each weighing 1 g, and was inoculated with 10^5 cells of *S. aureus* or *E. coli*. Samples were left undisturbed for 5 min to allow for the inoculum to soak in and the cells to attach. The inoculated samples were wrapped in UP and SIP, and then they were placed in a Petri plate and incubated at 4 or 20 °C. Every day, a sample was opened aseptically and homogenized and the number of colonies were counted. This study was performed for 6 days.

Statistics. Data reported here were expressed as the mean ± standard error (SE) of three samples in each experiment. One-way analysis of variation (ANOVA) and two-sample *t* test were performed using MiniTab, version 14.0 (MiniTab, Inc., State College, PA). A p value < 0.05 is considered statistically significant.

RESULTS AND DISCUSSION

The susceptibility of the microbes to 50 μL of subtilisin was determined by the zone of inhibition method (Table 2). There is a statistically significant difference (p < 0.01). After the antimicrobial activity was confirmed, subtilisin was immobilized on polycaprolactam to study its action on bacteria and fungi.

Standardization of Immobilization Conditions. The optimum pH condition, temperature, time, subtilisin concentration,

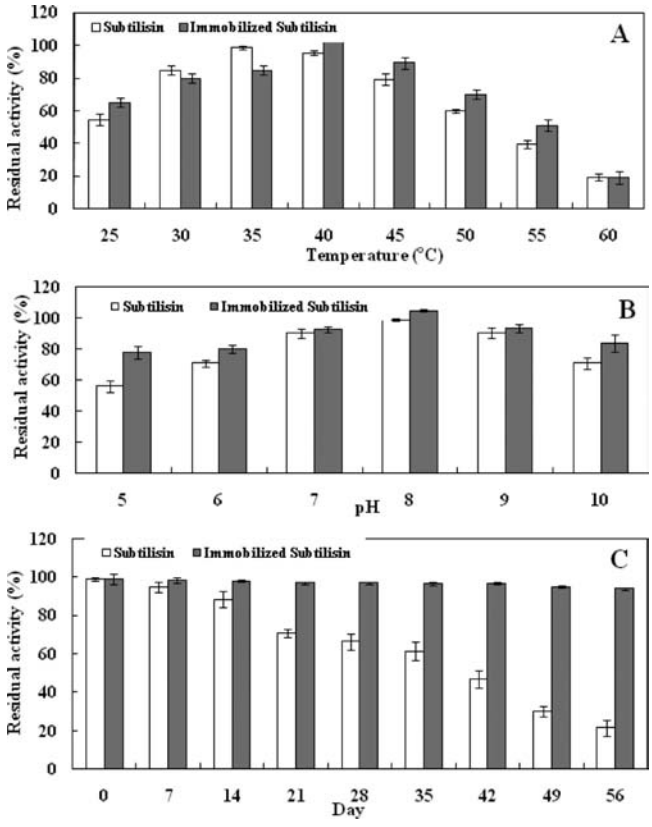

Figure 3. Activity of subtilisin before (□) and after (■) immobilization at different (A) pH conditions, (B) temperature conditions, and (C) time.

and glutaraldehyde concentration needed for maximum activity recovery of immobilized subtilisin are shown in Figure 1. The glutaraldehyde concentration of 0.5% resulted in maximum activity recovery (p < 0.05), namely, 66.67 ± 0.88%. The glutaraldehyde concentration beyond 0.6% decreased the activity recovery (Figure 1A). Above this concentration, the reaction could not be controlled, leading to polymerization of glutaraldehyde in the solution. These results are in agreement with earlier reports.[29] When an ideal concentration of glutaraldehyde is used, one molecule of glutaraldehyde becomes introduced into each of the primary amino groups present in the polymer. Apparently, it is relatively easy to form dimers of glutaraldehyde on the primary amino groups, but it is difficult to have higher degrees of polymerization in a controlled way, because of the fact that the amino of the support linked with the glutaraldehyde molecule (after the first glutaraldehyde amino reaction) may promote an increase in the reactivity of this glutaraldehyde molecule with other glutaraldehyde molecules present in the solution.[36]

The optimum pH for immobilization of subtilisin was nearly 8. At this pH, the activity recovered was 67.33 ± 1.20% (Figure 1B). At pH of 6 and 5, the percentage of activity recovered decreased to 39 ± 2.98 and 17 ± 1.52%, respectively. (Figure1B). Neutral pH helps in preventing the inactivation of the support.[36] The highest residual activity of 71 ± 4.05 and 64 ± 0.58% was observed when the subtilisin was immobilized at 4 and 10 °C (Figure 1C), respectively. An incubation time of 25 h (Figure 1D) and a subtilisin concentration of 600 μL (Figure 1E) were found to yield the highest activity recovery, namely, 74.33 ± 2.33 and 79.33 ± 1.86%, respectively. The optimized set of parameters was a glutaraldehyde concentration of 0.5%, pH of 8, temperature of 4 °C, 25 h of

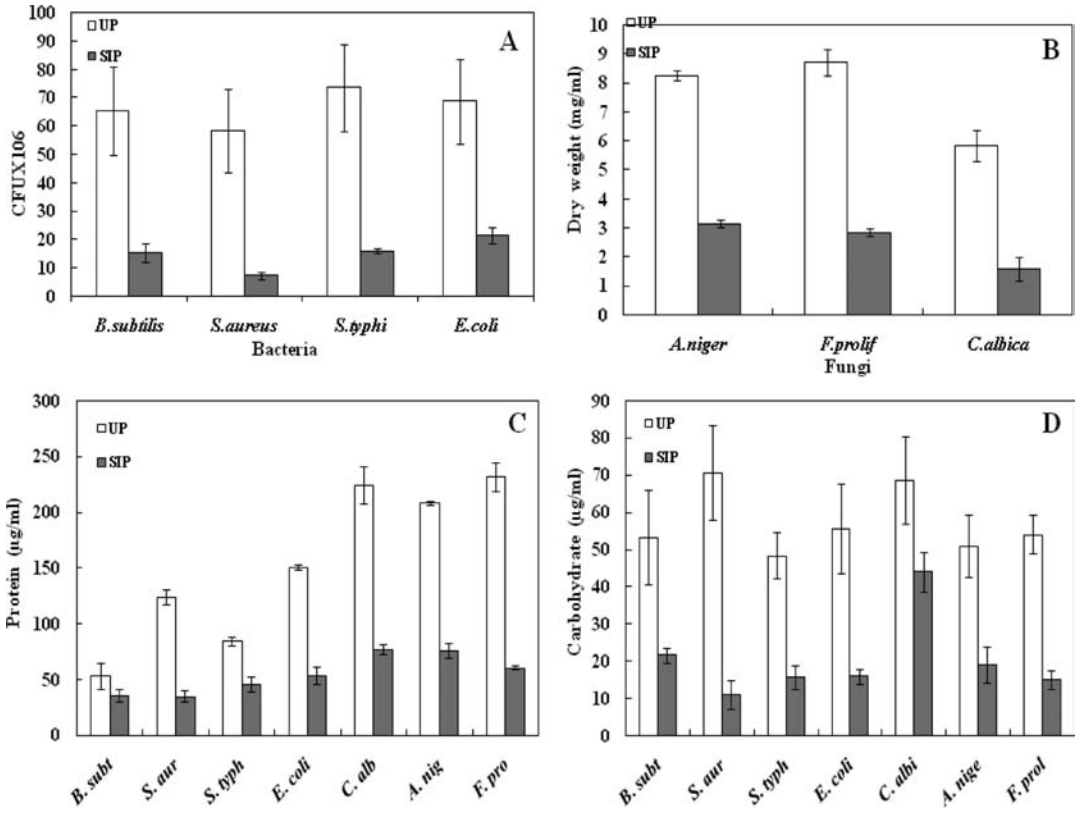

Figure 4. Antimicrobial activity and biofilm-prevention ability of UP and SIP: (A) antibacterial activity measured as colony forming units, (B) antifungal activity measured as dry weight, (C) protein concentration in biofilm, and (D) carbohydrate concentration in biofilm.

incubation, and a subtilisin concentration of 600 μL. These values were used in all future experiments.

FTIR Spectroscopic Analysis. The FTIR spectrum of UP and SIP are shown in Figure 2. The characteristic absorption because of the secondary amide is observed at a wavelength of 3300 cm^{-1}, which is similar to the observation of others.[37] The appearance of the amine link (−CH═N−) is confirmed by the presence of peaks at 1298 and 1576 cm^{-1}.[38] These results confirm the immobilization of subtilisin on polycaprolactam.

Characterization of SIP. The effect of pH and temperature on the activity of immobilized and free subtilisin is presented in panels A and B of Figure 3, respectively. SIP exhibited a maximum residual activity of 106.67 ± 4.41% at 40 °C (Figure 3A). The optimum temperature for maximum activity of free and immobilized subtilisin was 35 and 45 °C, respectively (Figure 3B). After immobilization, there was a change in the residual activity exhibited by subtilisin at different pH conditions (Figure 3B). SIP remained active and stable at higher pH values when compared to the free subtilisin ($p < 0.05$). SIP showed 104.67 ± 0.88, 93.33 ± 2.4, and 83.67 ± 5.78% residual activities at pH values of 8, 9, and 10 when compared to 98.67 ± 0.88, 90.33 ± 3.18, and 71.00 ± 3.79% residual activities exhibited by free subtilisin at the same pH value. This shift happened because the negatively charged support increased the concentration of proteins in the vicinity of the active site of the immobilized subtilisin, so that the pH within the immobilized subtilisin was lower than in the bulk solution. To counteract this effect, the optimal pH had shifted to a more alkaline region.[39] A study on the stability of SIP is essential to test its durability as a food packaging material. The immobilization of the subtilisin on glutaraldehyde-activated polycaprolactam resulted in a significant stabilization of the subtilisin when compared to the free subtilisin (Figure 3C). SIP retained a residual activity from 99 ± 2.65 to 94 ± 0.58% from day 1 to 56, whereas the activity of free subtilisin reduced from 99 ± 1.00 to 21.33 ± 4.10% at the end of 56 days (Figure 3C). The drop in the activity of the free subtilisin when compared to SIP at the end of the study period is large ($p < 0.001$). Glutaraldehyde means of immobilization are quite simple and efficient, and glutaraldehyde improves enzyme stability by multi-point or multi-subunit immobilization.[40] Multi-point covalent immobilization improves the enzyme stability by preventing its aggregation, proteolysis, and interaction with hydrophobic surfaces.[41] Thereby, the drawbacks of free enzymes that include instability, solubility, and susceptibility to inhibition could be overcome by this method.

Bacterial Adhesion Studies on SIP and UP. Experiments were carried out to determine the biofilm-prevention ability of subtilisin. The antibacterial activity of SIP was studied against Gram-positive bacteria, *B. subtilis* and *S. aureus*, as well as against Gram-negative bacteria, *E. coli* and *S. typhimurium*. These strains are common contaminants in meat products. The colony count of *S. aureus* on UP and SIP was 58 × 10^6 ± 14.88 and 7 × 10^6 ± 1.33, respectively, indicating 8 times reduction in the later ($p < 0.01$) (Figure 4A). The reduction in colonies of *B. subtilis*, *S. typhimurium*, and *E. coli* on SIP were 4, 4.56, and 3.24 times less than on UP, respectively (Figure 4A). The bactericidal activity of the subtilisin is the reason for this observed behavior. Also, SIP exhibited antifungal activity against *A. niger*, *F. proliferatum* and *C. albicans* (Figure 4B). There was a 3−4 times reduction in the dry weight of the biomass on SIP when compared to UP ($p < 0.01$).

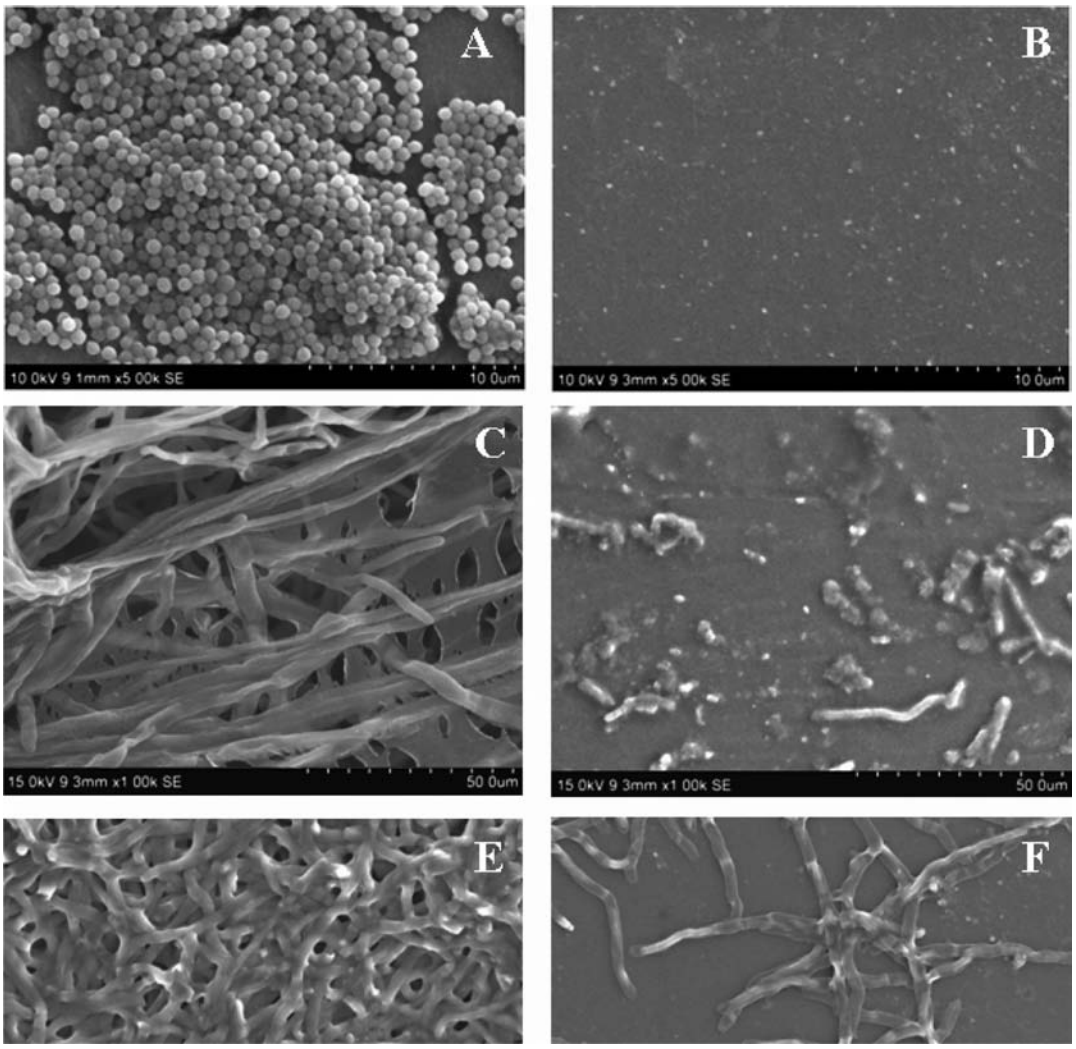

Figure 5. Scanning electron microscopic images: *S. aureus* adhered on (A) UP and (B) SIP, *A. niger* adhered on (C) UP and (D) SIP, and *C. albicans* adhered on (E) UP and (F) SIP.

The composition of bacterial EPS comprises polysaccharides, proteins, nucleic acids, lipids, and phospholipids.[42] Proteins and polysaccharides account for 75−89% of the EPS.[43] EPS imparts protection to microorganisms against adverse conditions, including a high concentration of biocides.[44] Moreover, within the EPS matrix, the molecules required for cell−cell communication and community behavior may accumulate at concentrations high enough to be effective.[45] Because the major component of the biofilm is protein and carbohydrate, experiments were performed to test the influence of subtilisin on the amount of these components.

Analysis of Biofilm Protein on SIP and UP. Biofilm protein formed by *S. typhimurium* and *E. coli* on UP were 84.33 ± 4.1 and 150.67 ± 2.4 μg/mL, respectively (Figure 4C). Whereas SIP showed a 1.8 and 2.8 times reduction in the biofilm protein produced by the same strains ($p < 0.05$). The protein content in the bacterial biofilm was lesser (50−150 μg/mL) than that observed in the fungal biofilm (200−240 μg/mL) (Figure 4C). The protein content was more (maximum of 232 μg/mL) when compared to the carbohydrate content (maximum of 70 μg/mL) in the biofilm. The proteinaceous nature of the adhesives suggests that subtilisin might control the biofilm. There are reports on the anti-adhesive effect of a low concentration of protease.[46] It may be hydrolyzing the protein present on the surface of the biomaterial as soon as the latter attaches, thereby preventing the formation of a "conditioning layer". This layer is the basis for microbial adherence, biofilm formation, and microbial proliferation. Also, antibacterial property of the subtilisin has resulted in killing of the bacterial cells, thereby reducing the number of bacterial cells involved in the proliferation, protein secretion, and biofilm formation.

Analysis of Biofilm Carbohydrate on SIP and UP. In addition to antibacterial activity, SIP exhibited slimicidal activity, which is shown by the reduced carbohydrate content (Figure 4D). Carbohydrate contents on UP and SIP were 70.67 ± 12.58 and 11 ± 3.79 μg/mL, respectively, indicating a 6.42 times reduction in its amount on the latter surface ($p < 0.01$). In addition, there was a 2.46−3.49 times reduction in the carbohydrate content in the bacterial biofilm and a 1.56−3.6 times reduction in the carbohydrate content in the fungal biofilm. There is evidence to prove that subtilisin acts on exopolysaccharides.[47] This is because of the esterase activity of the subtilisin and its ability to reduce the adhesion strength of two algal species.[48] The main drawback of subtilisin reported in the literature is the quick loss of its activity and stability. Therefore, immobilization of subtilisin as reported here is an alternative means for achieving good results.

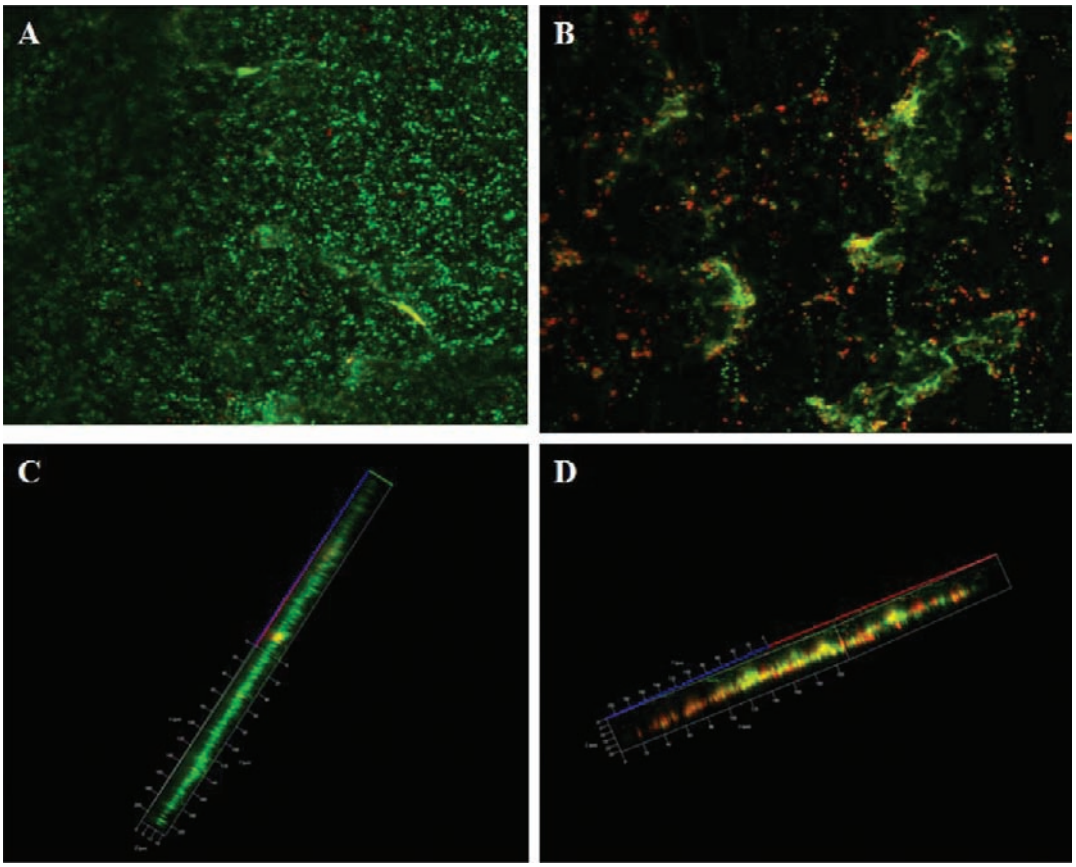

Figure 6. Confocal laser scanning microscopic images showing live (green) and dead (red) cells on (A) UP and (B) SIP after adhesion with *S. aureus* and the biofilm thickness on (C) UP and (D) SIP.

Contact Angle Analysis and Scanning Electron Microscopic Analysis. The static water contact angles of UP and SIP were 79.3 ± 1.9° and 72.0 ± 1.33°, respectively. Immobilization of subtilisin has made the hydrophobic polymer surface hydrophilic ($p < 0.05$). It has been generally observed that the hydrophobic polymer surface facilitates attachment of hydrophobic bacterial cells.

In the present study, UP (hydrophobic) showed a colony count of 58−73 × 10^6 colony forming units (CFU)/mL, whereas SIP (hydrophilic) showed a reduced colony count of 7−21 × 10^6 CFU/mL ($p < 0.05$). Reports state that most microbial pathogens are dependent upon hydrophobic interactions for successful colonization of host substrates.[49] An increase in the hydrophilicity of the membrane significantly reduces the fouling as a result of the reduced hydrophobic interaction between the protein and the membrane surface.[14,50]

Panels A and B of Figure 5 show SEM images of the surface of UP and SIP after the adhesion of *S. aureus*, respectively. A cluster of bacterial cells could be observed in the former (Figure 5A), whereas the latter is completely devoid of any attached bacterial cells (Figure 5B). Because SEM focuses on a very small area, the region that is focused did not contain any cells, whereas the colony count revealed the presence of live bacterial cells in the modified surface as well, although the count was much less. Immobilized subtilisin also reduced the mycelium and biofilm of *A. niger* (panels C and D of Figure 5) and *C. albicans* (panels E and F of Figure 5).

Confocal Laser Scanning Microscopic Analysis. Panels A and B of Figure 6 show the confocal images of UP and SIP after 24 h of adhesion of *S. aureus*, respectively. The UP was covered with live cells, as evidenced by the green color (Figure 6A), whereas predominantly dead cells (red color) and few live cells (green color) are observed on SIP (Figure 6B). The thicknesses of the biofilm in the case of UP and SIP were 14 and 7 μM, respectively (panels C and D of Figure 6, respectively). This indicates that SIP not only decreases the thickness of the biofilm formed on the polymer surface but also decreases the number of viable cells by damaging their cell membrane, as reported by others.[14,51] Sivakumar et al.[14] have reported that the attachment of *Vibrio natriegens* and the thickness of the biofilm could be considerably reduced on a dichlorochalcone-mixed paint-coated surface when compared to an ordinary paint-coated surface, because of the antibacterial property as well as the slimicidal activity of the compound. A CLSM has been previously used by investigators to detect biofilm formation[52] and also used successfully in the present study to detect bacterial adherence and cell wall disruption, as well as to measure the thickness of the biofilm. Therefore, one of the possible mechanisms of action of the immobilized subtilisin on bacteria is its cell-damaging activity.

Food Packaging Application. Antimicrobial action of SIP was tested on ham steaks. *S. aureus* and *E. coli* cell counts on ham steak samples wrapped with UP and SIP at 4 and 20 °C are shown in Figures 7 and 8. SIP acted better on *S. aureus* when compared to *E. coli* at 20 °C ($p < 0.05$), whereas SIP performed better on *E. coli* at 4 °C ($p < 0.05$). For the first 3 days, inhibition of the colony count was slow in the presence of SIP. However, large differences in the performance of UP and SIP were observed on

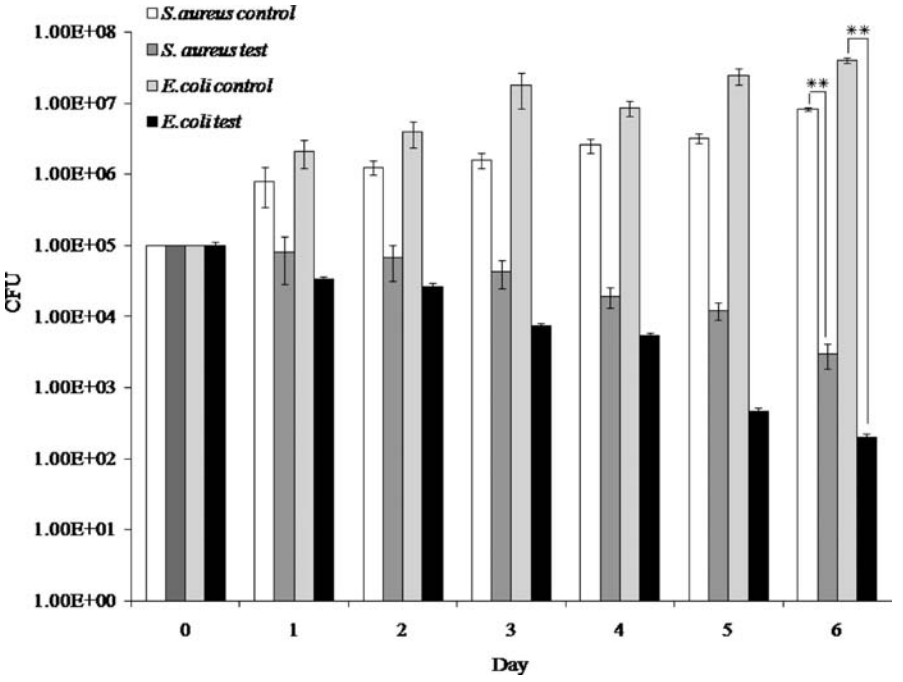

Figure 7. Growth of microorganisms on a food pack as a function of time at 4 °C (**, $p < 0.01$).

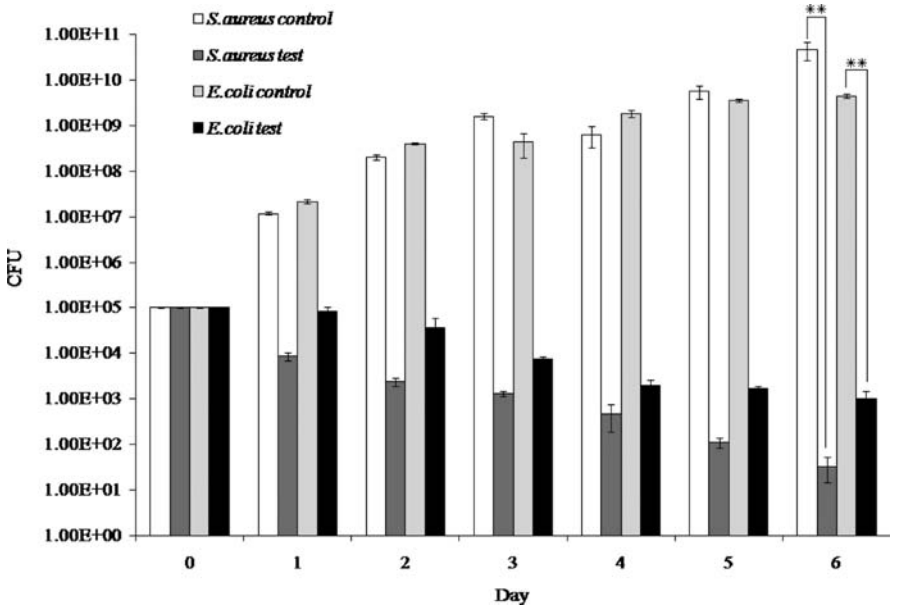

Figure 8. Growth of microorganisms on a food pack as a function of time at 20 °C (**, $p < 0.01$).

the 6th day. SIP was effective against both the Gram-positive as well as Gram-negative microorganisms and decreased the colony count of *S. aureus* from 10^5 to 10^3 and *E. coli* from 10^5 to 10^2 at 4 °C ($p < 0.01$). SIP exhibited a higher reduction of bacterial colony counts at 20 °C (3−4 times) when compared to 4 °C (1−2 times) (Figures 7 and 8), possibly because the activity of the immobilized subtilisin was higher at 20 °C (71% activity recovery) compared to 4 °C (69% activity recovery) (Figure 1). These results demonstrate that SIP could be used to control the growth of *S. aureus* and *E. coli* grown on food when stored for a short period of time.

To conclude, food contaminants comprise either a single bacteria or a mixture of different types of bacteria and fungi, along with EPS, which includes polysaccharides, glycoproteins, and proteins. Moreover, types of EPS produced by each microbe vary. The esterase and amidase activity of subtilisin acts on the peptidoglycan layer, lipopolysaccharides (LPS), phospholipids, and lipoproteins of the cell wall, imparting antimicrobial activity on both Gram-negative and Gram-positive cells. Therefore, as demonstrated here, a broad spectrum antibiotic, such as subtilisin, would solve this problem. Although microorganisms develop antibiotic resistance in the genetic level, the component of the cell membrane remains just the same. Therefore, an antimicrobial agent that acts on the microbial membrane would be an intelligent choice, as observed here. In packaged food, the food is

always in contact with the packaging material. If antibacterial activity is to be exhibited inside the food, SIP could be inserted as sachets and pads in between food substances. SIP imparted sustained activity on microbes. Because subtilisin is a food-grade protease, it is not harmful to humans. Therefore, SIP could be used as an environmentally benign antifouling agent in the food packaging industry.

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ABBREVIATIONS USED

UP, unimmobilized polycaprolactam; SIP, subtilisin-immobilized polycaprolactam; EPS, extracellular polymeric substances; FTIR, Fourier transform infrared; NCIM, National Collection of Industrial Microorganisms; CLSM, confocal laser scanning microscope; SRL, Super Religare Laboratories; NCL, National Chemical Laboratory; UV, ultraviolet; IU, international unit; DSA2, Digital Scrapbook Artist 2 Software; CFU, colony forming unit; YPD, yeast peptone dextrose; LPS, lipopolysaccharide

REFERENCES

(1) De Roever, C. Microbiological safety evaluations and recommendations on fresh produce. *Food Control* **1998**, *9*, 321–347.

(2) Restuccia, D.; Spizzirri, U. G.; Parisi, O. I.; Cirillo, G.; Curcio, M.; Lemma, F.; Puoci, F.; Vinci, G.; Picc, N. New EU regulation aspects and global market of active and intelligent packaging for food industry applications. *Food Control* **2010**, *21*, 1425–1435.

(3) Xavier, J. B.; Picioreanu, C.; Rani, S. A.; van Loosdrecht, M. C. M.; Stewart, P. S. Biofilm control strategies based on enzymatic disruption of the extracellular polymeric substance matrix—A modeling study. *Microbiology* **2005**, *51*, 3817–3832.

(4) Pavithra, D.; Doble, M. Biofilm formation, bacterial adhesion and host response on polymeric implants issues and prevention. *Biomed. Mater.* **2008**, *3* (3), 4003.

(5) Johansen, C.; Falholt, P.; Gram, L. Enzymatic removal and disinfection of bacterial biofilms. *App. Environ. Microbiol.* **1997**, *63*, 3724–3728.

(6) Leroy, C.; Delbarre, C.; Gillebaert, F.; Compere, C.; Combes, D. Effect of commercial enzymes on the adhesion of a marine biofilm forming bacterium. *Biofouling* **2008**, *24*, 11–22.

(7) Orgaz, B.; Kives, J.; Pedregosa, A. M.; Monistrol, I. F.; Laborda, F.; SanJose, C. Bacterial biofilms removal using fungal enzymes. *Enzyme Microb. Technol.* **2006**, *40*, 51–56.

(8) Loiselle, M.; Anderson, K. W. The use of cellulose in inhibiting biofilms formation from organisms commonly found on medical implants. *Biofouling* **2003**, *19*, 77–85.

(9) Flemming, H. C. Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. *Polym. Degrad. Stab.* **1998**, *59*, 309–315.

(10) Gomez-Suarez, C.; van der Borden, P. J.; Wingender, J.; Flemming, H. C. Influence of extracellular polymeric susbatnces deposition and redeposition of *Pseudomonas aeruginosa* to surface. *Microbiology* **2002**, *148*, 1161–1169.

(11) Zhang, T.; Ke, S. Z.; Liu, Y.; Fang, H. P. Microbial characteristics of a methanogenic phenol-degrading sludge. *Water Sci. Technol.* **2005**, *52*, 73–78.

(12) Lapidot, A.; Romling, U.; Yaron, S. Biofilm formation and the survival of *Salmonella typhimurium* on parsley. *Int. J. Food Microbiol.* **2006**, *109*, 229–233.

(13) Ploux, L.; Beckendorff, S.; Nardin, M.; Neunlist, S. Quantitative and morphological analysis of biofilms formation on self assembled monolayers. *Colloids Surf.* **2007**, *57*, 174–181.

(14) Sivakumar, P. M.; Prabhawathi, V.; Doble, M. Chalcones as an effective antibiofoulant against marine isolated microorganisms. *Colloids Surf., B* **2010**, *81*, 439–446.

(15) Sivakumar, P. M.; Prabhawathi, V.; Balaji, S.; Neelakandan, R.; Manoharan, P. T.; Doble, M. Effective antibacterial adhesive coating on cotton using ZnO nanorods and chalcone. *Carbohydr. Polym.* **2010**, *79*, 717–723.

(16) Quintavalla, S.; Vicini, L. Antimicrobial food packaging in meat industry. *Meat Sci.* **2002**, *62*, 373–380.

(17) Han, J. H. Antimicrobial food packaging. *Food. Technol.* **2000**, *54*, 56–65.

(18) Nicholson, M. D. The role of natural antimicrobials in food packaging biopreservation. *J. Plast. Film Sheeting* **1998**, *14*, 234–241.

(19) Siracusa, V.; Rocculi, P.; Romani, S.; Rosa, M. D. Biodegradable polymers for food packaging: A review. *Trends Food. Sci. Technol.* **2008**, *19*, 634–643.

(20) Bhaskar, N.; Sudeepa, E. S.; Rashmi, H. N.; Tamil Selvi, A. Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour. Technol.* **2007**, *98*, 2758–2764.

(21) Yen, Y. H.; Li, P. L.; Wang, C. L.; Wang, S. L. An antifungal protease produced by *Pseudomonas aeruginosa* M-1001 with shrimp and crab shell powder as a carbon source. *Enzyme Microb. Technol.* **2006**, *39*, 311–317.

(22) Rao, M. B.; Tanksale, A. M.; Ghatge, M. S.; Deshpande, V. V. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 597–635.

(23) London South Bank University. *Applications of Proteases in the Food Industry*; London South Bank University: London, U.K., 2004; http://www.lsbu.ac.uk/biology/enztech/proteases.html.

(24) Chellapandian, M.; Sastry, C. A. Immobilization of alkaline protease on nylon. *Bioprocess Biosyst. Eng.* **1994**, *11*, 17–21.

(25) Kunitz, M. Crystalline soyabean trypsin inhibitor II. General properties. *J. Gen. Physiol.* **1947**, *30*, 291–310.

(26) Tagg, J. R.; McGiven, A. R. Assay systems for bacteriocins. *J. Appl. Microbiol.* **1971**, *21*, 943–949.

(27) Fernandez-Lafuente, R.; Rossell, C. M.; Rodriguez, V.; Santana, C.; Soler, G.; Bastida, A.; Guisan, A. M. Preparation of activated supports containing low p*K* amino groups. A new tool for protein immobilization via the carboxyl coupling method. *Enzyme Microb. Technol.* **1993**, *15*, 546–550.

(28) López-Gallego, F.; Betancor, L.; Hidalgo, A.; Mateo, C.; Guisán, J. M.; Fernández-Lafuente, R. Optimization of an industrial biocatalyst of glutaryl acylase: Stabilization of the enzyme by multipoint covalent attachment onto new amino-epoxy sepabeads. *J. Biotechnol.* **2004**, *111*, 219–227.

(29) Monsan, P. Optimization of glutaraldehyde activation of a support for enzyme immobilization. *J. Mol. Catal.* **1978**, *3*, 371–384.

(30) Owens, D. K.; Wendt, R. C. Estimation of the surface free energy of polymers. *J. Appl. Polym. Sci.* **1969**, *13*, 1741–1747.

(31) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(32) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.

(33) Arkatkar, A.; Arutchelvi, J.; Bhaduri, S.; Uppara, P. V.; Doble, M. Degradation of unpretreated and thermally pretreated polypropylene by soil consortia. *Int. Biodeterior. Biodegrad.* **2009**, *63*, 106–111.

(34) Sudhakar, M.; Doble, M.; Murthy, P. S.; Venkatesan, R. Marine microbe-mediated biodegradation of low- and high-density polyethylenes. *Int. Biodeterior. Biodegrad.* **2008**, *61*, 203–213.

(35) Besse, N. G.; Audinet, N.; Beaufort, A.; Colin, P.; Cornu, M.; Lombard, B. A contribution to the improvement of *Listeria monocytogenes* enumeration in cold-smoked salmon. *Int. J. Food Microbiol.* **2004**, *91*, 119–127.

(36) Betancor, L.; López-Gallego, F.; Hidalgo, F.; Alonso-Morales, N.; Mateo, G. D. C.; Fernández-Lafuente, R.; Guisán, J. M. Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. *Enzyme Microb. Technol.* **2006**, *39*, 877–882.

(37) Ding, L.; Yao, Z.; Li, T.; Yue, Q.; Chai, J. Study on papain immobilization on a macroporous polymer carrier. *Turk. J. Chem.* **2003**, *27*, 627–637.

(38) Bahulekar, R.; Ayyangar, N. R.; Ponrathnam, S. Polyethyleneimine in immobilization of biocatalysts. *Enzyme Microb. Technol.* **1991**, *13*, 858–868.

(39) Mukherjea, R. N.; Bhattacharya, P.; Ghosh, B. K.; Taraphdar, D. K. Process engineering studies on immobilized trypsin using molecular sieves as carrier. *Biotechnol. Bioeng.* **1977**, *19*, 1259–1268.

(40) Estela, M. L.; Rui, M. B.; Balcão, V. M.; Malcata, F. X. Hydrolysis of whey proteins by proteases extracted from *Cynara cardunculus* and immobilized onto highly activated supports. *Enzyme Microb. Technol.* **2001**, *28*, 642–652.

(41) Mateo, C.; Abian, O.; Fernandez-Lorente, G.; Pedroche, J.; Fernandez-Lafuente, R.; Guisan, J. M. Epoxy sepabeads: A novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. *Biotechnol. Prog.* **2002**, *18*, 629–634.

(42) Sutherland, I. W. The biofilm matrix—An immobilized but dynamic microbial environment. *Trends Microbiol.* **2001**, *9*, 222–227.

(43) Tsuneda, S.; Aikawa, H.; Hayashi, H.; Yuasa, A.; Hirata, A. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiol. Lett.* **2003**, *223*, 287–292.

(44) Simões, M.; Lúcia, C.; Simões; Vieira, M. J. A review of current and emergent biofilm control strategies. *LWT—Food Sci. Technol.* **2010**, *43*, 573–583.

(45) Pearson, J. P.; Delden, C. V.; Iglewski, B. H. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **1999**, *181*, 1203–1210.

(46) O'Toole, G. A.; Kolter, R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol. Microbiol.* **1998**, *28*, 449–461.

(47) Molobela, I. P.; Cloete, T. E.; Beukes, M. Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria. *Afr. J. Microbiol. Res.* **2010**, *4*, 1515–1524.

(48) Tasoo, M.; Pettitt, M. E.; Cordeiro, A. L.; Callow, M. E.; Callow, J. A.; Werner, C. Antifouling potential of subtilisin A immobilized onto maleic anhydride copolymer thin films. *Biofouling* **2009**, *25*, 505–516.

(49) Doyle, R. J. Contribution of the hydrophobic effect to microbial infection. *Microbes Infect.* **2000**, *2*, 391–400.

(50) Khandwekar, A. P.; Patil, D. P.; Shouche, Y. S.; Doble, M. Surface entrapment modification of poly(caprolactone) by biomacromolecules and their blood compatibility. *J. Biomater. Appl.* **2010**, DOI: 10.1177/0885328210367442.

(51) Nielson, S. F.; Larsen, M.; Boesen, T.; Schonning, K.; Kromann, H. Cationic chalcone antibiotics. Design, synthesis, and mechanism of action. *J. Med. Chem.* **2005**, *48*, 2667–2677.

(52) Rice, S. A.; Koh, K. S.; Queck, S. Y.; Labbate, M.; Lam, K. W.; Kjelleberg, S. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J. Bacteriol.* **2005**, *187*, 3477–3485.