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Papain Immobilized Polyurethane Film as Antimicrobial Food Package

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***Abstract*—**Food contamination occurs during post process handling. This leads to spoilage and growth of pathogenic microorganisms in the food, thereby reducing its shelf life or spreading of food borne diseases. Several methods are tried and one of which is use of antimicrobial packaging. Here, papain, a protease enzyme, is covalently immobilized with the help of glutarldehyde on polyurethane and used as a food wrap to protect food from microbial contamination. Covalent immobilization of papain was achieved at a pH of 7.4; temperature of 4°C; glutaraldehyde concentration of 0.5%; incubation time of 24h; and 50mg of papain. The formation of -C=N- observed in the Fourier transform infrared spectrum confirmed the immobilization of the enzyme on the polymer. Immobilized enzyme retained higher activity than the native free enzyme. The modified polyurethane showed better reduction of *Staphylococcus aureus* biofilm than bare polymer film (eight folds reduction in live colonies, two times reduction in protein and 6 times reduction in carbohydrates). The efficacy of this was studied by wrapping it over S. *aureus* contaminated cottage cheese (paneer) and cheese and stored at a temperature of 4°C for 7days. The modified film reduced the bacterial contamination by eight folds when compared to the bare film. FTIR also indicated reduction in lipids, sugars and proteins in the biofilm.

***Keywords*—**Cheese, Papain, polyurethane, *Staphylococcus aureus.*

I. Introduction

MICROBIAL contamination of food occurs at its surface due to post process handling including packaging. This is one of the major causes of food borne illness and spoilage. Millions of people globally are affected by food borne diseases which may a times also becomes fatal causing death. Microbes adhere to the packaging material and get transferred to the packed food. Absence of food storage facilities in many countries also lead to food contamination and spoilage leading to economic as well as human loss.

Different polymers including low density polyethylene, high density polyethylene, poly propylene, polyurethane, linear low density polyethylene and polycaprolactam are commonly used for wrapping fresh and processed food as well as vegetables and fruits. Food may get spoilt during transportation or when stored in the super-market shelves due to several factors, including bacterial growth, build up of oxygen and/or moisture due to diffusion. Use of antimicrobial packaging plays a vital role in maintaining the life of the food. Antimicrobial agents coated on various surfaces prevent the biofilm formation [1]. Nanoparticles such as titanium oxide, silver and gold coated surfaces are toxic to organisms but could also affect the human [2]. Antibacterial sprays or dips have also been used. However, these have limited benefits because they may get degraded or diffuse in to the food and hence may not last longer duration. Peroxides, eugenol and nisin are few antimicrobials which are toxic to humans. So, in order to improve shelf life of food products use of enzymes, as an antimicrobial agent is desired. Papain, a protease enzyme exhibits antibacterial, antifungal, antibiofilm and anti-inflammatory activity.

Protease enzymes are widely used in various commercial products. They are also used for meat tenderization, cheese ripening and milk coagulation because of the peptidase and esterase activities [3].

In this paper covalent crosslinking of papain to polyurethane (most versatile material in the world today) and test its antibiofilm property is reported. 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 is reported here. Covalent immobilization prevents its aggregation.

Ninety percent of the cases of food poisoning each year are caused by organisms including *Staphylococcus aureus, Salmonella* spp., *Clostridum perfringens, Campylobacter, Listeria onocytogenes, Vibrio parahaemolyticus, Bacillus cerus, Entero-pathogenic* and *Escherichia coli. S. aureus* is a very common food borne pathogen that causes illness by producing a heat stable toxin. So it is taken as the model organism for performing the studies as well as elucidating the advantages of the techniques described here.

II. Materials and Method

A. Papain Estimation

The activity of papain was determined as per a reported procedure using casein as a substrate [3].

B. Minimum Inhibitory Concentration

Papain was tested for antimicrobial activity by microdilution broth assay using resazurin dye as an indicator against *S. aureus* with slight modifications as reported by Sarkar et al. [4]. *Staphylococcus aureus* NCIM (National Collection of Industrial Microorganisms) 5021, was purchased from the National Chemical Laboratory (NCL), Pune, India. It was stored in glycerol stock at -20°C and used when required.

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C. Immobilization of Papain on Polyurethane

Glutaraldehyde preactivated Polyurethane was prepared by suspending pieces (1x1 cm) in 0.5% of glutaraldehyde and 25 mM of phosphate buffer at a pH of 7.0 [5]. The polymer was kept under mild stirring for 15 h at 25°C. Then, they were taken out and washed with 25 mM phosphate buffer and again with Milli-Q water.

Polyurethane was incubated with 0.5% of glutaraldehyde solution and 0.1mM of papain in Phosphate buffer solution at a pH of 7 at 25°C for 1h [6]. The polymer was then removed and washed with phosphate buffer solution to remove excess of glutaraldehyde. Again it was incubated for 20h to achieve cross linking between the enzyme and the polymer.

The crosslinking and the immobilization of the enzyme on polyurethane were identified by Fourier Transform Infrared (FTIR) spectra recorded in the frequency range of 500-4000 cm^{-1} using a Perkin-Elmer PE 1600 FTIR spectrometer.

Contact angle is measure of the hydrophilicity of the polymer, the contact of bare PU and modified PU was measured using a Goniometer (Kruss germany) with Milli-Q water (Millipore grade). The images obtained were analyzed with a Digital Scrapbook Artist 2 Software (DSA2) with an accuracy of ±0.1°. The experiments were performed on five different locations and average value was reported here.

D. Biological Characterization of Coated and Uncoated Polymers

The bacterial suspension was inoculated from stock culture into 25 ml of nutrient broth and incubated at 37ºC for 16 h in a shaker (Scigeneis Pvt., Ltd, Chennai, India) at 120 rpm. 50ml of the culture was taken, centrifuged (Eppendroff, Germany) at 4ºC at 10000 rpm for 10min and diluted with phosphate buffer solution (10 mM) such that the OD was 0.1 (at 600 nm) which was equivalent to approximately $1x10^9$ cells/ml. This suspension was inoculated into two flasks containing nutrient broth along with bare and papain coated polymer (of size 1x1cm). They were then stirred for 24 h at 30°C and 120 rpm. The samples were removed with sterile forceps and the strongly bound biofilm formed on the surface was carefully removed using ultra sonication (Thosan Pvt., Ltd, Ajmer, India) into flasks containing 0.7% of saline solution [7]. The protein and carbohydrate in the biofilm were estimated as per Lowry's method [8] using crystalline bovine serum albumin and phenol sulphuric acid method using glucose as the standard respectively [9]. The live bacterial colonies in the biofilm was determined as per a standard procedure and represented as colony forming units (cfu/ml). All the experiments were repeated thrice and the statistical significance of the data was ascertained.

E. Food Pack Applications

Freshly purchased paneer (cottage cheese) and cheese samples were kept frozen at -20°C and thawed at 2°C for 1 day immediately before use. It was cut into small pieces, each weighing 1 g, was inoculated with 10^9 cells of *S. aureus* and were wrapped with bare and protein immobilized polymers, placed in a petri plate and incubated at 4°C. After 7 days the number of colonies formed on the samples was measured [10]. The biofilm developed on the bare and enzyme immobilized surfaces after the food pack application were characterized with FTIR and were also photographed to observe changes in their appearance and colour.

F. Statistics

All the analysis were repeated thrice on three independent samples and were reported as mean ± standard errors (SE). One way ANOVA and two samples t-test were performed using MIniTab Ver 14.0 (MiniTab inc., USA). A p value <0.05 was considered to be statistically significant.

III. Results and Discussion

A. Enzyme Immobilization

The papain immobilized polyurethane retained 88% of enzyme activity. After 30 days of storage, free enzyme retained 86% of its original activity, while on immobilization papain retained 97% of its original enzyme activity. These data indicates that this crosslinking process retains the enzyme activity as well as maintains its stability on a polymeric surface.

The FTIR spectrum of bare and papain immobilized polymers are shown in Fig. 1. The absorption band of the secondary amide is observed at a wavelength of 3300 cm^{-1}. The appearance of the amine peak at 1635 cm^{-1} confirms the presence of amide (C=N). These results confirm the immobilization of papain on polyurethane.

The contact angle of the bare PU and papain immobilized PU were 83±1.9º and 58±1.3º respectively, indicating that immobilization has made the surface very hydrophilic. PU is a very hydrophobic polymer. It is reported that hydrophilic surfaces generally prevent bacterial adhesion, which is desired for imparting anti-biofilm property to the surface.

B. Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentration (MIC) of control (Milli Q water is used as control) and papain which inhibited the growth of *S. aureus* as determined by microdilution broth assay method are 1.8 and 0.92 µM respectively indicating that papain has good antibacterial activity.

C. Biofilm Studies

The antibacterial activity of papain immobilized polymer was studied against the Gram positive bacteria, *S. aureus*. The number of live colonies of the organism on bare and papain immobilized polymers after 24 hrs were $55±13 \times 10^9$ and $40 ± 5 \times 10^1$ respectively, indicating the effectiveness of the enzyme in preventing the bacterial growth. There was an 8 log reduction in the live colonies on surface immobilised with papain when compared to the control PU film.

Biofilm consists of exopolysacharides (EPS) which comprises of polysaccharides, proteins, nucleic acids, lipids, and phospholipids. Proteins and polysaccharides account for 75 to 89% of the EPS. It imparts protection to microorganisms against antibacterials and biocides. Because the major component of the biofilm is protein and carbohydrate,

experiments were performed to test the effect of the enzyme on these components.

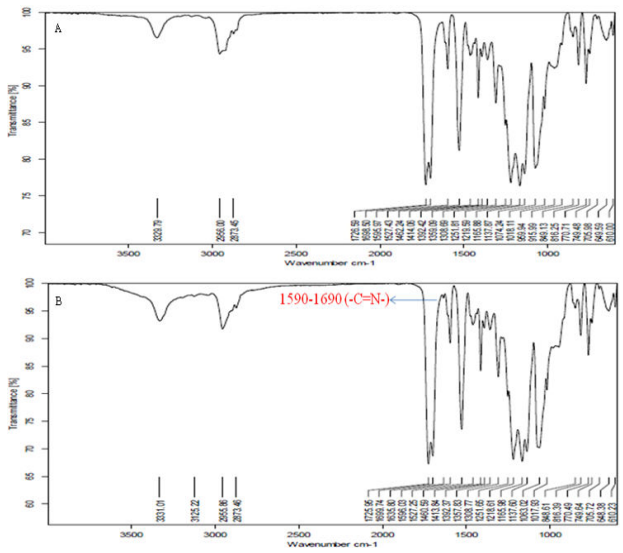

Fig. 1 FTIR spectra of (A) uncoated (B) papain immobilized polymer

The amount of protein on bare and enzyme immobilized surfaces were 155±11 and 78±8 µg/ml respectively. The corresponding carbohydrate values were 95±13 and 13±3 µg/ml respectively. Papain immobilized polymer has the least bacterial attachment. There is a direct correlation between the amount of live colonies present and the amount of protein and carbohydrate in the biofilm. The enzyme increases the membrane permeability as well as acts on the proteins ($p<0.01$) and the peptidoglycan present in the outer membrane of the bacteria leading to the loss of cell content through cell wall leakage, resulting in bacterial destruction. There are literatures reports which show that protease [11] hydrolyses the protein present on a polymer surface.

Papain immobilized polymer has the least carbohydrate content in their biofilm followed by bare surfaces. This is because of the esterase activity of the protein. The protein is able to break the ester bond. The immobilisation approach mentioned here also helps to retain the activity and stability of the enzyme.

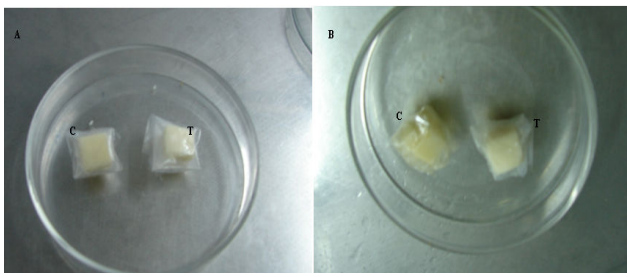

Fig. 2 Cheese wrapped in (C) bare and (T) papain immobilized PU films (a) on Day 1 (b) on 7th day

D.Food Pack Applications

Cheese wrapped with uncoated and papain immobilized polymers showed 22±4 $x10^{10}$ and 44±9 $x10^{1}$ CFU of *S.aureus* cells /ml respectively at the end of seven days. There was a 9 log reduction in the live colonies in the food wrapper with surface immobilized with papain coated polymer film when compared to the control PU film. Fig. 2 shows cheese wrapped with modified and unmodified film on day 0 and day 7. A change in colour could be seen in the 7 days old food wrapped with bare PU film, indicating that it has been spoilt due to the growth of *S. aureus.*

The FTIR spectra of the biofilm formed on the bare and enzyme immobilized surfaces are shown in Fig. 3. The absorption is represented in the y-axis and the wave numbers are given in the x-axis. FTIR is a good technique to monitor proteins, polysaccharides and oxygenated compounds. The changes in their amount could also be monitored with this technique.

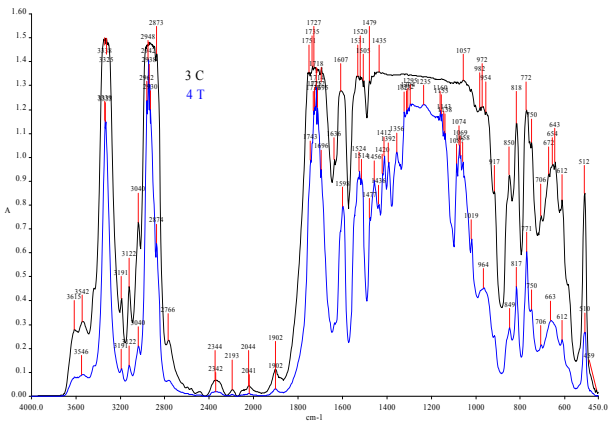

Fig. 3 FTIR spectra of biofilm formed on 3C) uncoated 4T) papain immobilized polymer that were wrapped with cheese for 7 days

Comparison of the two spectra indicates that there is a reduction in the amounts of polysaccharides (3000-3700 cm^{-1}), lipids (2700-3000 cm^{-1}), proteins (2500-3000 cm^{-1}) in the biofilm formed on papain immobilized surface than the bare surface. The absorption values corresponding to these peaks from the biofilm on the enzyme immobilized surface are less than the corresponding values from the bare surface.

Paneer (cottage cheese) wrapped with uncoated and papain immobilized polymers showed 30±3 $x10^{10}$ and 25±5 $x10^{1}$ CFU of *S. aureus* cells /ml. Fig. 4 shows the differences in the food wrapped with both the films (food wrapped with bare polymer has changed in colour at the end of seven days indicating that it has been spoilt. These results indicate that papain immobilized polymer could be used to effectively control the growth of *S. aureus* on the surface of the food (especially milk products) when stored for a short period of time. Papain being a food grade material may not be expected to be harmful for the consumers and may be assumed to be safe.

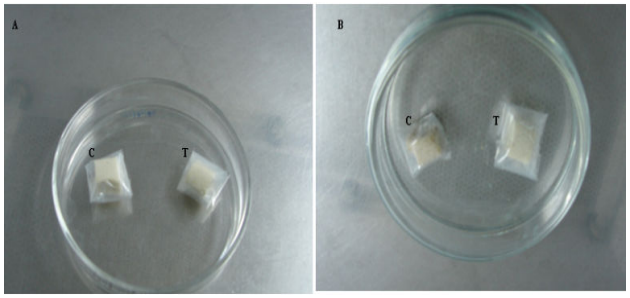

Fig. 4 Cottage cheese (Paneer) wrapped in (C) bare and (T) papain immobilized PU films (a) on Day 1 (b) on 7th day

IV. Conclusion

Food contaminants comprise of bacteria and fungi, protein and carbohydrate from the biofilm. The esterase and amidase activities of papain can act on the peptidoglycan layer, lipopolysaccharides (LPS), phospholipids, and lipoproteins of the cell wall, imparting antimicrobial activity on both Gram-negative and Gram-positive cells. So it can behave as a broad spectrum antibiotic and would solve this problem. Although microorganisms develop antibiotic resistance, the component of the cell membrane remains the same. So an antimicrobial agent that acts on the membrane could be a good 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, papain immobilized polymer could be inserted as sachets and pads inside the food. Because papain is a food-grade protease, it is not harmful to humans and may not spoil the quality of the food. It could be a used as an environmentally benign antifouling agent in the food. Of course, studies need to be done to see if the enzyme immobilised surface has led to changes in the taste and texture of the food that is wrapped. The long term stability and activity retention of the enzyme may also have to be studied in a systematic manner. This technique could be adopted to immobilise papain on other polymers used as food wrap as well. The current study is a green sustainable solution to address this issue. Other food grade esterases and proteases could also be immobilised on such polymers using the technique described here.

Food contaminants comprise either a single bacteria or a mixture of different types of bacteria and fungi as mentioned before. In addition the biofilm will contain EPS, which includes polysaccharides, glycoproteins, and proteins [12]. The type of EPS produced by each microbe will vary. Once the organism forms a biofilm, it becomes difficult to kill it, because of the protection offered by the EPS layer. The esterase and amidase activity of papain acts on the peptidoglycan layer, lipopolysaccharides (LPS), phospholipids, and lipoproteins of the cell wall. Thus, imparting antimicrobial activity on both Gram-negative and Gram-positive cells. Papain immobilisation also imparts hydrophilic properties to the surface which also aids in the prevention of the microorganism to the polymer surface, since hydrophilic surfaces prevent attachment of hydrophobic bacteria.

Biodegradable wrappers which degrade within a reasonable short period of time are preferred now to prevent accumulation of non-biodegradable polymers in the environment causing problems to flora and fauna. The immobilisation technique reported here could also be extended to biodegradable polymers also to acquire the benefit of the latter.

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