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ANTI-MICROBIAL ACTIVITIES OF SOYA PROTEIN ISOLATE (SPI)/ CLOISITE C30B (MMT) NANOCOMPOSITE FILM

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ABSTRACT

In the present research program, the anti-bacterial properties of Soya Protein blended with Cloisite 30 B have been investigated. The nanocomposite films were prepared blending it with Cloisite 30 B. The composites were characterized by Fourier Transmission Infra Red Spectroscopy (FTIR), X-Ray Diffraction (XRD) analysis. The morphology of the nanocomposites was ascertained from the SEM studies. The polymers were tested for anti-microbial activity against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli). The SPI/C30B nanocomposite films strongly inhibited the growth of a wide variety of microorganisms including Gram-positive bacteria, Gram-negative bacteria. The anti-microbial studies were carried out against food borne pathogens. The biodegradability of the nanocomposites has also been reported.

Key Words: Soya protein, MMT, Nanocomposites, Anti-microbial activity

INTRODUCTION

Soy protein isolate (SPI) is an abundant, inexpensive and renewable natural material. It is composed of almost exclusively of two globular protein fractions differentiated by sedimentation coefficient: 7S (b-conglycinin) and 11S (glycinin) [1]. Both fractions have the ability to form films by a two-step process. During the preheating step, proteins are unfolded and polymerized into soluble aggregates, followed by a cooling step and subsequent surface dehydration, which results in the formation of a film network through disulfide cross-linking and hydrophobic bonds [2,3]. In general, protein films are effecttive lipid, oxygen, and aroma barriers at low to intermediate relative humidity (RH). The water exclusion properties of SPI films are relatively poor due to the hydro philic nature of soy proteins and to substantial amounts of added hygroscopic plasticizers [4,5]. Numerous studies have concentrated on improving the mechanical and water-excluding properties of soy protein-based films through physical, chemical and enzymatic treatments or compositing with hydrophobic materials in order to develop alternative resources for bioplastics in packaging applications [6,7]. However, no investigation of SPI film as a controlled delivery system has been reported.

The concept of combining the biodegradability with bioreactivity of natural polymers is attracting scientists from diverse areas. A good example is the proposal of blends of several protein based blends[8–10] as an alternative to the most common biodegradable polymers applied in the biomedical fields such as polylactic acid[11,12], polyhydroxybutyrate[11,13] and polyglycolic acid[11,12]

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However, the number of biopolymers in clinical use is rather smaller and still presenting several weaknesses[13]. A potential solution to overcome these difficulties may rely on the development of new materials such as new protein-based thermoplastics (from vegetable or animal origin). Their high chemical versatility[14-16]and similarity to tissue constituents[17] may lead to the introduction of novel biomaterials into the clinical areas. Furthermore proteins are very versatile materials both by source and because of a wide variety scale of possible modifications. So their properties can be tailored towards the diverse requirements of a specific application. As a result proteins may be regarded as an eventual ideal template suitable for being used as biomaterials. Temporary replacement implants, tissue-engineering scaffolding, membranes for promoting wound healing, and drug delivery carriers are the most promising target applications. In this preliminary work, new casein and soybean thermoplastic formulations were developed by extrusion compounding. The effects of inert (alumina, used as a model system) and bioactive (tricalcium phosphate, a bone-like ceramic) ceramic reinforcements over the composites' mechanical properties, its degradation behavior and bioactivity character were examined.In this prospect soy protein isolate is a very promising biomaterial for anti- bacterial and anti-fungal activity.

Pharmacology studies have revealed that montmorillonite (MMT) adsorbed bacteria such as Escherichia coli (E. coli), Staphylococcus aureus (S. aureus) and immobilized cell toxins [18-21]. Some researchers found that natural clay minerals showed no anti-bacterial effect but could adsorb and kill bacteria when materials with anti-microbial activity were intercalated. There are certain number of reports about modified MMT with anti-bacterial activity such as cetylpyridinium-exchanged MMT, MMT-carrying copper and silver ions as effective bacteriostasis materials. [22-28] Anti-microbial polymers have been used as coatings in many areas such as food processing, biomedical devices, filters and additives for antifouling paints. The use of cationic anti-microbial polymers can eliminate bacterial infection of implanted devices such as catheters. This renders them antiseptic and thus less able to transmit bacterial infections. They are used in the textile industry to form anti-microbial fibers and as disinfectants and preservatives in pharmaceuticals[29].

During the last decade Nayak and coworkers have carried out extensive research work on soy protein to be used as degradable films and molded products (30-39). This communication reports the degradation of SPI cross-linked with furfural, a bio-based heterocyclic aldehyde and blended with montmorillonite, MMT. The spectral, thermal, XRD and biodegradability properties of the nanocomposites have also been reported.

EXPERIMENTAL

Materials

Soya Protein, Nano clay Cloisite 30B, was purchased from Southern Clay Co.(USA).The cultures of L.monocytogenes, *S.aureus, B.cereus, S.sonnei, S. typhimurium, E.coli, and V. vulnificus bacteria were* maintained in the Microbiology Laboratory, Bio-Lab , Bhubaneswar, Odisha.

Preparation SPI-C30B (MMT) Nanocomposites

The formulation consisted of Soy Protein Isolate (SPI), glycerol and MMT (0% - 15%, dry basis). All three types of clays (Cloisite 30B) were used at four different levels (0, 5, 10, and 15%). The ingredients were mixed and left at room temperature for 2 hours for hydration. Nanoclay solutions with two clay compositions were prepared by dispersing appropriate amounts of clays solution and vigorously stirring . The mixtures were stirred continuously for 2 hrs and then cast onto level Teflon-coated glass plates. After drying at room temperature for at least 72 h, the films were peeled from the plates

CHARACTERIZATION

FTIR Spectral Analysis

The Fourier Transmission Infrared Spectra (FTIR) were obtained from the cross linked products through a Perkin Elmer Spectrum RX1 FTIR spectrometer at Hanyang University, South Korea.

X-ray Diffraction (XRD)

The change in gallery height of the blend was investigated by WAXD experiments, which were carried out using a X-ray diffractometer (BEDE D-3 system) with Cu Ka radiation at a generator voltage of 40 kV and a generator current of 100 mA. Samples were scanned from $2\theta = 1–10^{o}$ at a scanning rate of 2^{o}/min.

Scanning Electron Microscopy (SEM)

The morphology of the fracture surface (cross-sectional surface) of the nanocomposite films were visualized using a field emission scanning electron microscope (JEOL 6400F, Japan Electron Optics Ltd., Tokyo, Japan) oper- ating at 5 kV. Small pieces (0.5 × 0.5 cm) of bio nano- composite films were frozen in liquid nitrogen, cut using a sharp razor blade and mounted on specimen stubs with 2 sided carbon tape. The fracture surfaces of the films were sputter coated with a thin layer (~8 - 10 nm) of gold-palladium (Au-Pd) using a sputter-coater (Hummer II, Anatech Ltd., Union City, CA). After coating the samples were viewed under the scanning electron microscope.

Anti-microbial Susceptibility Test

The disc diffusion method was used to screen the anti-microbial activity. *In vitro* anti-microbial activity was screened by using Mueller Hinton Agar (MHA) obtained from Hindustan Scientific PVT limited, Cuttack (Odisha). The MHA plates were prepared by pouring 15 ml of molten media into sterile Petri plates. The plates were allowed to solidify for 5 min and 0.1% inoculum (0.5 McFarland standard) suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. 50 µl concentration of test sample was loaded on 0.5 cm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. For each bacterial strain, negative controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter and the result obtained was tabulated) and Ampicillin (10 mcg/disc) were used.

Biodegradation Test under Soil Environment

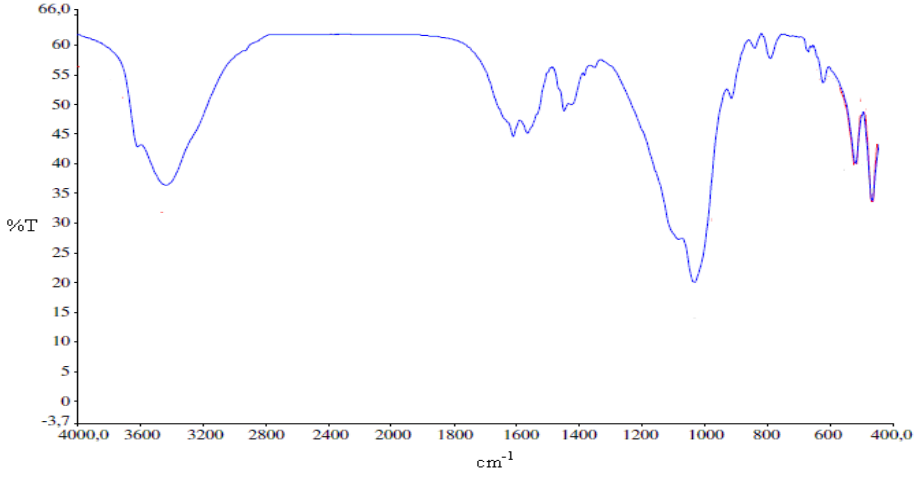
Biodegradability of the samples was studied by evaluating weight loss of the blends over time in soil environment. Samples of 30 x 30 x 1 mm were weighed and then buried in boxes containing alluvial soil. The soil was maintained at approximately 20% moisture in weight and the samples were buried at a depth of 15 cm. A control box was also maintained, consisting of only samples and no soil. The buried samples were dug out once a month, washed in distilled water, dried in vacuum oven at $50 \pm 2\ ^{o}C$ for 24 h, equilibrated in a desiccator for at least a day. The samples were then weighed before returning them to soil.

RESULTS AND DISCUSSION

Fourier Transmission Infra Red Spectroscopy (FTIR)

C 30B/ soya protein biocomposite showed peaks at 1612 cm^{-1} (N–H bending), 1566 cm^{-1} (N–H bending), 1450 and 1425 cm^{-1} (C–H bending), and also absorbancies due to structural O–H stretching at 3621 cm^{-1}, H–O–H deforming (absorbed water) at 1634 cm^{-1}, and Al–O vibrations at 915, 624 842, and 792 cm^{-1}confirm the presence of C 30B in the dispersion. The Si–O stretching peaks can be seen at 1086 and 1034 cm^{-1} and finally Si–O bending peaks at 520 and 467 cm^{-1}.

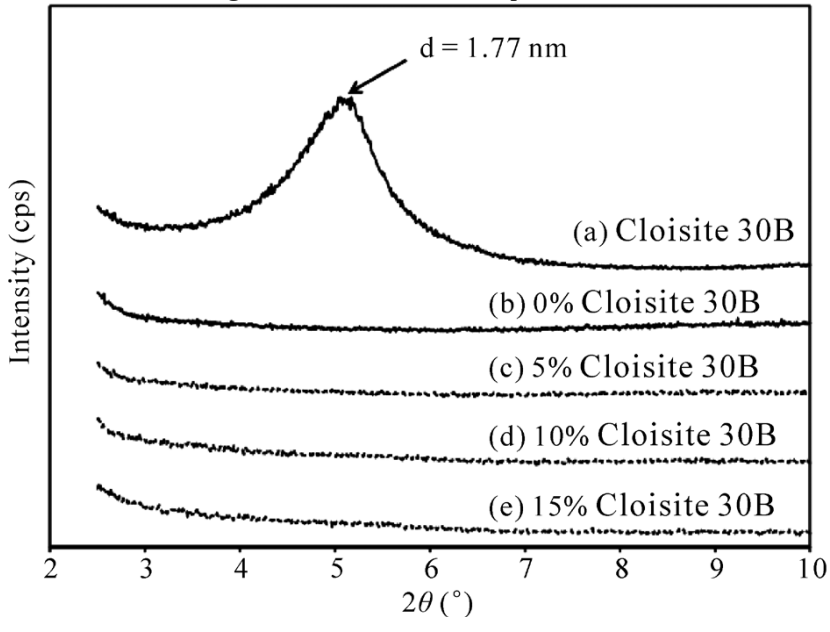
Fig- 1. FTIR spectra C 30B/SPI nanocomposites film



X-Ray Diffraction Analysis

XRD patterns of Cloisite 30B and SPI-Cloisite 30B (0%, 5%, 10%, and 15%) nanocomposite powders are shown in **Figure- 2**. The d-spacing of Cloisite 30B corre- sponding to the diffraction peak at a 2θ angle of 5.0˚ was calculated to be 1.77 nm. There was no diffraction peak in the 2θ range of 2.5˚ to 10˚ for the nanocomposites at all MMT contents of Cloisite 30B. Absence of diffraction peaks for SPI-MMT bio-nanocom- posites suggests that the layers of MMTs have a d-spacing of at least 3.2nm**.**

Fig-2. XRD of SPI nanocomposite film



Sem

SEM images of the fracture surface (cross-sectional surface) of SPI-MMT nanocomposite films with 5% and 15% contents of Cloisite 30B are shown in **Figure 3**. The white strands in the SEM images correspond to MMT platelets. At a MMT content of 0%, and 10% MMT platelets were well dispersed in the nanocomposite films (**Figures 3(a)** and **3(c)**). This suggests exfoliation of MMT in the

nanocomposite film with MMT content of 5%. The fracture surface of the films with both Cloisite 30B became rougher as the MMT content increased to 15% (**Figures 3(b)** and **3(d)**). In agreement with the TEM results of intercalated structures, larger aggregates of Cloisite 20A were found in nanocomposite films with MMT content of 15% (**Figure 3(d)**). Based on the XRD, TEM, and SEM results, it can be concluded that extrusion of SPI and modified MMTs resulted in nanocomposites with exfoliated structures at lower MMT content (5%). At higher MMT content (15%), the structure of nanocomposites ranged from intercalated for Cloisite 20A to disordered intercalated for Cloisite 30B.

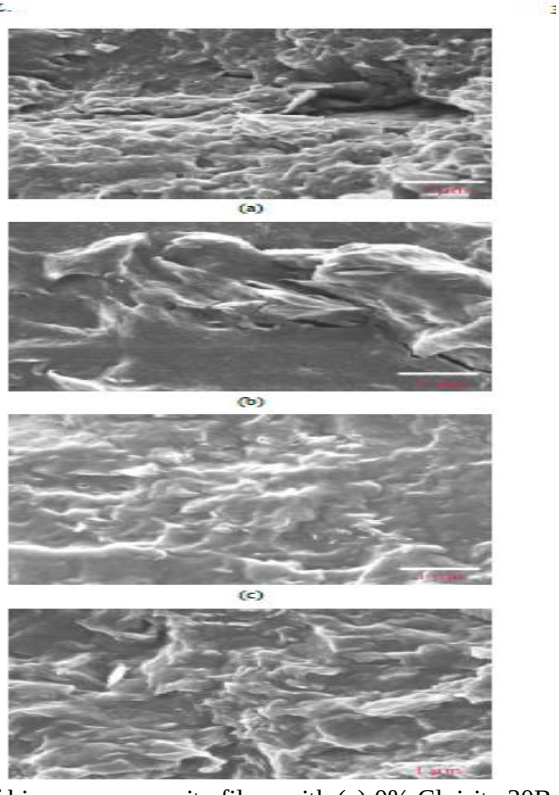


Figure- 3. SEM images of bio-nanocomposite films with (a) 0% Cloisite 30B; (b) 5% Cloisite 30B; (c) 10% Cloisite 30B; and (d) 15% Cloisite 30B.

Anti-bacterial Activities of SPI/C30B Film

A preliminary study has been carried out to compare the anti-bacterial activity of SPI/C30B film samples . The study was carried out against *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus* using the inhibition zone method. The results are shown in **Table- 1**. It was observed that film improved the anti-bacterial activity while the inhibition zone diameter for SPI/C30B film ranged between 9 and 11 mm against indicated bacteria, the in-hibition zone increased up to 17 mm (against *B. subtilis*) . Although the difference is not significant, activity of gram-positive bacteria seems to be more pronounced; increase in the inhibition zone diameter is 4 - 5 mm in gram-negative ones whereas it is 5 - 7 mm in gram-positive ones. The samples showed an increasing antibacterial activity for all of gram-negative and gram positive bacterias; a minimum of 2 mm increase was observed consistently when the sample percentage was increased . Average film weight (thickness) also effected the degree of anti-microbial activity of SPI/C30B samples and 3 - 4 mm in-crease was observed when the average film weight was increased .

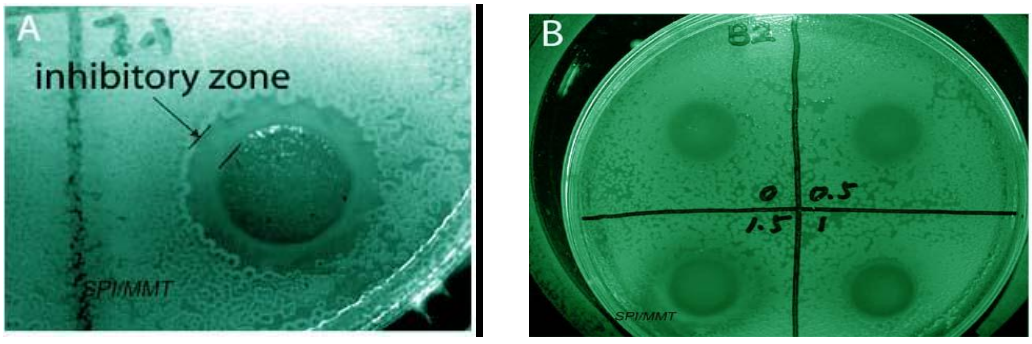


Fig-4.(A) Inhibitory zone (anti-microbial effect) of *E. coli* growth on a bacterial plate induced by a SPI/C30B film measured. (**B**) Inhibitory zones of *E. coli* growth around films with different concentrations of added solutions used to prepare the films.

Table-1. Anti-bactarial activities of SPI/C30B minimal inhibitory concentration (MIC) mg/ml

Bacteria	**MIC (mg/ml)**
Escherichia coli	18.02±1.00
Enterobacter cloacae	23.02±1.07
Enterobacter cloacae	25.00±0.97
Enterococcus faecalis	29.75±1.84
Proteus vulgaris	29.09±1.78

Anti-fungal Activity

Further the SPI/C30B film were found to be highly toxic against clinically isolated fungal species. At a concentration of 50 µl sample revealed a higher anti-fungal activity against *C. albicans*, *Candida kefyr*, *Aspergillus niger* whereas intermediated activity were showed against *C. tropicalis*, *C. krusei*, *A. flavus*, *A. fu-migatus*. The inhibitory activities of all the sample compound are reported in **Table-2**. The data results were compared with the standard antimicrobics of Ketocona-zole (30 mg) and Itraconazole (30 mg).

Table-2. Anti-fungal activities of SPI/C30B film minimal inhibitory concentration (MIC mg/ml)

Fungus	MIC (mg/ml)
C. albicans	15.22±0.04
C. tropicalis	13.20±0.00
C. krusei	17.16±0.02
C. Kefyr	11.23±0.04
A. flavus	18.22±0.31

Biodegradation Test Under Soil Environment

The biodegradability test of SPI/ furfural nanocomposites was carried out with and without organoclay using soil burial test. Figure- 4 shows the changes in weight ratio (degraded sample/initial sample) with time for SPI/ Furfural and SPI/ Furfural /organoclay nanocomposites. In the soil environment, water diffused into the polymer sample causing swelling and enhancing biodegradation. The blends containing higher percentage of organoclay degraded rapidly in the initial 8 weeks and a gradual decrease of weight occurred during the next 8 weeks.

CONCLUSION

Soya protein isolate is a non-toxic and most abundant food-based natural polymer. In our work Soya protein isolate / clay nanocomposite films were prepared and characterized. From the FTIR spectra the

different pendant groups present in the composites have been ascertained. The morphology as well as compatibility of blends has been studied using XRD. Thus suggesting that C 30B particle can be well dispersed in SPI and the fabricated membrane can be considered as homogenous and dense with no obvious phase separation has been studied using SEM. The soil burial test revealed that the nanocomposites degraded at a faster rate with increasing organoclay content. Among the SPI/C30B films studied, all Soya protein isolate films exhibited prominent inhibitory effect on all 7 pathogenic bacteria. All SPI/C30B films showed distinctive inhibition zones against all pathogenic bacteria tested and the inhibition zones were considerably thicker than those produced by Soya protein isolate formate films. Also, the inhibitory effects of Soya protein isolate citrate films were remarkably higher for *Staphylococcus aureus* and *Vibrio vulnificus* as indicated by thicker inhibition zones accounting for more than 4 mm. The Soya protein isolate films were the only films with antimicrobial effects against *Bacillus cereus*, and *Vibrio vulnificus*. The higher inhibitory activity shown by all Soya protein isolate films can be attributable to complete solubility which could make them more reactive against bacterial cells.

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