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Exploring 2,2'- Bipyridine as an Additive for Soy Protein Isolate Biopolymeric Films

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Abstract: **2,2'-bipyridine (BIPY) (1% w/w with respect to soy protein isolate (SPI)) was incorporated in SPI to prepare BIPY reinforced SPI films. Prepared film was characterized structurally by FTIR spectrophotometer, which indicates shifting of –C=O band from 1641 cm^{-1} to 1632 cm^{-1} wavenumbers, indicating towards successful incorporation of BIPY in SPI matrix. Interestingly, the water resistance of BIPY incorporated SPI film was increased by four folds as compared to neat SPI films. However, the antibacterial properties of BIPY incorporated SPI films were negligible despite the reported antibacterial properties of BIPY. Docking studies were performed for SPI in order to evaluate their binding affinity with BIPY. After docking, the ligands in form of BIPY were evaluated according to their binding affinity and the interaction pattern between SPI and BIPY. The docking results indicated the absence of hydrogen bonding between SPI and BIPY. It has been reported in the literatures that BIPY also exerts some health-related issue due to which extra precaution is needed to prepare BIPY incorporated SPI films.**

Index Terms: **2,2'-bipyridine, Dimensional stability, FTIR, Soy protein isolate, Water uptake.**

I. Introduction

Soy protein can be one the best material to be fabricated as the suitable ecofriendly alternative to commodity plastic material and is currently explored by many groups of researchers (Song *et al.*, 2011). Soy protein isolates (SPI) with 90-92% protein, derived from soy protein powder, are explored for variety of applications, such as, edible films, antimicrobial packaging films etc. (Eswaranandam *et al.*, 2006; Perez-Perez *et al.*, 2004). SPI shows some major qualities of polymeric films, along with some limitations. One limitation of SPI based film is low water resistance, that can be increased by adding hydrophilic/hydrophobic plasticizers (Liu *et al.*, 2006) or by fabricating benzilic acid or salicylic acid incorporated SPI film (Bai *et al.*, 2010; Kumar and Zhang, 2008a). Another limitation is that the mechanical properties of SPI based films also deteriorate at high humidity. However, the incorporation of aromatic acids in SPI eventually improves the water resistance and mechanical properties of SPI films as compared to control film (Bai *et al.*, 2010; Kumar and Zhang, 2008a; Kumar *et al.*, 2009). Aromatic acids such as benzilic acid and salicylic acid also improve the water resistance of modified SPI film by creating a lotus-like effect (Bai *et al.*, 2010; Kumar and Zhang, 2008a). Recently, Kumar *et al.*, in 2020 have studied the effect of incorporation of mandelic acid, nalidixic acid and benzilic acid in SPI based bioplastics. They have shown that the incorporation of all the three acids improve the mechanical properties and water resistance of the SPI film but mandelic acid and benzilic acid do not give any zone of inhibition when subjected to antibacterial studies (Kumar and Zhang, 2008a; Kumar *et al.*, 2016) unlike nalidixic acid incorporated SPI films (Kumar *et al.*, 2020).

2,2'-bipyridine or birpyridyl (BIPY) is an aromatic compound which contain N- containing heterocyclic groups. It acts as a strong ligand which exhibits antimicrobial properties. BIPY is known for its chelating abilities, and can coordinate several metal ions (Agwara *et al.*, 2010; Kae *et al.*, 2000; Kumar *et al.*, 2008b; Obaleye *et al.*, 2018; Osowole *et al.*, 2008). In this research paper, we have focused on the potential of BIPY as a potential additive for SPI to create hydrophobic BIPY incorporated antibacterial SPI films. Homology modeling and docking studies was used to find out the patterns of interaction between SPI receptors and BIPY as a ligand. To perform the experiment, we have taken the 43 amino acid sequence of soy protein from the literature (Odani *et al.*, 1987). Based on the

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sequence reported by Odani *et al.*, we have tried the 3D modeling of SPI with BIPY. The basis of this study is that aromatic structure of BIPY can interact well with SPI and can give the hydrophobic films.

II. Materials and Methods

A. Materials

Soy protein isolate with a protein concentration of 90.27 % (on dry basis) was procured from Zhenghou Ruikang Enterprise Co., Ltd. (Zhengzhou, China). 2,2'-bipyridine or bipyridyl was purchased from Sigma. Glycerol and sodium hydroxide pellets were procured from Fisher Scientific and Titan Biotech Ltd., respectively. *E. Coli /*BL21strain was purchased from Gbiosciences. IMTECH Chandigarh, India provided the *L. monocytogenes* MTCC 839. Luria Bertani (LB) powder was procured from HiMedia.

B. Preparation of Neat and 2,2'-Bipyridine Incorporated SPI Film

For this study, 6% SPI film was used to prepare neat film or control film. Firstly, we have prepared neat SPI film by adding 3 g of SPI in 50 ml of distilled water at 60^{o}C in stirring condition. pH of the SPI suspension was maintained between 9-9.5. Prior to preparation of SPI suspension, 0.9 g of glycerol (30 % w/w with respect to SPI) was added as plasticizer. The SPI suspension was stirred at 60^{o}C for 60 min. Afterwards solution was transferred in vacuum desiccator for the removal of any trapped air bubble. The SPI suspension was poured on silicon coated glass plates with a dimension of 15cm x 10cm after removal of air bubbles and dried at 55-60^{o}C for 24 h. Further the dried films were kept in a desiccator having an RH of 75%, which conditions film for peeling off from the glass plate.

BIPY incorporated SPI film was prepared using above mentioned method with certain modifications. For the preparation of 1% BIPY (w/w of SPI) incorporated film, 30 mg of BIPY was added to the stirring SPI suspension after 30 min from the starting of above mentioned method. Rest of the procedure was same as adopted in the preparation of neat film. BIPY incorporated SPI film is designated as SPI-BIPY.

C. Antimicrobial Studies

For antibacterial studies *E. coli* (Gram negative bacteria) and *L. monocytogenes* (Gram positive bacteria) was used. Both the bacteria was revived in 10 ml Luria Bertani (LB) broth, respectively and incubated overnight at shaker incubator. After that optical density (OD) for both the culture was maintained at 0.4 by dilution. From each culture, 10 µl ($3.2x10^{4}$) bacteria were spread on LB agar plate, respectively. Afterwards neat and BIPY incorporated SPI films (1cm x 1cm) were placed on each plate, respectively. Experiment was done in triplets. Plates were incubated at 37^{o}C in static incubator overnight and observed afterwards.

D. In-Silico Analysis of SPI with 2,2'-Bipyridine

For this study, the sequence of soy protein containing 43 amino acids was taken as reported in the literature (Odani *et al.*, 1987). The polypeptide chain is composite of **SKWQHQQNSCRKQLQGVNLTPCEKHIMEKIQGRGDD DDDDDDD** sequence of amino acids. The 3-D (three dimensional) structure of soy protein is an essential element for the *In-Silico* interaction analysis. Therefore we employed a homology modelling approach by using the Modeller 9.21 software (https://salilab.org/modeller/9.21/release.html) to predict the five structure models of the soy protein. The templates were selected with the help of the BlastP (Protein Blast tool) of NCBI (https://www.ncbi.nlm.nih.gov). There were four templates which indicated the best PDB (protein data bank) structure based on identity threshold value (PDB id: 1W2Q, 3OB4, 2CDN, and 1P4S). With these templates, five models were generated and among those the fifth model generated was used to analyse the *In-silico* interaction analysis. The selected model was analysed earlier on the basis of allowed region through RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php), which is the software used to generate a Ramachandran plot. Among the generated models, the fifth model was selected as it contains 95 % residues in allowed region of the Ramachandran plot. We have followed the same methodology as mentioned in our earlier research paper to analyze the *In-silico* interaction analysis (Kumar *et al.*, 2020). Active sites for the docking analysis were further predicted by the CASTp (Computed Atlas of Surface Topography of proteins) (http://sts.bioe.uic.edu/castp/index.html?1bxw) online server. CASTp is based on recent theoretical and algorithmic results of computational geometry. The two pockets were analysed for *In-silico* interaction using AutoDock Vina tools for 2,2'-bipyridine. The ligand with pockets were analysed for polar interaction between receptor soy protein and ligand with the help of Ligplot v.4.5.3 software (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

E. Characterizations

FTIR study was performed using FTIR spectrophotometer from Perkin-Elmer, USA at IIT Patna, India. Neat and BIPY incorporated SPI films were scanned under infrared spectroscopy, from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} at room temperature. All spectra were reported after an average of 32 scans.

BIPY incorporated SPI samples for transmittance study was prepared by cutting the films in a dimension of 4 cm x 1 cm. Prepared samples were scanned using UV–Visible spectrophotometer (Motras Scientific) at different wavelengths between 400 nm to 700 nm.

The water uptake of prepared BIPY incorporated SPI films were tested by following ASTM D570-81. Sample films were cut in a dimension of 1 cm x 1 cm and preconditioned at 60°C for 24 h followed by cooling in 0% RH desiccator for 30 min. Preconditioned samples were weighed (W_0) and immersed into 10 ml distilled water for 24h. Afterwards the samples were picked out and the surface water was removed out with tissue paper and the final weight (W_1) was taken. Experiment was performed in triplets and the average difference of initial and final weight was recorded as the value of water uptake. The percentage water uptake is given by formula given below.

$$Water\ Uptake(\%) = \frac{W_1 - W_0}{W_0} \text{ x } 100$$

III. RESULTS AND DISCUSSION

A. Visual Inspection and Transmittance Studies

Fig. 1 shows the visual appearance of as prepared SPI-BIPY films. From visual observation, it appears that the BIPY powder which is white crystal, when dissolved in SPI suspension becomes pink in colour. The SPI-BIPY film prepared from the BIPY incorporated SPI suspension also appeared pink and the film lacked dimensional stability. However, over the period of time, the SPI-BIPY film becomes pale yellow in color. It was also not possible to increase the concentration of BIPY, as the film tends to break in multiple pieces at increased concentration of BIPY. This result indicates towards the possible incompatibility of BIPY with SPI above 1% of BIPY.

Fig. 1. Visual appearance of as prepared BIPY incorporated SPI films

Table 1 shows the transmittance data of SPI – BIPY films. Data depicts that incorporation of BIPY in SPI films, decreases the transmittance of as-prepared films with respect to neat SPI film at 400 nm, 500 nm and 700 nm. This decrease can due to the presence of BIPY in SPI-BIPY films.

Table 1. Optical transmittance of neat and BIPY incorporated SPI film at 400nm and 700nm

Sample designation	Transmittance (%) at wavelength		
	400 nm	500 nm	700 nm
SPI	7.099	22.68	26.36
SPI-BIPY	5.049	12.726	22.83

B. FTIR Spectra

Fig. 2 illustrates the FTIR spectra of neat and BIPY incorporated SPI films. Neat SPI film shows a broad N–H stretching band between 3200 and 3400 cm^{-1} attributed to amide A band, BIPY incorporated film also shows these bands. The peaks at 1539 cm^{-1} resulted due to N-H bending which is attributed to amide II bands of protein. –C=O band in SPI film is represented at 1641 cm^{-1}. In BIPY incorporated SPI film, –C=O band shows a slight shift from 1641 cm^{-1} to 1632 cm^{-1}, it can be due to ring structure carbonyl stretching upon incorporation of BIPY, which also indicates towards successful incorporation of BIPY in SPI film.

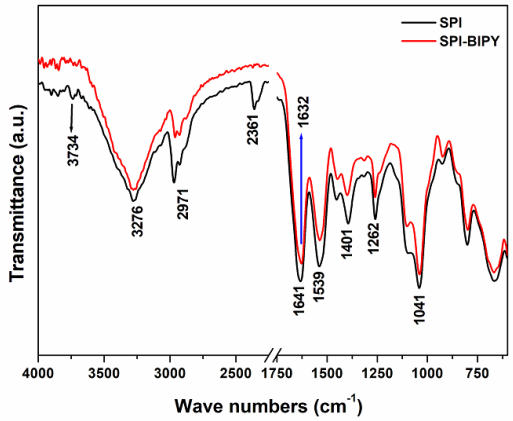

Fig. 2. FTIR spectra of neat and BIPY incorporated SPI film

C. Water Uptake of Neat and BIPY Incorporated SPI Films

Fig. 3 demonstrates the water uptake properties of neat and BIPY incorporated SPI film. Data shows that the incorporation of BIPY increased the water sensitivity of SPI film by almost 4 folds when compared to the neat SPI film. That means the hydrophobicity of SPI film is increased upon incorporation of BIPY like mandelic acid and benzilic acid incorporated SPI film (Kumar and Zhang, 2008a; Kumar *et al.*, 2016).

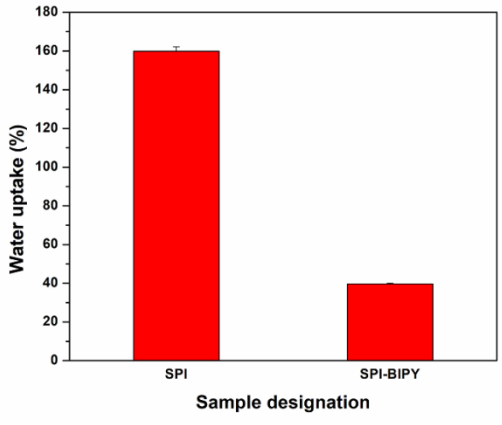

Fig. 3. Water uptake properties of BIPY incorporated SPI films

D. Interaction between 2,2'-Bipyridine and SPI

Fig. 4 shows the interaction pattern between 2,2'-bipyridine and SPI. In both pocket i.e., pocket 1 and pocket 2 we could see the absence of hydrogen bonding. However, there is some sort of interaction in form of co-ordinate bond and hydrophobic interaction as evident from Fig. 4. The low value of binding affinity confirms the above said interaction between 2,2'-bipyridine and SPI.

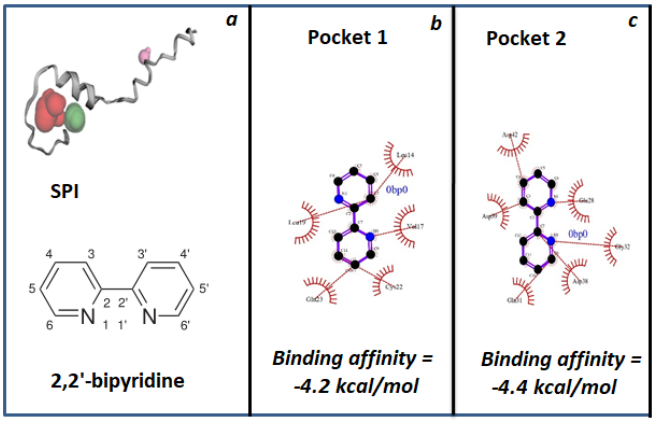

Fig. 4. 3D structure of soy protein with best three pockets as predicted by CASTp Server. Here red and green indicate pocket 1 and pocket-2, respectively (a). 2D diagram showing the types of contacts formed between 2,2'-bipyridine and soy protein in the two pockets along with binding affinity (b)

E. Antimicrobial Properties of Neat and BIPY Incorporated SPI Films

Fig. 5 shows the effect of neat and BIPY incorporated SPI films against *E. coli* and *L. monocytogenes*, respectively. From the images it is evident that BIPY doesn't show any zone of inhibition for antibacterial properties against both the bacteria. However, there is inhibition of growth in BIPY incorporated SPI films. The SPI film is completely covered by the growth of *L. monocytogenes*, on the other hand on BIPY-SPI film there is negligible growth of *L. monocytogenes* unlike *E. coli*.

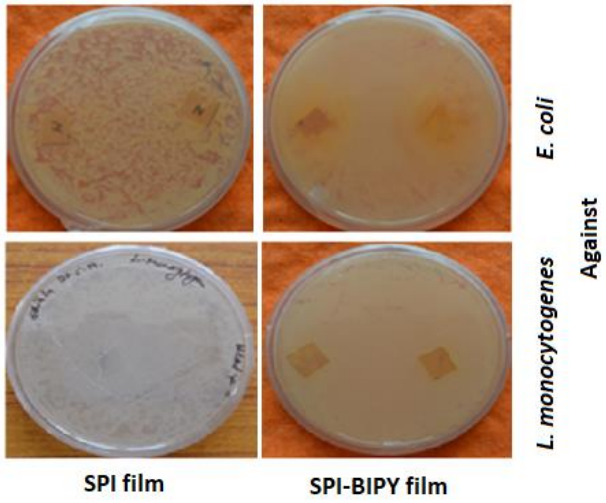

Fig. 5. Antimicrobial properties against neat (left) and BIPY incorporated SPI (right) film against *E. coli* and *L. monocytogenes*

IV. Conclusions

BIPY incorporated SPI film were prepared at 1% concentration of BIPY. It was observed that the prepared film loses its color and dimensional stability over time. And it was also not possible to increase the concentration of BIPY as the films surface was cracking. Though the BIPY reinforced SPI film showed high water resistance but it was also causing some health-related hazards during preparation of SPI-BIPY films. The results from molecular docking indicate the lower binding energy of 2,2'-bipyridine with SPI with some sort of interaction in form of co-ordinate bond and hydrophobic interaction. However, SPI-BIPY films showed better antibacterial properties when compared to neat SPI film which showed negligible antibacterial properties. So we may conclude that, the work relating to incorporation of BIPY in SPI films needs high attention so as to prepare BIPY incorporated SPI film showed high water resistance and good flexibility with utmost care due to toxic nature of BIPY.

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