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Soy Protein Isolate Film by Incorporating Mandelic Acid as Well as Through Fermentation Mediated by *Bacillus Subtilis*

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Abstract: Soy protein isolate (SPI) biopolymeric films were prepared by adding different contents of mandelic acid (1 to 5% wrt SPI) to glycerol plasticized SPI by solution casting method. Also, SPI was fermented by *Bacillus subtilis* to get fermented SPI films by solution casting. Molecular mass determination of mandelic acid incorporated and fermented SPI films was carried out by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Mandelic acid incorporated and fermented SPI films were characterized by Fourier-transform infrared spectroscopy (FT-IR), dynamic mechanical analysis (DMA), tensile strength, water uptake and optical transmittance studies. Results indicated that incorporation of mandelic acid in SPI resulted in high tensile strength (8.03 MPa) and high α-relaxation (T_α) as well as low water uptake. On the other hand, films cannot be prepared from fermented SPI with SPI contents of 8% and 12%. However, film from fermented SPI with 16% SPI content could be prepared but it exhibited low tensile strength (3.18 MPa) and low T_α as well as high water uptake. The resulting mandelic acid incorporated SPI films were also subjected to antimicrobial studies. At all the concentration of mandelic acid, we can easily observe the antimicrobial effect in mandelic acid incorporated SPI films unlike fermented SPI films. This work will be helpful in fabricating antimicrobial SPI film from renewable resources.

Keywords: Soy protein isolate; mandelic acid; fermentation; film; tensile properties; antimicrobial properties

1 Introduction

Soy protein isolate (SPI), obtained from the renewable resources, is widely explored as environmentally safe industrial products for packaging applications and as commodity plastics. Basically, SPI is a waste product of soy oil industry and biodegradable SPI films could be used as commodity bioplastics as well as environment-friendly packaging materials [1]. Biopolymeric and edible SPI films with several acid additives such as citric acid, malic acid, lactic acid, tartaric acid [2], mandelic acid [3], and adipic acid [4] have been prepared by solution casting or compression molding methods. Ferulic acid, a phenolic acid, present in plants has been reported to be used as crosslinking agent in preparation of SPI and gelatin based edible films [5,6]. Other phenolic compounds have also been used as crosslinking agents in SPI films such as rutin, epicatechin, caffeic acid or gallic acid [7,8]. Recently, Alves et al. assessed the potential of ferulic acid (1.0 g l^{-1} and 4.0 g l^{-1}) in soy protein-based edible coating formulations with an aim to increase the quality and shelf life of fresh-cut apples [9]. Ferulic acid incorporated SPI films demonstrated antimicrobial properties. The mechanical and other important properties of the fabricated SPI films can be altered by the addition of different acids (synthetic or natural), antimicrobial compounds and method adopted for the fabrication of film [3,10].

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Bacteriocins are also the bacteria-originated antibacterial proteins which inhibit the growth of other bacteria. SPI films can be prepared by adding bacteriocin or it can be generated *in-situ* via fermentation process [10-13]. Nisin, a type of bacteriocin, is produced by *Lactococcuslactis* fermentation and is recognized as a safe preservative so it can be added in certain food products [14]. Kim et al. conducted experiments to economically develop an antimicrobial edible film from defatted soybean meal by inoculating with bacteriocin-like substance (BLS)-producing bacteria and to verify whether the film could be used as a packaging material in the food industry [13]. It has been reported in the literatures that the presence of cross-linking agent or antimicrobial agent or acids as an antimicrobial agents can effect SPI films strength and/or toughness, water resistance and other physical properties [15]. During fermentation, there are chances of formation of low molecular mass protein fractions in SPI by microorganisms which may affect film forming ability of SPI and also the physical properties of the prepared fermented films [13].

Mandelic acid is an alpha hydroxy acid with one aromatic ring and can be excreted in the urine if ingested orally. Recently, mandelic acid has been explored as an additive for soy protein films prepared by compression molding technique [3]. This study attempts to incorporate mandelic acid (with inherent antibacterial nature) into SPI films, obtained from agricultural resources, with an objective to prepare bactericidal bio-films by solution casting method. Also, we have tried to ferment SPI by *Bacillus subtilis* and subsequently prepare SPI film by solution casting method. Mandelic acid incorporated and fermented SPI films were characterized by mechanical, thermomechanical, transmittance and water uptake studies. Molecular mass of mandelic acid incorporated SPI and fermented SPI were determined by SDS-PAGE. Finally, the antibacterial properties of the as-prepared SPI films were determined.

2 Materials and Methods

2.1 Materials

Soy protein isolate (SPI) containing 90.27% of protein on dry basis was purchased from Zhenghou Ruikang Enterprise Co., Ltd (Zhengzhou, China) and was used as a source to prepare SPI films. Mandelic acid (mp: 119-121^{o}C, mol. wt: 152.15) was purchased from Sigma-Aldrich and used as received. Glycerol (Gy) (bp: 181-182^{o}C, mol. wt: 92.01, and density: 1.182 g cm^{-3}) was purchased from Fisher Scientific. Sodium chloride and sodium hydroxide pellets were purchased from Titan Biotech Ltd. KH_2PO_4, K_2HPO_4, and NH_4Cl were purchased from Merck, Fisher Scientific and LobaChemie, respectively. *Bacillus subtilis* was procured from CSIR, Institute of Microbial Technology, Chandigarh, India (MTCC Code 441). *E. Coli/*BL21strain was procured from Gbiosciences. Luria Bertani (LB) powder was purchased from HiMedia.

2.2 Preparation of Soy Protein Isolate Film

In this method, 8g of SPI was added slowly in 100 ml of distilled water. The pH of the resulting SPI suspension was adjusted between 9.5 to 10.0 by adding required amount of 1N NaOH. After that glycerol (30% w/w with respect to SPI) was added to the SPI suspension and stirred continuously at 60^{o}C for about 1h. The resulting SPI suspension was kept for 3-4 hours in a vacuum desiccator so as to remove the air bubbles. The SPI suspension was then poured on the glass plates of 15 cm × 10 cm dimensions and kept at 50-60^{o}C for 24h. The thickness of the edge for casting the SPI suspension was 0.5 cm. After the designated time, the dried SPI film was kept at a RH of 75% (maintained by using saturated salt solution of NaCl) for 12h at 25 ± 1^{o}C and subsequently it was peeled off from the glass plate. The thickness of the peeled off SPI films was 0.25 ± 0.03 mm. The function of the saturated salt solution was to assist the SPI film to absorb moisture so that the film could be easily peeled off from glass plate.

2.3 Preparation of Mandelic Acid Incorporated SPI Films

The SPI suspension with 30% glycerol (30% w/w with respect to SPI) was prepared according to the method as discussed above. Mandelic acid (0-5% with respect to SPI) was added to the as-prepared SPI

suspension. For adding mandelic acid in SPI, mandelic acid was first dissolved in 5 ml distilled water in microwave oven and then added to the SPI suspension. After the addition of mandelic acid, the pH of the SPI suspension decreased due to acidic nature of the additive (Tab. 1). Final pH of the mandelic acid incorporated SPI suspension was maintained around 10.5 by the addition of 1N NaOH. The resulting mandelic acid incorporated SPI suspension was stirred continuously at 60^{o}C for about 30 minutes. Again, the resulting suspension was kept for 3-4 hours in a vacuum desiccator to remove the air bubbles. The suspension was then poured on the glass plates of 15 cm × 10 cm dimensions and kept at 50-60^{o}C for 24 hours. The thickness of the edge for casting the SPI suspension was 0.5 cm. After the designated time, the dried film was kept at a RH of 75% and subsequently it was peeled off from the glass plate. The thickness of the prepared SPI films was 0.25 ± 0.03 mm. The resulting mandelic acid incorporated SPI films were designated as S-0M, S-1M, S-2M, S-3M and S-4M, and S-5M where the numeral values with M denotes the percentage (w/w) of mandelic acid in SPI (Tab. 2). The scaling-up of this method could be easily done by taking glass plates of larger dimensions with higher amount of SPI suspension.

Table 1: pH of the SPI suspension after incorporating mandelic acid

Mandelic Acid (M)	Initial pH of SPI Suspension	pH after addition of Mandelic Acid in SPI	Final pH adjusted after adding 1N NaOH
S-0M	10.5	NA	NA
S-1M	10.5	7.7	10.5
S-2M	10.5	7.4	10.5
S-3M	10.5	7.4	10.5
S-4M	10.5	6.4	10.5
S-5M	10.5	3.02	10.5

Table 2: Designation and amount of additives in mandelic acid and fermented SPI films

Sample Designation	SPI (g)	Water (ml)	Glycerol (g) (30% wrt SPI)	Mandelic Acid (g) (wrt SPI)
S-0M	8	100	2.4	0
S-1M	8	100	2.4	0.08
S-2M	8	100	2.4	0.16
S-3M	8	100	2.4	0.24
S-4M	8	100	2.4	0.32
S-5M	8	100	2.4	0.40
S-8NF	8	100	1.2	0
S-8F	8	100	1.2	0
S-12F	12	100	1.8	0
S-16F	16	100	2.4	0

*1M to 5M represent weight of mandelic acid wrt SPI; NF and F represent non-fermented and fermented SPI, respectively.

2.4 Fermentation of SPI by Bacillus Subtilis

For the fermentation of SPI, the fermentation media was prepared containing SPI, KH_2PO_4 (500 mg/l), K_2HPO_4 (1000 mg/l) and NH_4Cl (500 mg/l) in 50 ml distilled water. The concentration of SPI in the fermentation media was varied from 8% to 12% and finally to 16% [13]. The fermentation media were

autoclaved at 121°C for 20 minutes. After that the media were inoculated with *Bacillus subtilis* culture (0.5 O.D) and incubated at 37°C for 33 hours. Autoclaved SPI suspension with 8% SPI powder was taken as control.

2.5 Preparation of Fermented SPI Film

The fermented SPI suspension containing 8%, 12% and 16% SPI were centrifuged at 5000 rpm for 5 minutes in order to remove the cell mass from the SPI suspension. To the supernatant, 15% glycerol (w/w with respect to SPI) was added to it with pH being adjusted to 10.5 by 1N NaOH. The suspension was then poured on the glass plates of 15 cm × 10 cm dimensions and kept at 50-60°C for 24 hours. The thickness of the edge for casting the SPI suspension was 0.5 cm. After the designated time, the dried film was kept at a RH of 75% and subsequently it was peeled off from the glass plate. The thickness of the peeled off SPI films was 0.25 ± 0.03 mm. As discussed in Section 2.2, the function of the saturated salt solution was to assist the SPI film to absorb moisture so that the film can be easily peeled off from glass plate. The resulting fermented SPI films were designated as S-8F, S-12F, S-16F where the numeral denotes the percentage of SPI in water and F denotes the fermented SPI (Tab. 2). The same procedure was also performed for the casting of non-fermented SPI film with 8% SPI content in autoclaved SPI suspension and it is designated as S-8NF, here NF denotes non-fermented SPI film.

2.6 Molecular Mass Determination by SDS-PAGE

Molecular mass of SPI, mandelic acid incorporated SPI and fermented SPI was determined by using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Protein samples (10 μg μl^{-1}) were prepared in a buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 5% 2-mercaptoethanol and 0.25% bromophenol blue. Standard markers having molecular mass of 3.5kDa (insulin), 6.5 kDa (aprotinin), 14.3 kDa (lysozyme), 20.1kDa (soybean trypsin inhibitor), 29 kDa (carbonic anhydrase), 43 kDa (ovalbumin), 66 kDa (bovine serum albumin, BSA), 97.4 kDa (phosphorylase) and 205 kDa (myosin) were used for comparing the molecular mass of protein samples. Amount between 10μl to 40 μl of each protein samples was applied in the wells of the stacking gel. 5% stacking gel (pH 6.8) and 8% separating gel (pH 8.8) were used. The gels were stained with 0.25% coomassie blue, R-250 after the completion of electrophoresis and destained until the background stain was removed.

2.7 Antimicrobial Studies

Approximately, one million *E. coli* cells (0.01 OD at 600 nm) were put into molten LB agar. It was then allowed to solidify at room temperature to get plate with uniform *E. coli* cells. Mandelic acid incorporated SPI films as well as fermented SPI films were placed on the LB plates with uniform *E. coli* cells. The LB agar plate was then incubated at 37°C for overnight and the growth of bacteria in LB agar plates containing neat SPI film as well as mandelic acid incorporated SPI films and fermented SPI films were analyzed.

2.8 Characterizations

The Fourier transform infrared (FT-IR) spectroscopy of the neat, mandelic acid incorporated and fermented SPI films were recorded with FT-IR spectrophotometer from Perkin-Elmer, USA at IIT Patna, India. The FT-IR spectra of the solid film samples were taken at room temperature. The samples were scanned from 4000 to 400cm^{-1} with a resolution of 4 cm^{-1}. All spectra were reported after an average of 32 scans.

The tensile tests were carried according to ASTMD 882 on a Universal Tensile Testing machine from Zwick, Germany at IIT Patna, Bihar, India. The tensile specimens with dimension of 80 mm × 10 mm with thickness of around 0.25 ± 0.03 mm were cut from samples prepared by solution casting. The tensile strength and elongation at break values of the neat, mandelic acid incorporated and fermented SPI films

were carried out and the tensile tests were carried at a cross head speed of 10 mm/min. Five test samples from each formulation were tested and the average values were reported.

Dynamic mechanical analysis (DMA) was performed on a dynamic mechanical analyser (DMA8000, PerkinElmer, Buckinghamshire, UK) with dual cantilever at a frequency of 1 Hz. Neat and modified SPI films with dimensions 50 mm × 10 mm (length × width) were used for testing at the temperature ranging from -50 ^{o}C to 200^{o}C. The heating rate was maintained at 2 ^{o}Cmin^{-1}. The relaxation temperature, α_r, was determined as the peak value of the loss angle tangent (tan δ).

The optical transmittances of the SPI films with and without mandelic acid as well as fermented SPI films were measured with an ultraviolet–visible spectrophotometer (UV-160A, Shimadzu). For the transmittance studies, the SPI films were cut into small stripes of 4cm length and 1cm breadth. The strips were kept in glass cuvette and their transmittance was taken at varying wavelength ranging from 400 nm to 800 nm.

The water uptake properties of SPI films with and without mandelic acid as well as fermented SPI films were determined according to ASTM D570-81. The films were cut in small square pieces (1 cm × 1 cm) and preconditioned at 60^{o}C in hot air oven for 24 hours. After that it was cooled in desiccators at 0% RH and weighed (W_0). The film pieces were then immersed in distilled water for 24 hours. After that it was taken out and dried with tissue paper so as to remove the excess water from the surface of the wet film and then weighed (W_1).

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

3 Results and Discussion

3.1 Visual Inspection of Mandelic Acid Incorporated SPI Films

Fig. 1 shows the photograph of mandelic acid incorporated SPI films as well as fermented SPI films with an average thickness of 0.25 ± 0.03 mm. The photograph was taken with 10 megapixel camera. SPI film incorporated with mandelic acid showed the change in colour from yellow to brown [8,16]. We could not prepare the autoclaved and fermented SPI film at 30% plasticizer content unlike that of SPI films incorporated with mandelic acid, so we have to decrease the contents of plasticizer in case of autoclaved and fermented SPI films. After optimising the contents of plasticizer, we could prepare the autoclaved and fermented films at 15% plasticizer content having dark brown colour. Also fermented SPI film could not be prepared at 8% and 12% SPI unlike 16% SPI and this may be attributed to the significant reduction in the high molecular mass protein fractions as observed from SDS-PAGE (discussed in the later section). From visual inspection, it was evident that mandelic acid incorporated SPI film exhibited homogeneous surface whereas autoclaved and fermented films clearly showed non-uniformity in the surface.

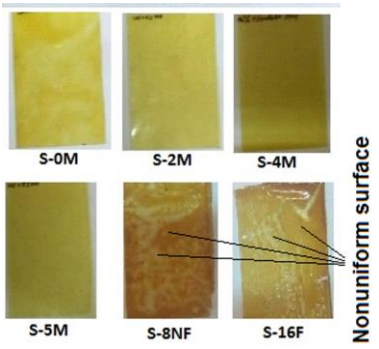

Figure 1: Photograph of mandelic acid incorporated as well as fermented SPI films

3.2 Growth Curve of SPI by Bacillus Subtilis

Fig. 2 shows the growth curve of *B. subtilis* in 8%, 12% and 16% SPI. It has been observed that with the increase in the concentration of SPI from 8% to 16%, viscosity of the SPI suspension increased. The viscosity of SPI suspension was indirectly correlated with the flowability nature of the SPI suspension. Till 12% of SPI, we could observe the flowability character of SPI suspension but at 16% the flowability character of SPI suspension could be observed only after 24 hours of incubation. The lag phase as observed from Fig. 2 was 0-6 hours and 0-5 hours for S-8F and S-12F, respectively. After lag phase, there was exponential phase for both S-8F and S-12F followed by death phase. We have observed another stationary phase from 24 -30 hours. Kim et al. also observed second stationary phases but at higher incubation time i.e., from 36-42 hours [13]. They observed fast bacterial growth after 24 hours of incubation but in our study we have observed fast bacterial growth after 5 hours which may be attributed to the use of SPI (having 90-92% protein content) instead of defatted soybean meal (having 48-50% protein content) as well as very high concentration (20%) of defatted soy bean meal.

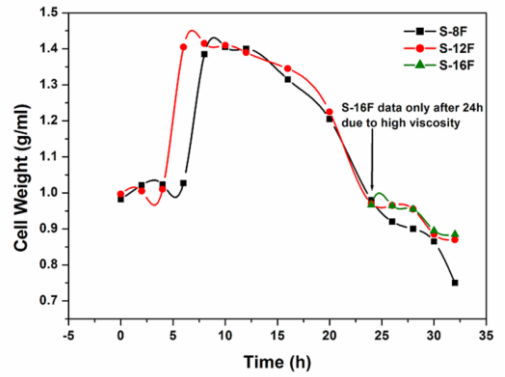

Figure 2: Growth curve of *B. subtilis* in 8, 12 and 16 % SPI

3.3 Molecular Mass Determination of Neat, Mandelic Acid Incorporated and Fermented SPI Films by SDS-PAGE

An analysis of total protein from SPI showed mixture of high and low molecular mass proteins shown in the form of a continuous band in the gel. However, two distinct molecular mass fractions of 34-38 kDa and 68 kDa were resolved, which signified that the protein fractions of these molecular masses were predominant in soy protein (Fig. 3, all lanes) [17,18]. The band of very high intensity at 34-38 kDa represented the acidic subunit of 11S-RG protein. α, α1 and β subunit of 7S-RG were denoted by 80 kDa, 68 kDa and 48 kDa molecular mass bands, respectively. There were no differences in the molecular mass bands of mandelic acid incorporated SPI and neat SPI [8].

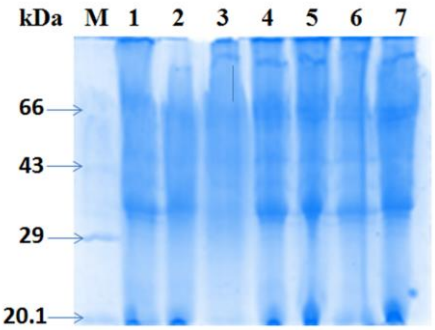

Figure 3: SDS-PAGE of mandelic acid incorporated SPI films. (Showing lane 1,10 µl of S-0M; lane 2,10 µl of S-1M; lane 3,blank; lane 4,10 µl of S-2M; lane 5,10 µl of S-3M; lane 6,10µl of S-4M; lane 7,10 µl of S-5M, lane M, Marker)

On the other hand, all the major bands of SPI, except 34-38 kDa, disappeared in the fermented soy protein unlike as reported by Kim et al. (Fig. 4) [13]. SDS-PAGE images of the fermented films at all the contents (10 µl to 40µl) showed drastic reduction in the molecular mass of 8% and 12% fermented SPI. Similarly, SDS-PAGE analysis of 8% autoclaved SPI film showed the absence of all significant molecular mass bands except that of 48kDa. However, there was appearance of band at ~13 kDa which signified the formation of bacteriocin as reported by Park et al. and Kim et al. [12,13].

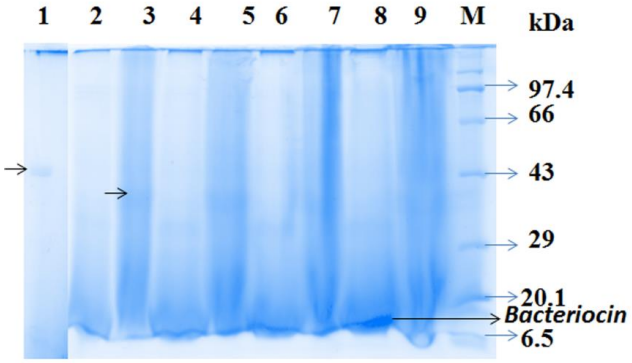

Figure 4: 12.5 % SDS-PAGE showing the band of bacteriocin in fermented SPI films (Showing lane 1, 10 µl of S-8NF; lane 2,10 µl of S-8F; lane 3, 10 µl of S-16F; lane 4, 10 µl of S-12F; lane 5, 20 µl of S-16F; lane 6,20 µl of S-8F; lane 7, 30 µl of S-16F; lane 8, 20 µl of S-12F; lane 9, 40 µl of S-16F, lane M, Marker)

3.4 FT-IR and Water Uptake of Neat, Mandelic Acid Incorporated and Fermented SPI Films

Fig. 5 shows the FT-IR spectra of the mandelic acid incorporated SPI films. A broad N-H stretching and O-H stretching band was observed between 3200 cm^{-1} and 3400 cm^{-1} in SPI films in absence of mandelic acid (S-0M). This peak was assigned to amide A of soy protein films [19]. The amide II and amide III bands of soy protein were observed at 1538 and 1260cm^{-1}, respectively [20]. The carbonyl band (C = O) in soy protein was represented at1631 cm^{-1}. With the increase in the concentration of mandelic acid, the intensity of all the prominent peaks decreased except that of peak at 699 cm^{-1} which was assigned to the aromatic group [3]. With the increase in the concentration of mandelic acid, number of aromatic groups in SPI increased, hence the intensity of the said peak increased. There was narrowing of N-H stretching band and O-H stretching band between 3200 $cm^{-1}$and 3400 cm^{-1} which indicated the presence of less amino and hydroxyl groups for hydrogen bonding. This signified that the water uptake of the mandelic acid incorporated SPI film should decrease as it has been discussed in the next to next paragraph.

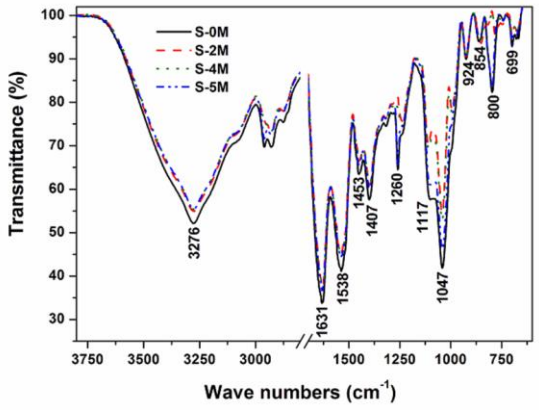

Figure 5: FT-IR spectrum of the mandelic acid incorporated SPI films

Fig. 6 shows the FT-IR spectra of the fermented soy protein films. In the native SPI films (S-0M), the same peaks were observed as discussed in previous paragraph. In the autoclaved and fermented soy protein all the prominent peaks decreased except that of peak between 3200 cm^{-1} and 3400 cm^{-1}. There was decrease in intensity as well as narrowing of band at 3276 cm^{-1} in autoclaved SPI films unlike fermented SPI films. This signified that the water uptake of the autoclaved SPI film should decrease and water uptake of the fermented SPI film should increase and same results were observed from water uptake studies (discussed in the next paragraph). There was broadening of -N-H bending band between 3200 cm^{-1} and 3400 $cm^{-1}$for fermented soy protein which indicated the availability of more amino and hydroxyl groups for hydrogen bonding [19]. There was also the appearance of a band at 854 cm^{-1} which was assigned to -CH_2 bending of bacteriocin formed after fermentation of SPI.

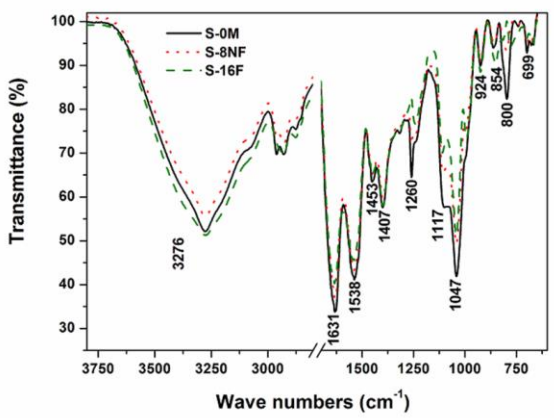

Figure 6: FT-IR spectra of the fermented soy protein films

Tab. 3 shows the decrease in water uptake with increase in percentage of mandelic acid. Soy protein film without mandelic acid (S-0M) showed high water uptake (661.3 ± 69.9%). After the addition of mandelic acid, water uptake decreased from 430.6 ± 22.2% to 124.2 ± 4.5%. The values of water uptake for mandelic acid incorporated solution casted SPI film was much higher compared to compression molded mandelic acid incorporated SPI film [3]. The aromatic groups present in mandelic acid were responsible for low water uptake in mandelic acid incorporated SPI film. The water uptake of autoclaved SPI films was around 341.1 ± 33.8%, however S-16F disintegrated once it was immersed in water for 24 hours which was attributed to predominance of low molecular mass protein fractions of fermented SPI. The water uptake results were in agreement with the FT-IR result (discussed above) and the theoretical conclusion of FT-IR matched with the experimental water uptake values.

Table 3: Water uptake of mandelic acid incorporated and fermented SPI films

Sample	Water Uptake (%)
S-0M	661.3 ± 69.9
S-1M	430.6 ± 22.2
S-2M	438.2 ± 48.5
S-3M	429.7 ± 28.3
S-4M	124.2 ± 4.5
S-5M	138.8 ± 7.4
S-8NF	341.1 ± 33.8
S-16F	Disintegrated

3.5 Tensile strength of Neat, Mandelic Acid Incorporated and Fermented SPI Films

Tab. 4 shows the tensile properties of soy protein films at different contents of mandelic acid. Soy protein film without mandelic acid (S-0M) exhibited tensile strength of 6.50 ± 0.29 MPa and elongation at break as 80.33 ± 4.29%. S-4M showed the increase in tensile strength as well as elongation at break from 6.50 ± 0.29 MPa to 8.03 ± 0.12 MPa and 80.33 ± 4.29% to 125.06 ± 5.18%, respectively. Also, S-4M showed highest value of modulus i.e., 179.68 ± 6.11 MPa. In the literatures, it has been reported that caffeic acid, nisin and adipic acid incorporated SPI film exhibited tensile strength of 6.5 MPa, 5.11 MPa and 6.2 MPa, respectively [2,4,21]. The values of tensile strength and elongation at break were reported to be lower for compression molded mandelic acid incorporated SPI film [3]. At 5% of mandelic acid, the tensile strength of SPI film increased but elongation at break decreased due to structural rigidity exhibited by aromatic rings of the mandelic acid.

As discussed in previous paragraph, soy protein film exhibited tensile strength of 6.50 ± 0.29 MPa and elongation at break as 80.33 ± 4.29% at 30% plasticizer content. S-8NF showed the decrease in tensile strength from 6.50 ± 0.29 MPa to 5.50 ± 0.09 MPa and decrease in elongation at break from 80.33 ± 4.29% to 16.82 ± 0.84% but the plasticizer content was 15% instead of 30%. It has been well reported in the literature that as the content of plasticizer decreased the elongation at break decreased [22,23]. For S-16F there was again decrease in tensile strength from 6.50 ± 0.29 MPa to 3.18±0.08 MPa even though the plasticizer content was reduced from 30% to 15%. The decrease in tensile strength may be attributed to decrease in molecular mass of fermented soy protein as evidenced from SDS-PAGE results discussed in Section 3.3.

Table 4: Tensile strength of mandelic acid incorporated and fermented SPI films

Sample	Tensile Strength (MPa)	Elongation (%)	Young's Modulus (MPa)
S-0M	6.50 ± 0.29	80.33 ± 4.29	89.90 ± 3.69
S-1M	6.46 ± 0.26	82.65 ± 3.96	92.22 ± 3.05
S-2M	7.15 ± 0.33	93.46 ± 6.84	100.63 ± 2.93
S-3M	7.46 ± 0.40	108.43 ± 4.12	93.39 ± 5.55
S-4M	8.03 ± 0.12	125.06 ± 5.18	179.68 ± 6.11
S-5M	7.53 ± 0.34	36.40 ± 2.02	141.63 ± 6.67
S-8NF	5.50 ± 0.09	16.82 ± 0.84	110.89 ± 3.05
S-16F	3.18 ± 0.08	13.11 ± 1.02	47.62 ± 1.47

3.6 DMA of Neat, Mandelic Acid Incorporated and Fermented SPI Films

Fig. 7 shows the temperature dependence of the loss factor (tan δ) of the SPI films. In the Figure, α-relaxation (T_α) values observed at high temperature were attributed to the protein-rich domains similar to that reported in the literatures [22,24]. At low content of mandelic acid (3%), there was an increase of about ~ 16°C in the T_α value unlike 4% mandelic acid incorporated SPI film. Also at low content of mandelic acid,the lower value of T_α was assigned to plasticizer rich domain. As the content of mandelic acid increased from 1% to 4%, the value of T_α increased from -50°C to -16°C. This indicated that the incorporation of mandelic acid decreased the molecular mobility in the protein materials. The fermented sample (S-16F) exhibited lower T_α values compared to unfermented SPI sample which may be attributed to low molecular mass of fermented sample. Again in S-8NF, T_α value at low temperature (-24°C) and high temperature (123°C) were assigned to the plasticizer-rich domains and SPI-rich domains, respectively. Again the increase in both the values for S-8NF as compared to S-0M may be negligible considering the fact that that the plasticizer content in this case has been decreased from 30% to 15% and lower plasticizer content will give the structural rigidity in the SPI film.

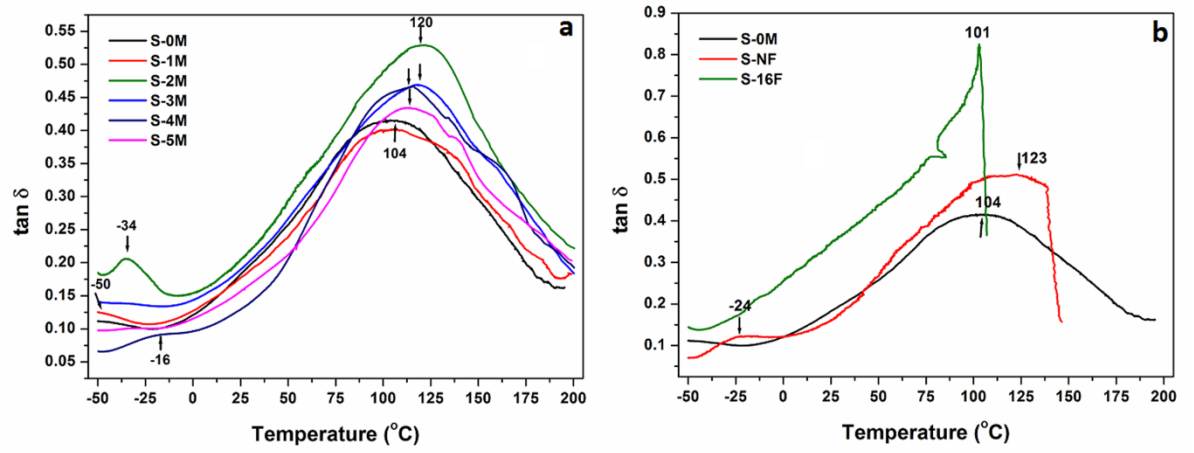

Figure 7: Tan delta curve of (a) mandelic acid incorporated and (b) fermented SPI films

The storage modulus curves of the mandelic acid incorporated and fermented SPI films are presented in Fig. 8. As the temperature decreased, the storage modulus for all the SPI films decreased. S-4M samples showed higher storage modulus than SPI film without mandelic acid. The storage modulus for S-4M was highest at 25ºC (shown by line) and it can be correlated with highest tensile strength for S-4M as discussed in section 3.5. Storage modulus for autoclaved and fermented SPI films showed lower storage modulus than native SPI films.

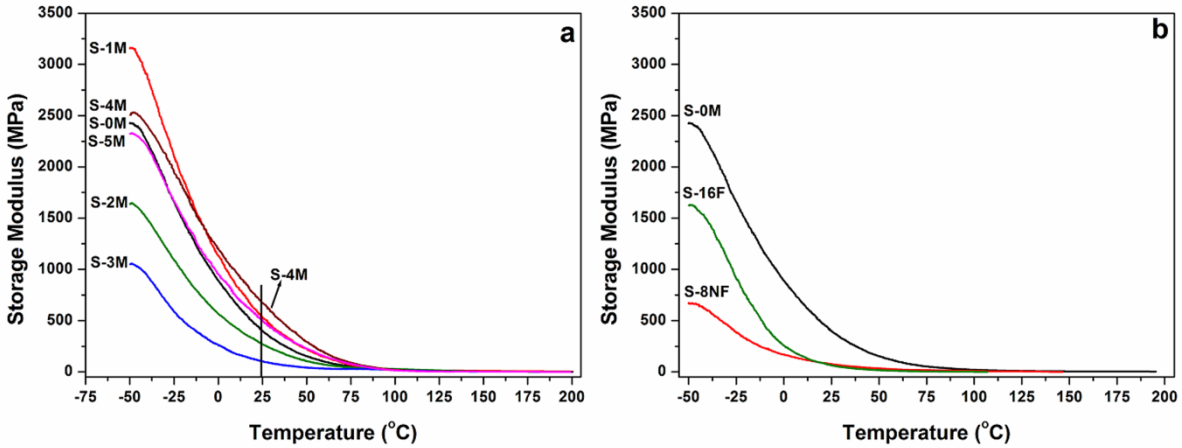

Figure 8: Storage modulus of (a) mandelic acid incorporated and (b) fermented SPI films

3.7 Optical Transmittance of Neat, Mandelic Acid Incorporated and Fermented SPI Films

Fig. 9(a) shows the transmittance of neat and mandelic acid incorporated SPI films. The percentage of transmittance increased with the wavelength showing highest transmittance at 800 nm for each film. However, with the introduction of mandelic acid i.e., for sample S-5M the percentage transmittance decreased when compared to S-1M. On contrary, the transmittance of S-3M increased when compared to S-1M. Fig. 9(b) shows the transmittance of fermented SPI films. The percentage transmittance of SPI film was high compared to autoclaved and fermented SPI films and this may be attributed to nonuniformity in the surface of the prepared SPI film unlike mandelic acid incorporated SPI film.

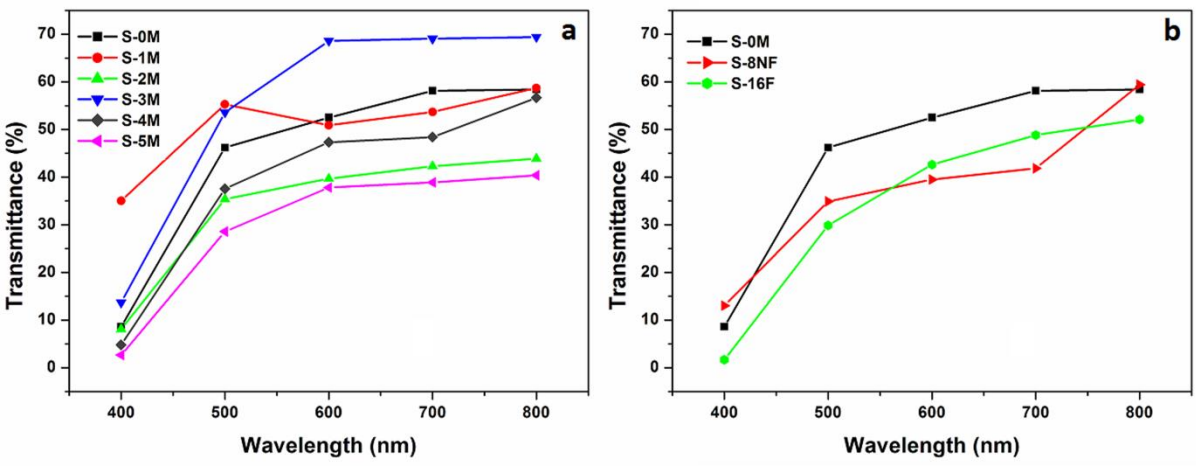

Figure 9: Transmittance of (a) mandelic acid incorporated and (b) fermented SPI films

3.8 Antimicrobial Properties of Neat, Mandelic Acid Incorporated and Fermented SPI Films

Fig. 10 shows the antimicrobial properties of mandelic acid incorporated SPI films. There was no antibacterial property contributed by S-0M while the mandelic acid (S-1M to S-5M) added SPI film showed antibacterial property as there was no microbial growth on the films after 24 hours and the SPI film remained smooth as evidenced in the photograph. However, in S-8NF and S-16F there was no antibacterial property seen after 24 hours. We can easily see the growth of microbes on the surface of the S-NF and S-16F film indicating heterogeneous surface of SPI film. It has been reported by Lin et al., that the presence of phenolic groups in compound are responsible for the antimicrobial activities and in mandelic acid there is also phenolic group [25]. The aromatic group present in mandelic acid potentially disturb the function of *E. coli* cell membranes. This in turn retarded the growth and multiplication of bacteria.

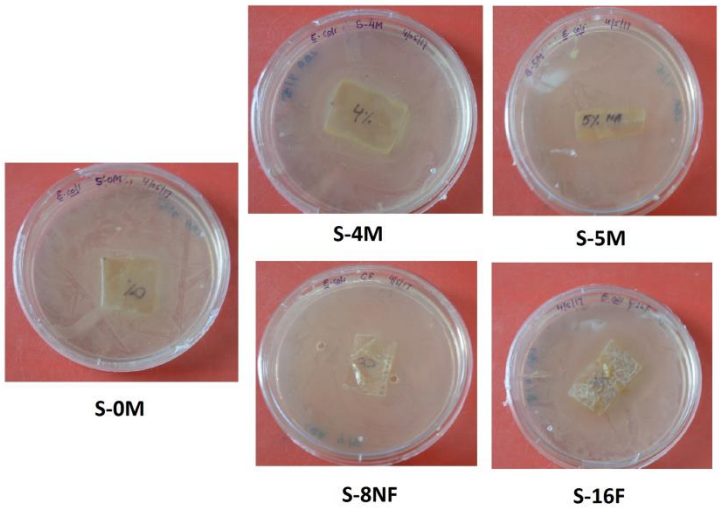

Figure 10: Antibacterial properties of mandelic acid incorporated and fermented SPI films

4 Conclusions

Different contents of mandelic acid (0-5%) were successfully added in SPI to prepare homogeneous film of SPI as well as fermented SPI films which showed nonuniformity in the surface. Molecular mass profile of mandelic acid incorporated SPI film showed similar molecular mass profile unlike fermented

SPI. We could not prepare the fermented SPI films at low concentration of SPI due to predominance of lower molecular mass protein fractions. The effect of the mandelic acid contents, till 4% on SPI films, resulted in increased tensile strength (8.03 ± 0.12 MPa) and storage modulus (highest at 25^{o}C) as well as low water uptake (124.2 ± 4.5%). Mandelic acid incorporated SPI films compared to neat SPI films showed low water uptake due to the presence of aromatic groups in the mandelic acid. On the other hand, the fermented SPI films showed decrease in tensile strength and storage modulus as well as disintegration when completely immersed in water. The study showed that 4% mandelic acid imparted better properties in SPI films than native SPI films. No growth of *E. coli* on the surface of mandelic acid incorporated SPI films suggested the antibacterial nature of the SPI films. However, the bacterial growth was evident on the fermented SPI films. The use of above mentioned soy based film in form of bioplastic may lead to the prevention of problems and diseases caused by continuous usage of synthetic based plastics.

Note: The part of this work has been delivered at the oral presentation in the APA 2017 conference held at New Delhi, India from 23rd Nov. to 25th Nov. 2017.

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