IJIRSET

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Synthesis of an Active Antimicrobial Packaging Material Based On the Bio-Switch Model

Pushpa Agrawal[1], Amrutha Anandaraman[2], Shruti Ramaprasad[3]

Professor & Dean, P.G. Biotechnology and Chemical Engineering, R.V. College of Engineering, Mysore Road, Bangalore, India[1]

U.G. Student, Department of Biotechnology, R.V. College of Engineering, Mysore Road, Bangalore, India[2],

U.G. Student, Department of Biotechnology, R.V. College of Engineering, Mysore Road, Bangalore, India[3],

ABSTRACT: In response to the dynamic changes in current consumer demand and market trends, the area of active packaging is becoming increasingly significant. An antimicrobial active packaging material can be made by incorporating and immobilizing antimicrobial agents into food packaging material and applying a bio-switch concept. The lignocellulose-based film was synthesized by incorporating antimicrobial agents such as lysozyme and EDTA. The sugarcane bagasse was digested by *Aspergillus tamarii*, lysozyme from egg white was partially purified and, optimized concentrations of antimicrobial agents were incorporated during the casting of antimicrobial lignocellulose films. The lysozyme at 0.15-0.2 mg/ml along with 15 Mm EDTA at 0.2 v/v was incorporated into the lignocellulose films and the antimicrobial effect of the active antimicrobial lignocellulose films was tested on *E. coli*. Surface characterization of the developed active films indicated smooth and homogenous topography by atomic force microscopy and they showed increased hydrophobicity with the incorporation of glycerol as plasticizer. Tensile strength tests indicated increased tensile strength with the addition of glycerol and the tensile strength of the developed active packaging material was comparable to that of conventional polyethylene packaging material

KEYWORDS: Active packaging, Antimicrobial, Bio-switch, Lignocellulose, Sugarcane bagasse

I. LITERATURE SURVEY

Conventional food packaging materials provide basic protection and barrier function but the demand for minimally processed, easily prepared and ready-to-eat ‘fresh’ food products, globalization of food trade, and distribution from centralized processing pose major challenges for food safety and quality. Active packaging is defined and classified by the extra functions added to the packaging systems which used to have only barrier and protective functions. Packaging is termed as “active” when it performs some desired role other than to provide an inert barrier to the external environment [1]. The extra functions include oxygen scavenging, antimicrobial activity, moisture scavenging, ethylene scavenging, ethanol emitting and so on. The research focuses on antimicrobial food packaging which acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or the packing material itself [2]. The packaging field is still dominated by petroleum-derived polymers, such as polyethylene and polystyrene, despite global concerns about the environment. [3]. Lignocellulose is a common type of natural fiber which has the potential to be used as base packaging material. Cellulose, hemicellulose and lignin form ~90% of the total dry matter. Lignocellulose also contains lesser amounts of minerals, oils, and other components [4]. The potential use of incorporating lignocellulosic agricultural residues, such as soy stalk, corn stalk, wheat straw, sugarcane bagasse and perennial grasses, like switchgrass and miscanthus, as reinforcement into a biodegradable polymer matrices comprising a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(butylenes-adipate-coterephthalate) blend has also been recently investigated [5].

IJIRSET

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

II. INTRODUCTION

Antimicrobial packaging materials have to extend the lag period and reduce the growth rate of microorganisms to prolong the shelf life and maintain food safety [6]. Lysozyme is one of the most frequently used antimicrobial enzymes incorporated into packaging materials [7]. This enzyme shows antimicrobial activity mainly on gram-positive bacteria by splitting the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan in their cell walls. When lysozyme is combined with EDTA the outer membranes of gram-negative bacteria are destabilized by this agent and the antimicrobial spectrum of lysozyme increases significantly [8].

Biodegradable films generally consist of at least two major components: a high molecular weight film forming polymer and a plasticizer [9]. Plasticizers are used in films to increase film flexibility and processibility [10]. The most commonly used plasticizers for polymer-based films are glycerol, glycols and other hydroxyl compounds [11]. In the present study, glycerol was chosen as the plasticizer to be used as, glycerol is the by product of biodiesel manufacture and there will be a novel utilization of the residual glycerol in the design of the packaging material. The mechanism of action of the antimicrobial film is based on the bioswitch model. In a bioswitch system, an antimicrobial agent is released on command when there is microbial growth [12]. The basic concept is that a change in the environment such as pH, UV light, or temperature occurs and the antimicrobial agent responds accordingly. This system could increase the stability and specificity of preservation and reduce the amount of chemicals needed in foods. Many bacteria will digest polysaccharide when they grow and so if bacterial contamination occurs, the growth of the bacteria will release the antimicrobial component and could inhibit subsequent microbial growth.

The main objectives of the present study are:

- Microbial digestion of sugarcane bagasse to obtain lignocellulose
- Optimization of concentrations of antimicrobial agents to be incorporated into lignocellulosic film
- Synthesis of an active antimicrobial lignocellulosic film
- Testing and characterization of synthesized active antimicrobial lignocellulosic film

III. MATERIALS AND METHODS

As per the experimental requirements, sugar cane bagasse was obtained from Cane Crush sugarcane juice centre (Bangalore, India). Glycerol, which is a by product of bio diesel manufacture, was obtained from the bio diesel plant in R.V College of Engineering in order to use it as a plasticizer. The fungal and bacterial cultures were procured from The Microbial Type Culture Collection and Gene Bank (MTCC, Chandigarh, India). The fungal strain of *Aspergillus tamarii* (Catalogue No: 8841) was isolated from plant debris (V.K Morya) as given in the MTCC database. The bacterial strain of *Escherichia coli* (Catalogue No: 1622) was isolated (V.Kumar) as given in the MTCC database. The antimicrobial reagents, namely, lauric acid (**W261408)** and EDTA (**E9884)** were purchased from Sigma Aldrich (Bangalore, India). Lysozyme was prepared from eggs purchased in Big Bazaar (Bangalore, India). Potato Dextrose Broth (catalogue number: M403) and Agar-Agar, Type 1 (catalogue number: RM666) was obtained from HiMedia Laboratories (Mumbai, India).

Digestion of sugarcane bagasse by Aspergillus tamarii

Aspergillus tamarii was procured from MTCC. Based on literature survey, the optimum growth conditions were established as 25ºC in Potato dextrose agar (PDA) media Petri plates for 72-96 hours. The cultures were inoculated in the solidified media and plates were incubated in inverted position. Sugarcane bagasse was chopped into 30-50 mm pieces and was soaked in boiling water for 10 min and the excess water was drained out. About 25 g of moistened bagasse was transferred into 500 ml flask and used as the biodegradation substrate. To the flask was added, 10 ml of nutrient medium containing 5% glucose and 0.21% ammonium sulphate. The flask containing the substrate was autoclaved at 120 °C for 15 min and the digestion of sugarcane bagasse was carried out by inoculating the fungal culture of *Aspergillus tamarii* into the flask. The flask was incubated at 25 °C for 15 days.
After incubation, the contents of the flask were subject to centrifugation at 10,000 rpm for 5 min and the supernatant obtained was used as lignocellulose base film material.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Preparation of partially purified lysozyme by ethanol precipitation method

Two eggs were taken and the egg whites were separated carefully from the egg yolks without disturbing the egg yolks. The egg whites were diluted with two volumes of 0.05M NaCl solution. In order to precipitate all the proteins other than lysozyme, the pH of the mixture was set to 4 by carefully adding several drops of 1 N acetic acid and then diluted with an equal volume of 60% (v/v) ethanol. After six hours of incubation at room temperature with 30% ethanol, the mixture was centrifuged at 15000 x g for 15 minutes at 4°C. The precipitate was discarded and the supernatant containing lysozyme was dialyzed for 21 h at 4°C by three changes of 2000mL distilled water. The above procedure was repeated thrice. In order to confirm that the enzyme isolated was lysozyme, 1 ml of the dialyzed supernatant from each of the three batches was taken and 100 µl of 1x SDS loading dye was added to the sample. The sample was boiled for 5 min and then cooled on ice. The sample was then run on a 15% SDS PAGE. To estimate the lysozyme obtained from the ethanol precipitation of egg white, Lowry's protocol for protein estimation was followed.

Optimizing the concentrations of antimicrobial agents

In order to optimize the concentrations of the anti microbial agents and to establish the most effective anti microbial agent, zone inhibition tests were carried out with different concentrations of lysozyme, lauric acid and a combination of lysozyme and EDTA.

E. coli in LB broth was grown to an optical density within the range of 0.62 to 1.0 at 600 nm. LB media supports *E. coli* growth OD600 under normal shaking incubation conditions (250 rpm). Using a sterile spreader, 100 µl of *E. coli*-LB broth was spread evenly over the face of a sterile pre-prepared LB-agar plate.

Varying concentrations of lysozyme (0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml and 0.2 mg/ml respectively) were prepared. Sensi discs were soaked in the different concentrations of the lysozyme solution for half an hour. The sensi discs were then placed on the plate.

Similarly, varying concentrations of lauric acid (0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml and 0.2 mg/ml respectively) were applied on a second plate using sensi discs. A third agar plate was prepared which contained sensi discs that had varying concentrations of lysozyme (0.05 mg/ml 0.1 mg/ml, 0.15 mg/ml and 0.2 mg/ml respectively), each incorporated with EDTA (15mM used as 0.2%v/v).

These agar plates with the different anti microbial agents were then incubated for 48 hours, at 37°C. After incubation, the diameters of the zones of inhibition were measured using a vernier calliper. The data was interpreted using the graphical method and the optimum concentration was determined. The test was conducted in triplicates with control sensi discs.

Lignocellulose film preparation

Lignocellulose films from sugarcane bagasse were prepared by adding 10 ml of the supernatant obtained after the digestion of sugarcane bagasse by *Aspergillus tamarii,* in 90 ml of 20% ethanol with continuous stirring using the magnetic stirrer. After the solution was homogenous, 3.8 ml glycerol was added as plasticizer and the mixture was heated slowly to a mild boiling. 5ml of the film mixture was measured and poured into the Petri dishes and acrylic slabs. They were then were placed for 24 hours in an oven set at 50°C.

For preparation of antimicrobial lignocellulose films, 10 mL of antimicrobial solution consisting of 0.2 mg/ml crude lysozyme and 15mM EDTA used as 0.2v/v was added to 80 ml of 20% ethanol film base solution with 10 ml of the lignocellulose supernatant. 3.8% of the plasticizer was added and the antimicrobial lignocellulose films were cast similar to the control lignocellulose films.

Liquid culture test of antimicrobial lignocellulose films

For the liquid culture test to estimate the effectiveness of the film incorporated with the antimicrobial reagent, each film was cut into squares (5 cm x 5 cm). Three sample squares were each immersed in 20 mL of LB broth in a 25 mL universal bottle. The medium was inoculated with 200µL of Escherichia coli in its late exponential phase, and then transferred to an orbital shaker and rotated at 37°C at 200 r.p.m. The culture was sampled periodically (0, 4, 8, 12, 16, 20, 24, 28 hours) during the incubation to obtain microbial growth profiles. The same procedure was repeated for the control film with no antimicrobial agent. The optical density (O.D.600) was measured at λ = 600nm using a spectrophotometer.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

IV. RESULTS AND DISCUSSION

Confirmation of partially purified lysozyme

To confirm the presence of lysozyme extracted from ethanol precipitation of egg white, SDS-PAGE was carried out. Dialyzed lysozyme extracts from three batches were loaded on to 15% SDS-PAGE gel, electrophoresed and stained with Coomassie brilliant blue.

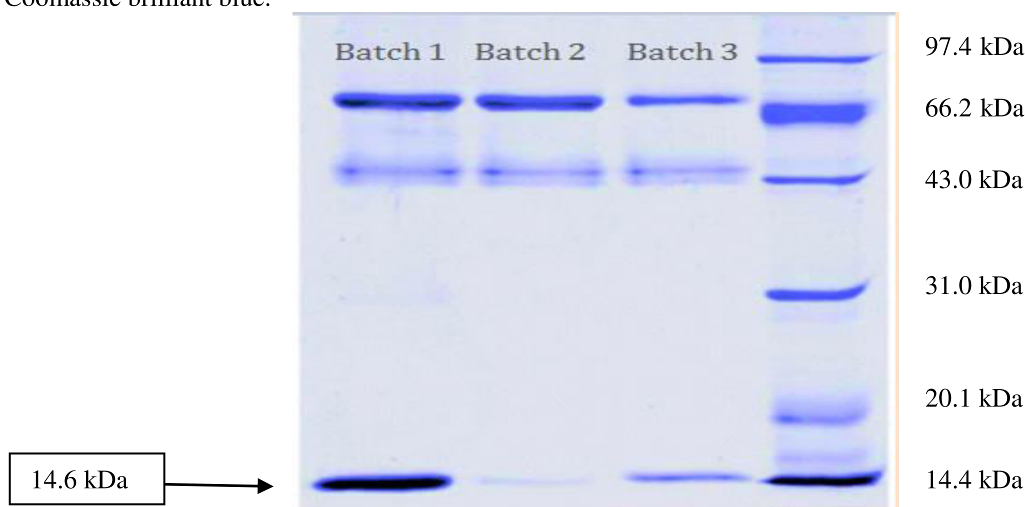

Figure 4.1: Confirmation of lysozyme using SDS-PAGE

Inference: After carrying out SDS-PAGE for three batches of partially purified lysozyme, the band corresponding to the extracted lysozyme was observed. As per literature survey, the molecular weight of lysozyme is 14.6 kDa and upon comparing the band obtained to the 100 kDa protein ladder, the presence of lysozyme was confirmed.

Protein content estimation of partially purified lysozyme

To estimate the total content of lysozyme extracted from the ethanol precipitation method, the Lowry's protocol for protein estimation was conducted. The concentration of standard BSA used is 1 mg/ml and reagents I and II were prepared as mentioned in the methodology.

Extrapolated values of amount of protein obtained from each batch:

Table 4.2 (b): Total amount of lysozyme obtained after ethanol precipitation in each batch

Batch No.	Total Lysozyme (µg)
1	820
2	713
3	680

Optimization of concentration of antimicrobial agents by zone of inhibition test

To incorporate optimum concentrations of antimicrobial agents in the lignocellulose film, zone diffusion method was followed. Different concentrations (0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml and 0.2 mg/ml) of lauric acid, lysozyme and lysozyme with EDTA solutions were prepared and sensi discs were soaked in the respective solutions. After placing the sensi discs on the sterile surface of the LB-Agar plates inoculated with *E.coli* and incubating for 48 h at 37 °C, the zones of inhibition were formed and the diameter of the zones of inhibition were measured using the vernier calliper. All the antimicrobial zone diffusion methods were carried out in triplicates.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Diameter of zones of inhibition with lysozyme against *E. coli*

Table 4.3 (a): Diameter of zones of inhibition with lysozyme against *E. coli*

SI No.	Concentration (mg/ml)	Diameter of Zone of Inhibition (cm)	Area of Inhibition (cm^2)
1	0.05	2.4	4.52
2	0.10	2.5	4.91
3	0.15	2.8	6.15
4	0.20	2.75	6.00

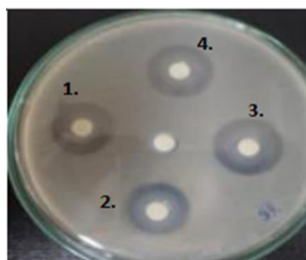

Figure 4.3 (a): Zones of inhibition with lysozyme against *E. coli*

Inference: The control sensi disc placed at the centre of the Petri dish with no lysozyme showed almost negligible zone of inhibition. After tabulating the values of the diameters of zone of inhibition for different concentrations of lysozyme, lysozyme at 0.15 mg/ml concentration was found to provide the highest diameter of zone of inhibition.

Diameter of zones of inhibition with lauric acid against *E. coli*

Table 4.3 (b): Diameter of zones of inhibition with lauric acid against *E. coli*

SI No.	Concentration (mg/ml)	Diameter of Zone of Inhibition (cm)	Area of Inhibition (cm^2)
1	0.05	2.1	3.56
2	0.10	2.2	3.80
3	0.15	2.4	4.52
4	0.20	2.4	4.52

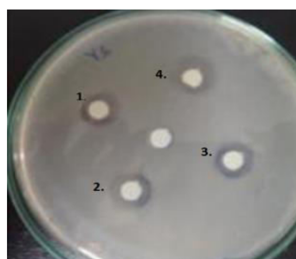

Figure 4.3 (b): Zones of inhibition with lauric acid against *E. coli*

Inference: The control sensi disc placed at the centre of the Petri dish with no lauric acid showed almost negligible zone of inhibition. After tabulating the values of the diameters of zone of inhibition for different concentrations of lauric acid, lauric acid at 0.15 mg/ml as well as 0.20 mg/ml concentrations were found to provide the highest diameter of zone of inhibition.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Diameter of zones of inhibition with lysozyme and EDTA against *E. coli*

Table 4.3 (c): Diameter of zones of inhibition with lysozyme and EDTA against *E. coli*

SI No.	Concentration (mg/ml)	Diameter of Zone of Inhibition (cm)	Area of Inhibition (cm^2)
1	0.05	2.1	3.56
2	0.10	2.2	3.80
3	0.15	2.4	4.52
4	0.20	2.4	4.52

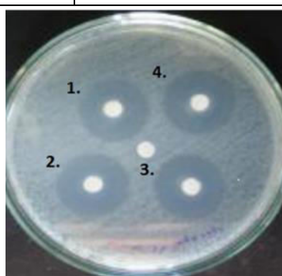

Figure 4.3 (c): Zones of inhibition with lysozyme-EDTA against *E. coli*

Inference: The control sensi disc placed at the centre of the Petri dish with no lysozyme & EDTA showed almost negligible zone of inhibition. After tabulating the values of the diameters of zone of inhibition for different concentrations of lysozyme with EDTA, lysozyme with EDTA at 0.15 mg/ml as well as 0.20 mg/ml concentrations were found to provide the highest diameter of zone of inhibition. Therefore, this was most effective pair of antimicrobial agents, and hence lysozyme at a concentration of 0.2 mg/ml with 15 mM EDTA used as 0.2 v/v will be incorporated into the lignocellulose films.

Liquid culture test of antimicrobial lignocellulose films

In order to test the antimicrobial effectiveness of the synthesized lignocellulose films, a liquid culture test was conducted. The results of the test were graphically plotted as below, representing the optical density of the culture medium at varying time intervals.

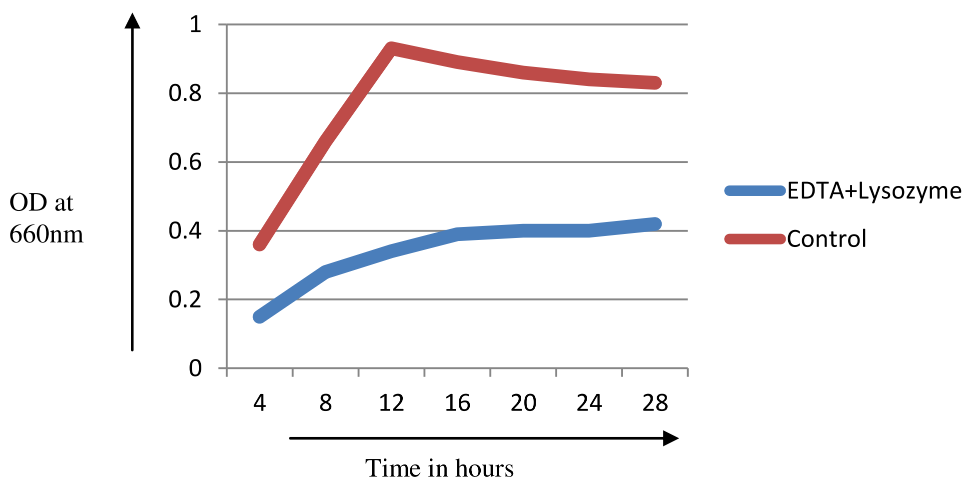

Figure 4.4: Inhibition of *E.coli* by antimicrobial lignocellulose film in liquid culture

Inference: Antimicrobial lignocellulose film containing lysozyme-EDTA gives an inhibitory effect towards the growth

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

of *E. coli.* Figure 4.4 shows the inhibition of *E. coli* by the antimicrobial film in liquid culture. Clearly, combination of lysozyme and EDTA shows a large reduction of stationary growth phase. At the stationary growth phase, the cell concentration in the control film flask ($O.D_{600nm}$ = 0.93) was almost two and a half times higher than the cell concentration in the medium containing the lignocellulose film incorporated with lysozyme and EDTA ($O.D_{600nm}$ = 0.39). From the above it can be concluded that lysozyme combined with EDTA enable inhibition of both bacteria growth and that the synthesized antimicrobial lignocellulose films are active in nature.

Atomic Force Microscopy

The morphology of the lignocellulose films was analyzed by and atomic force microscopy (AFM) and the high resolution images are shown in Figures 5.1(a) and 5.1(b).

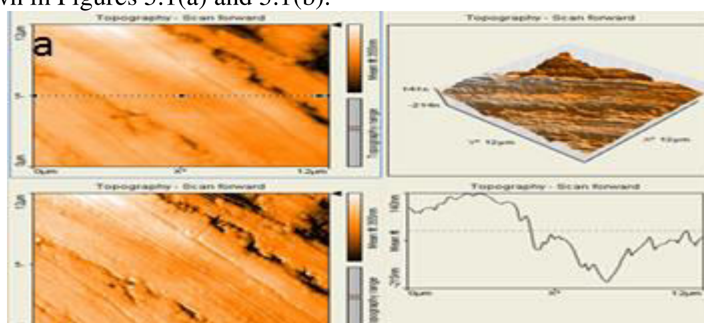

Figure 5.1 (a): Surface morphology of control film

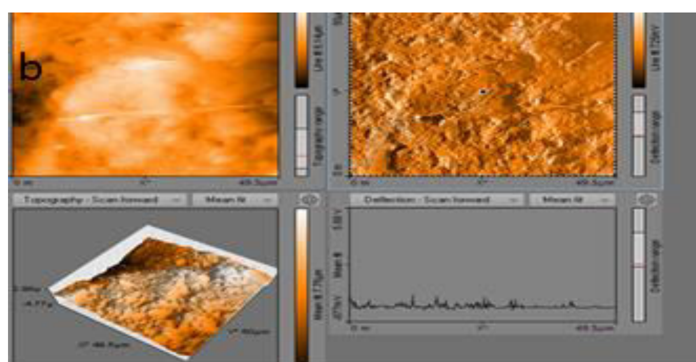

Figure 5.1 (b): Surface morphology of film with plasticizer

Inference: The micrographs proved that the surfaces of the lignocellulose films displayed homogeneous structures with typical granular morphology. It was also noted that the *z*-axis scale of the control sample as shown in Figure 5.1.1(a), i.e. the lignocellulose film synthesized without the plasticizer was bigger than that in the plasticized lignocellulose films, which meant that the roughness of the lignocellulose films was decreased in the presence of plasticizers.

Water Contact Angle

The interaction of the lignocellulose films with water was investigated by contact angle measurement using a goniometer, and the data is shown in Table 5.1

Table 5.1: Water contact angle for control and plasticized lignocellulose films

Sample	Contact Angle (°C)
Lignocellulose film without glycerol	36.4
Lignocellulose film plasticized with glycerol	74.4

IJIRSET

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Inference: It was observed that the contact angle of the plasticized lignocellulose film was increased by 104.4% in the presence of glycerol. The smooth and homogenous structure of the films lowers the interfacial free energy, thereby preventing water to penetrate into the films, thus increasing the contact angle. The increase of contact angle in plasticized lignocellulose films is beneficial for the utilization in packaging.

Tensile Strength Test

Tensile testing was performed on a Zwick UTM/Z005, fitted with a 200 N load cell with the crosshead speed of 0.5 mm/min, and the initial distance between the grips was 20 mm.

Four samples each of control film, i.e. lignocellulose film not plasticized with glycerol, and lignocellulose films were tested to check the consistency of the results. The measurements were performed under room temperature and the results were obtained graphically and were also tabulated.

The stress-strain curves of one set of the control lignocellulose film and plasticized lignocellulose film are shown in Figure 5.2.

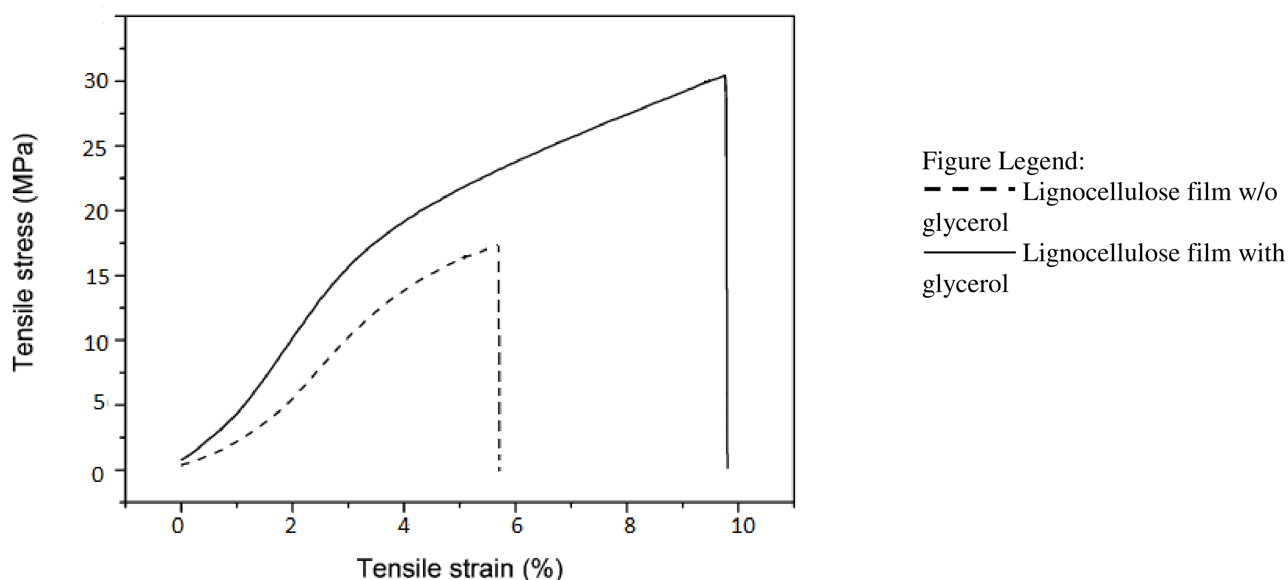

Fig 5.2: Stress strain curves for control and plasticized lignocellulose films

Table 5.2: Tensile strength and percentage elongation of control and plasticized lignocellulose films

Properties / Film	Formulation	Thickness (mm)	Tensile Strength (MPa)	% Elongation
Lignocellulose film without glycerol	F-1	0.54 $\pm$ 0.007	18.1118	5.63
	F-2	0.55 $\pm$ 0.012	18.05	5.34
	F-3	0.545 $\pm$ 0.012	18.64	5.79

IJIRSET

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

	F-4	0.53 ± 0.001	18.33	5.70
Lignocellulose film with glycerol	F-5	0.54 ± 0.002	27.16	9.71
	F-6	0.545 ± 0.004	27.07	9.63
	F-7	0.55 ± 0.006	27.49	9.54
	F-8	0.55 ± 0.010	27.96	9.69

To compare the mechanical properties of the synthesized antimicrobial lignocellulose film and to the existing polyethylene packaging material, the average tensile stress and percent elongation at break of the polyethylene material at 0.5 mm thickness was determined to be 29.9 MPa and 10.05 % respectively.

Inference: Tensile strength of the control lignocellulose film was relatively weak. In comparison to the control film, the tensile stress of the plasticized lignocellulose film was increased by almost 50%. The interface between the lignocellulose matrix and glycerol play an important role in the improvement of the mechanical properties of the lignocellulose films. In addition, it can be observed that all lignocellulose films showed thermoplastic-like behaviour, with stress increasing rapidly at small strains and more slowly after a yield point.
Furthermore, on comparison of the tensile strength values of both the synthesized lignocellulose film and polyethylene packaging material, it was observed that the tensile strength values of the lignocellulose films are almost similar to that of polyethylene. Thus, there is negligible difference the strengths of the two materials.

V. CONCLUSION

A translucent active antimicrobial lignocellulose material that is flexible has been successfully developed. The microbial digestion of sugarcane bagasse by *Aspergillus tamarii* yielded an 88% (22g from 25g of starting sugarcane bagasse) conversion to lignocellulosic biomass. An active antimicrobial lignocellulose film was synthesized by incorporating an optimized concentration of 0.2 mg/ml of partially purified lysozyme, prepared by ethanol precipitation of egg white, with 15mM EDTA.
It was found that the film was effective towards inhibition of microbial activity, i.e. , i.e. at the stationary growth phase, the cell concentration in the flask containing the control film without the antimicrobial agent ($O.D_{600nm} = 0.93$) was almost two and a half times the cell concentration in the medium containing the lignocellulose film incorporated with lysozyme and EDTA ($O.D_{600nm} = 0.39$).
The developed films were proven to have a smooth and homogenous topography after characterizing their surfaces using atomic force microscopy. The range of z-axis values for the lignocellulose film without glycerol was from 0 to 1295.67 nm whereas the range of z-axis values for the glycerol plasticized lignocellulose films was from 0 to 107.91 nm. This clearly indicates that roughness of the lignocellulose films was decreased with the inclusion of glycerol. The active antimicrobial lignocellulose film that which has been synthesized is an effective packaging material for moisture sensitive food articles due to the increased hydrophobicity expressed by the film in the presence of glycerol as a plasticizer.

IJIRSET

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 5, May 2014

Furthermore, the tensile strength of the developed film is comparable to that of the conventional polyethylene packaging material. Tensile strength tests indicated increased tensile strength with the addition of glycerol and the tensile strength of the developed active packaging material is comparable to that of conventional plastic packaging material. Lignocellulose films with an average thickness of 0.54 mm displayed an average tensile strength of 27.42 MPa and this is comparable to the average tensile strength of polyethylene material which is 29.9 MPa. Most importantly, the film that has been developed overcomes the drawback faced by the existing polyethylene packaging which are pollutants to the environment as the lignocellulosic films are biodegradable.

REFERENCES

[1] Hotchkiss J. H., "Food-packaging interactions influencing quality and safety"*Food Additives Contaminants*, vol. 14, 1997, pp 601-607.
[2] Appendini P. and Hotchkiss, J. H., "Immobilization of lysozyme on synthetic polymers for the application to food packages*",* Presented at Ann. Mtg. of Inst. of Food Technologists, New Orleans, La. 1996.
[3] Vermeiren L., Devlieghere F. and Debevere, J., "Effectiveness of some recent antimicrobial packaging concepts", *Food Additives and Contaminants*, vol.19, 2002, pp. 163 -171.
[4] Taherzadeh M. J. and K. Karimi, "Pre-treatment of lignocellulosic wastes to improve ethanol and biogas production: A review*", International Journal of Molecular Sciences*, vol. 9(9), 2008, pp. 1621-1651.
[5] Nagarajan V., Mohanty A. and Misra M., "Sustainable green composites: value addition agricultural residues and perennial grasses*",* *ACSSustain. Chem.Eng.*, vol. 1, 2013, pp. 325–333.
[6] Han, J.H., "Modeling the inhibition kinetics and the mass transfer of controlled releasing potassium sorbate to develop an antimicrobial polymer for food packaging*",* Ph.D. dissertation, Purdue Univ., West Lafayette, Ind.1996.
[7] Buonocore G. G., Del Nobile M. A., Panizza A., Bove S., Battaglia G., and Nicolais L., "Modeling the lysozyme release kinetics from antimicrobial films intended for food packaging application*", Journal of Food Science,* vol. 68, 2003, pp. 1365-1370.
[8] Branen J. K. and Davidson P. M., "Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin", *International Journal of Food Microbiology*, vol. 90, 2004, pp. 63-74.
[9] Banker G S., "Film coating theory and practice", J. Pharm. Sci, 1966, vol. 55, pp.89.
[10] Stuchell YM, Krochta JM., "Enzymatic treatment and thermal effects on edible soy protein films*", Journal of Food Sci*., vol. 59, 1994, pp. 1332–1337.
[11] Banker G. S., "Film coating theory and practice", *J. Pharm. Sci*, 1966, vol. 55, pp. 81-89.
[12] Dawson P. L., Harmon L., Sotthibandhu A., Han I. Y., "Antimicrobial Activity of Nisin-Adsorbed Silica and Corn Starch Powders", *Food Microbiology*, vol. 22, 2005, pp. 93.