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Biodegradation of Polystyrene (PS)-Poly(lactic acid) (PLA) Nanocomposites Using *Pseudomonas aeruginosa*

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Abstract: Poly(lactic acid) (PLA) was synthesized using condensation polymerization of *L*-lactic acid using a controlled ultrasonic cavitation technique. Polystyrene (PS) was used to prepare the PS:PLA and PS:PLA:organically modified montmorillonite (OMMT) composites. PS was dissolved in benzene (10:90) and kept overnight for dissolution. Meanwhile, surface modification of montmorillonite was done using a column chromatography technique and referred to as OMMT. The *d*-spacing was found to be 22 Å after modification due to sufficient column length and diameter with good retention time during ion exchange. PLA and OMMT were kept in hot air oven at 100 ºC for 30 min to remove the moisture. The mixtures of 10%, 15%, 20%, 25%, and 30% of PS:PLA:OMMT were subjected to ultrasonic irradiation (50 Hz) for homogenization and to form a biodegradable polymer nanocomposite sheet (5×5 cm^2). The amount of OMMT loading was from 0.5-5 mass%. These composites were subjected to degradation in minimal medium using *Pseudomonas aeruginosa* bacteria at controlled conditions, and the polymer is a major source of carbon. The degradation was confirmed using scanning electron microscopy, extracellular protein content change, biomass production, and % degradation with respect to time (up to 28 days) after incubation.

Keywords: poly(lactic acid) (PLA), organically modified montmorillonite (OMMT), polystyrene (PS), *Pseudomonas aeruginosa.*

Introduction

The promoting interest of biodegradable polymers is its growing concern raised by the recalcitrance and unknown environmental fate of many of currently used synthetic polymers. Thus, biodegradable polymers are one of the available options for the waste management of polymers in the environment which includes i) water-soluble and ii) water-insoluble. In recent years, considerable attention has been focused on biodegradability of polymeric materials mainly because a worldwide problem of pollution of the environment by the waste polymers. Two possible approaches to reduce the 'vices of polymeric materials' are (a) to develop a biodegradable commodity plastics and, (b) to identify potential micro organisms and define a protocol for effective biodegradation of polymeric materials. The present study is an effective attempt to investigate the potential of *Pseudomonas aeruginosa* (*P. aeruginosa*) for polymer degradation. This bacterial culture is isolated from a polymeric sheet under degradation due to bacterial colonization. The bacteria obtained from degraded polymer nanocomposite were cultured and brought in pure form, which then identified as *P. aeruginosa*. The potential of *P. aeruginosa* was studied for polymer degradation at laboratory conditions to which polystyrene (PS): poly(lactic acid) (PLA): organically modified montmorillonite (OMMT) nanocomposites were taken as a test material. PS is a class of non degradable commodity plastic used widely as a raw material in various industries, which is degraded in this study using PLA and OMMT. Biocomposites are usually fabricated with biodegradable or nonbiodegradable polymers as a matrix and natural fibres as a reinforcing agent.[1-7] Biodegradation is considered as a type of degradation, which involves biological activity. Many of living organisms, especially bacteria and fungi can play major role. Biodegradable polymer is define as 'a degradable polymer wherein the primary degradation mechanism is through the action and metabolism of microorganisms'.[8-10] The lactic acid based polyesters are well known thermoplastic biodegradable polymers and would appear to meet these strict requirements. Lactic acid is a naturally occurring nontoxic material which is a fermented product of renewable sources (like corn). PLA has an excellent physical properties, which mimic conventional thermoplastics and is a true biodegradable polymer. In a typical compost environment decomposition of PLA takes place rapidly to yield carbon dioxide, water and biomass.

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The plant growth stimulation activity also been claimed of lactic acid oligomers having less than 10 monomer units.[11-18] Non degradable plastics accumulate in the environment at a rate of 25 million tonnes per year. Extensive use of non biodegradable thermoplastics and the rate at which they accumulate in the environment makes humankind to realise the necessity to find its environmental impact. As the polymer usage is unavoidable, ways have to be found to (1) enhance the biodegradability of the polymers by blending them with biodegradable natural polymers such as poly(lactic acid) (PLA) starch or cellulose *etc*. (2) Mixing with prooxidants so that they are easily degraded and (3) *P. aeruginosa* and improve microorganisms that can efficiently degrade these polymers. In order to attempt the third option the mechanism of biodegradation should be understood.[19-31]

Experimental

Materials. *L*-Lactic acid (90% pure) as a monomer (Merck Specialities (P) Ltd, Mumbai MS, India), Tin chloride dihydrate (National Chemical Laboratory, Pune, MS, India), dichloro methane (s d fine chemical Ltd Mumbai, MS, India), xylene (RFCL Ltd, New Delhi India), *n*-hexane (Qualigens fine Chemicals, Mumbai MS, India), high impact polystyrene (HIPS) granules (Jain Irrigation system (P) Ltd, Jalgaon, MS India), montmorillonite (MMT) as filler reinforcing agent (Southern Clay, Texas), silicone oil (s d fine-chemical, Mumbai, MS, India), *P. aeruginosa* (NCBI accession number: JF732909) as a bacterial culture obtained from Department of Microbiology, School of Life sciences, North Maharashtra University Jalgaon.

Methods.

Synthesis of Poly(lactic acid) Using Polycondensation: Synthesis of poly(*L*-lactic acid) was done in a four neck round bottom flask equipped with nitrogen gas inlet, thermo well, magnetic stirrer, Dean Stark apparatus and a condenser. The flask was charged with *L*-lactic acid (38.204 g) and xylene (40 mL) which then heated at 145-150 ºC and allowed to reflux for 5 h so as to remove the water from reaction. After 5 h, reaction was stopped and allows cooling. Tin chloride dihydrate as the catalyst (0.067 g, 0.2 wt%) was added into the reaction mixture. The reaction was continued at the same temperature. The reaction mixture was again cool, which then dissolved in dichloro methane and precipitated in *n*-hexane under controlled stirring. The precipitate was filtered and dried in hot air oven. The material was white semicrystalline of PLA.

Surface Modification of MMT: 5 g of MMT was dispersed in deionised water (100 mL) with vigorous stirring to obtained an aqueous suspension of purified MMT. The aqueous suspension was kept at room temperature for 24 h. A stoichiometric amount of interfacial agent octadecylamine (7.7 g) was mixed with conc HCl (2.9 mL) and transfered into an anion exchange column of sufficient length (35 cm) and diameter (5 cm) packed with cellulose. The time of flow of mixture was approximately 25-35 mL/min. The entire slurry of surface modified clay was passed through nylon filter and collected at receiver. Precipitate was washed several times by flushing the column with hot distilled water so as to remove unreacted HCl and octadecylamine. This collected matter was freeze dried to yield a modified organoclay,[32] which was further referring as OMMT.

Preparation of Biodegradable Polymer Composites Using Ultrasonication Technique: PS was used for preparation of two different compositions that is PS: PLA and PS: PLA: OMMT. PS was dissolved in benzene (10: 90) and kept overnight for proper dissolution. PLA and OMMT were dried in hot air oven at 100 ºC for 15 min to remove the moisture, which they added in to the polymer solution under controlled ultrasonic (50 KHz) cavitation technique for 30 min. The homogenous mixture was poured in a petry dish coated with silica oil (5 cm) and covered with aluminum foil so as to protect it from dust and free oxygen. The petry dish was kept overnight for solvent evaporation. Subse-

Table I. Compositions of PS: PLA: and PS: PLA: OMMT Composites

Sr. No.	Benzene (mL)	Polystyrene (g)	PLA (g)	OMMT (g)	PLA (%)/OMMT (phr)
1.	45.00	5.00	0.50	0.00	10 %
2.			0.75		15 %
3.			1.00		20 %
4.			1.25		25 %
5.			1.50		30 %
6.			0.50	0.030	0.50 phr
7.			0.75	0.045	0.75 phr
8.			1.00	0.075	1 phr
9.			1.25	0.120	2 phr
10.			1.50	0.300	5 phr

quently, the sheet was peal out from petry dish and dried in an oven at 80 °C for 30 min. Composites of different ratios of PS: PLA (10%, 15%, 20%, 25%, and 30%) and PS: PLA: OMMT (0.5, 0.75, 1, 2, and 5 phr) were prepared (Table I).

X-Ray Diffraction (XRD). The XRD patterns were collected on 'Philips Diffractometer' at the wavelength λ=1.54 Å (CuK_{α}, a tube voltage 40 kV and tube current 25 mA). Bragg's equation, defined as wavelength (λ)=2$d$sinθ, was used to compute the crystallographic spacing (d) for the unmodified and modified montomorillonite. The term in the equation suggests; λ wavelength, δ basal spacing, sinθ diffraction angle.

Confirmation of Surface Modification Using FTIR. FTIR spectra of MMT and OMMT in powder form were recorded on Shimadzu FTIR-8000 spectrophotometer (Tokyo, Japan), to study functional groups attached at the time of surface modification. The samples were scanned from 800-4000 cm^{-1}.

Biodegradation Study.

Organism and Its Maintenance: The bacterial strain of *P. aeruginosa* was maintain on nutrient agar and routinely sub-culture after every three months, which was preserved at 4 °C. An active culture was used for degradation assay by inoculating the bacteria in a sterilized nutrient broth and incubated at room temperature for 24 h. The cells of *P. aeruginosa* were harvested in centrifuge at 4 °C and washed 2-3 times with a sterile saline. Culture of 1%, 0.1 O.D. (absorbance at 600 nm) was prepared in a sterile saline and used for further experimentation.

Assay Media: Minimum salt medium was used *in vitro* polymer degradation assay, which includes KH_2PO_4 (0.79 g/L), K_2HPO_4 (0.7 g/L), $MgSO_4.7H_2O$ (0.7 g/L), NH_4NO_3 (1 g/L), NaCl (5 mg/L), $FeSO_4{\cdot}7H_2O$ (2 mg/L), $ZnSO_4{\cdot}7H_2O$ (2 mg/L), and $MnSO_4{\cdot}H_2O$ (1 mg/L) with pH 7±0.2 was maintained. Media was steam sterilised at 121 °C (15 psi) for twenty minutes in an autoclave. Composites (0.5 cm^2) were surface sterilized with $HgCl_2$ (0.5%) solution and sub sequentially washed with distilled water in a sterile environment under laminar airflow.

***In vitro* Polymer Degradation Assay:** In sterile minimal salt medium, pre-weighed surface sterilized polymer composites were added in a (5%) solution of 0.1 O.D, at a wavelength of 600 nm was inoculated and incubated in a shaking incubator at room temperature for 28 days. After every 7 days a set of degraded assay was analyzed for its bacterial biomass, change in extracellular protein, pH and percent degradation.

Determination of Biomass: Incubated media was vortexed (shaking) for uniform mixing and biomass medium was used for detection of absorbance at 600 nm in UV-visible spectroscopy (UV mini-1240, Shimadzu Japan) with sterile minimal solution as a reference sample.

Determination of Protein: Incubated media was centrifuged at 10,000 rpm for 10 min for biomass settling. Supernatant liquid was analyzed at 280 nm in UV-visible spectrophotometer with sterile minimal solution as a reference sample.

Detection of Change in pH: The pH changes with change in metabolism activity. The pH was measured using pre calibrated pH meter (EQ-614 A).

Determination of % Degradation: Polymer composites were preweigh after degradation and washed with distilled water (D/W). The samples were dried in hot air oven at moderate temperature (65 °C) to evaporate moisture after this composites were weigh on ultra sensitive balance (Sartorius, CP 225D). Change in polymer weight was determined terms of in percent degradation.

$$\% \text{ Degradation} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} \times 100$$

Scanning Electronic Microscopy (SEM): Surface of film and bacterial growth in terms of degradation was performed on scanning electron microscope (SEM) (CEMECA model SU-1500, France). After incubation with bacterial culture the pieces of polymer were taken out from the culture and rinsed repeatedly with distilled water. The films were dried at 35 °C and used for evaluation of biodegradation efficacy. The same sample was used for SEM analysis.

Results and Discussion

Figure 1 shows the X-ray diffraction patterns of a) unmodified and b) organically modified MMT (OMMT) clay. Na^+-MMT showed characteristic diffraction peak at 2θ=6°, which was assigned to the interlayer platelet spacing (001 diffraction peak) of MMT with a d-spacing 16 Å. When the surface of MMT was modified as mentioned in the above text, peak was shifted towards the lower diffraction angle *i.e.* 2θ=2.8°, which relates with interlayer spacing of 22 Å. Overall, the improvement in the basal spacing suggests that column chromatography was an effective technique for increment in d-spacing of Na^+-MMT. The improvement in d-spacing of OMMT (22 Å) was due to ion exchange col-

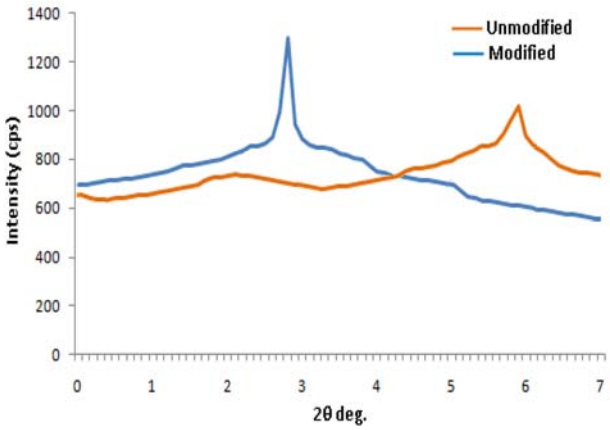

Figure 1. XRD patterns of modified and unmodified MMT.

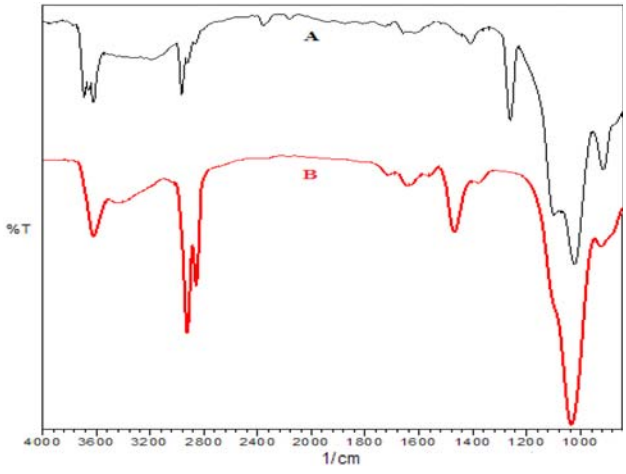

Figure 2. FTIR spectra of (A) MMT and (B) OMMT.

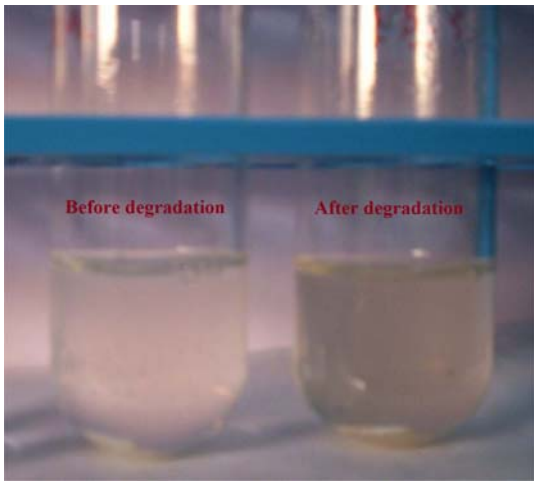

Figure 3. Photograph of before and after degradation of composite.

umn of sufficient length (35 cm) and diameter (5 cm) that provides maximum retention time for proper exchange of ions between MMT and ion exchange resin. Figure 2 shows the FTIR spectra of the MMT and OMMT. The initial montomorillonite shows a characteristic peak at 1033 cm^{-1} originating from Si-O-Si. A clear shoulder is observed at 914 cm^{-1} which corresponds to -Si-OH groups. Also weak peaks at 3616 and 1637 cm^{-1} are visible. The peak at 3616 cm^{-1} corresponds to free -OH groups. Ion exchanged clay shows clear -CH_2- peaks at 2920 and 2854 cm^{-1}, in addition to the characteristic peaks of montomorillonite can be seen.

In vitro submerged cultured microbial degradation of PS: PLA and PS: PLA: OMMT composites were carried out using of *P. aeruginosa*. Active bacterial culture in sterile basal mineral salt medium with different pre weighed polymer composites as carbon source were incubated at different (7, 14, 21, and 28 days) days at room temperature and analyzed for bacterial growth (biomass production), extracellular protein secretion by bacterium during incubation, change in pH of medium due to metabolic reaction of bacteria during degradation and degradation of polymer composites. Change in bacterial growth was observed visibly (Figure 3) with change in culture density (turbid and clear solution with degradation suggests the growth of bacteria in test medium solution with degradation). All compositions of polymer composites show such visible change, *i.e.* turbidity in medium after incubation. Spectrophotometrically bacterial growth density observed for its absorbance wavelength at 600 nm (Figure 4) which suggests increase in bacterial growth from 7 to 21 days. After 21 days, reduction in bacterial growth was observed as compared to 28 days. This might be due to lyses of bacterial cell therefore 21 days was the optimum growth period for such degradation *P. aeruginosa* supports maximum growth at 20% and 25% of PS: PLA polymer composites and 5 phr PS: PLA: OMMT nanocomposite. To make nutrient available bacteria secrete enzyme protein outside the cell which bind to complex nutrient and solubilise

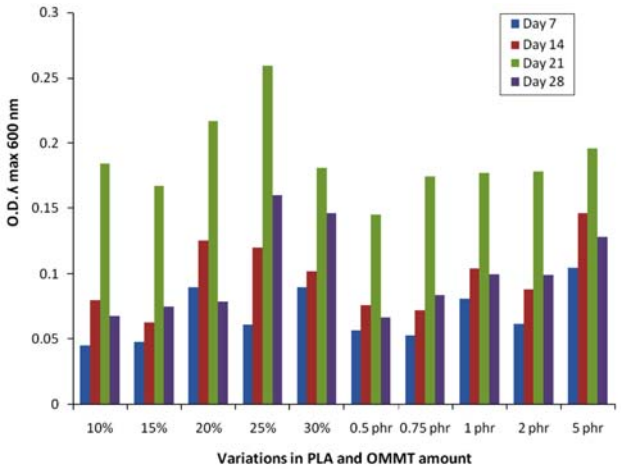

Figure 4. Bacterial growth density observed for its absorbance at 600 nm.

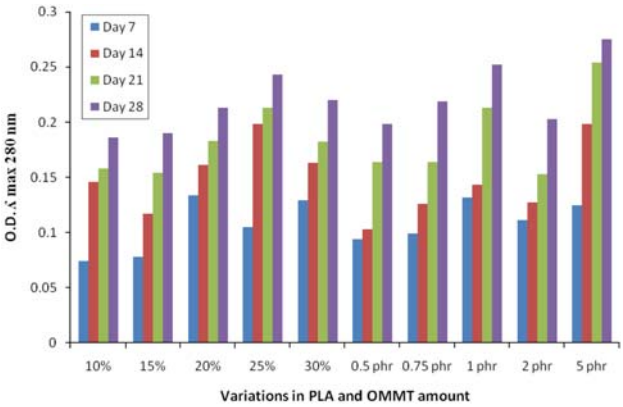

Figure 5. The change in extracellular protein of polymer nanocomposites at 280 nm.

it, and make available to the bacterium. The change in extracellular protein excretion by bacterium in degradation process was measured by its absorbance in UV-visible spec-

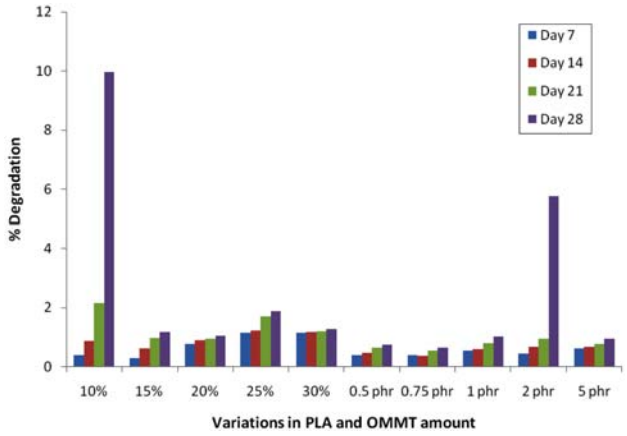

Figure 6. % degradation polymer composites.

trum at wavelength 280 nm revealed the maximum protein excretion at 28 day of incubation. Figure 5 shows maximum protein release in tube containing 5 phr PS: PLA: OMMT nanocomposite and 25% PS: PLA composite. However, protein excretion seemed to be for other composites constant. The change in pH of medium was exerted sometime due to extracellular metabolite production by bacteria. Such a change in pH of media was observed; however no significant changes could be seen, suggesting that pH modulating metabolites might not have produced by the organism during biodegradation assay. Figure 6 suggests the % degradation by bacterium under optimal stabilized condition. The maximum degradation supported at 10% and 25% PS: PLA composites and 2 phr PS: PLA: OMMT nanocomposite (9.9% and 5.7%), respectively, while it was seemed to be lesser in other composites. In this case optical micrographs helped to see the real degradation potential of bacterial strain in all the studied cases.

Morphological Study. Figures 7(a)-(e) and 8(a)-(e) show PS: PLA composites and PS: PLA: OMMT nanocomposites before degradation. These respectively samples were referred for the changes occur on surface of composites after degradation. Figures 9(a)-(e) and 10(a)-(e) show the SEM of PS: PLA composites and PS: PLA: OMMT after 28

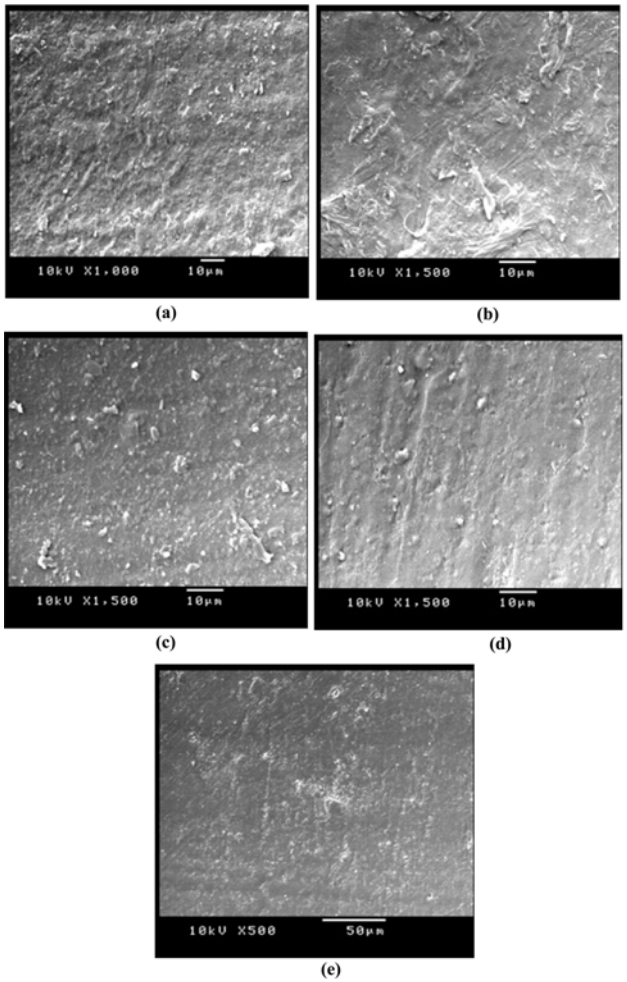

Figure 7. SEM of PS: PLA polymer composites before degradation (a) 10%, (b) 15%, (c) 20%, (d) 25%, and (e) 30% composite.

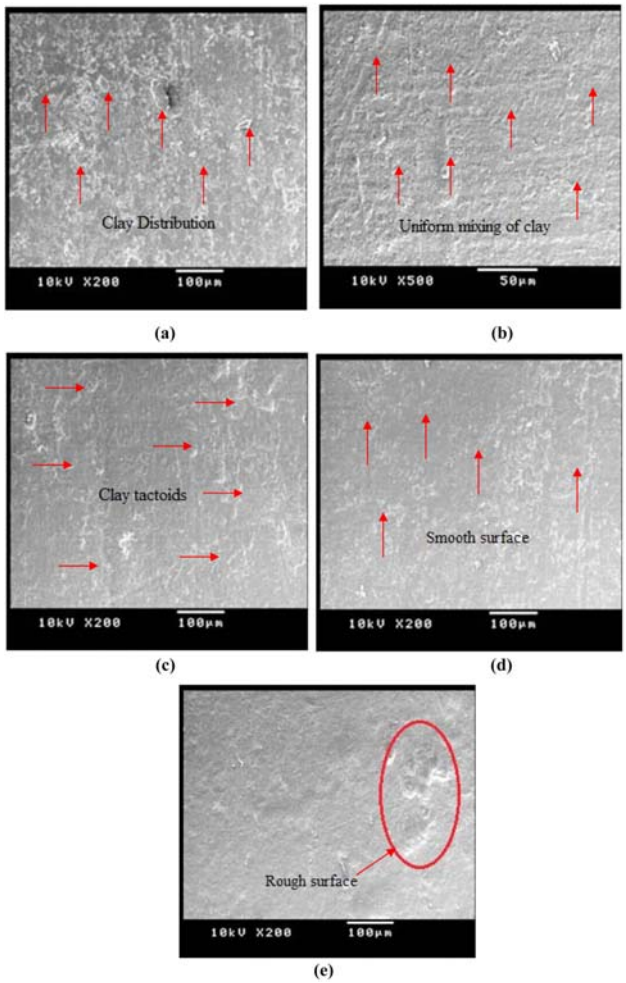

Figure 8. SEM of PS: PLA: OMMT polymer nanocomposites before degradation (a) 0.5, (b) 0.75, (c) 1, (d) 2, and (e) 5 phr.

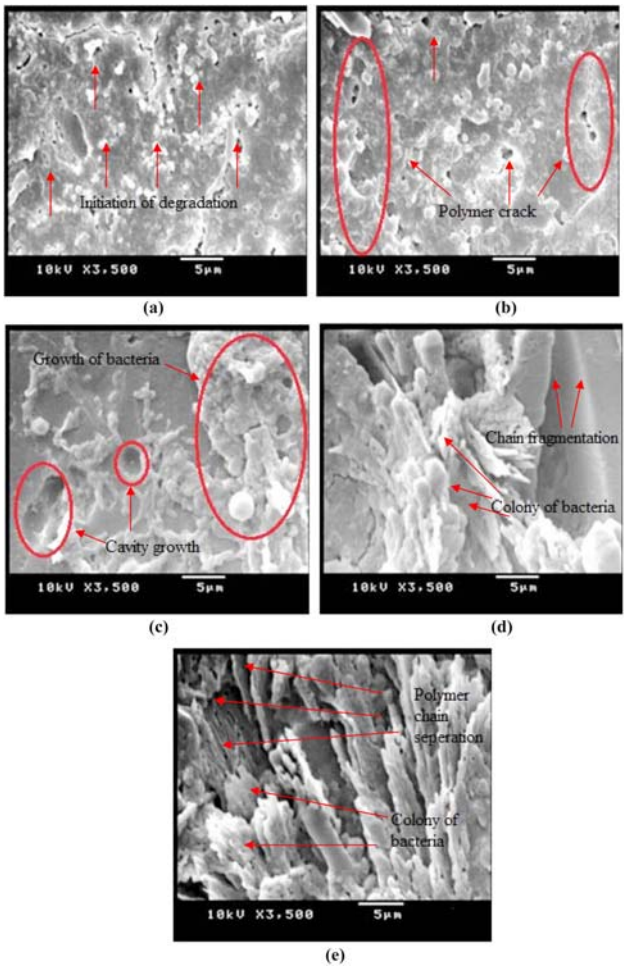

Figure 9. SEM of PS: PLA polymer composites after (28 days) degradation (a) 10%, (b) 15%, (c) 20%, (d) 25%, and (e) 30% composite.

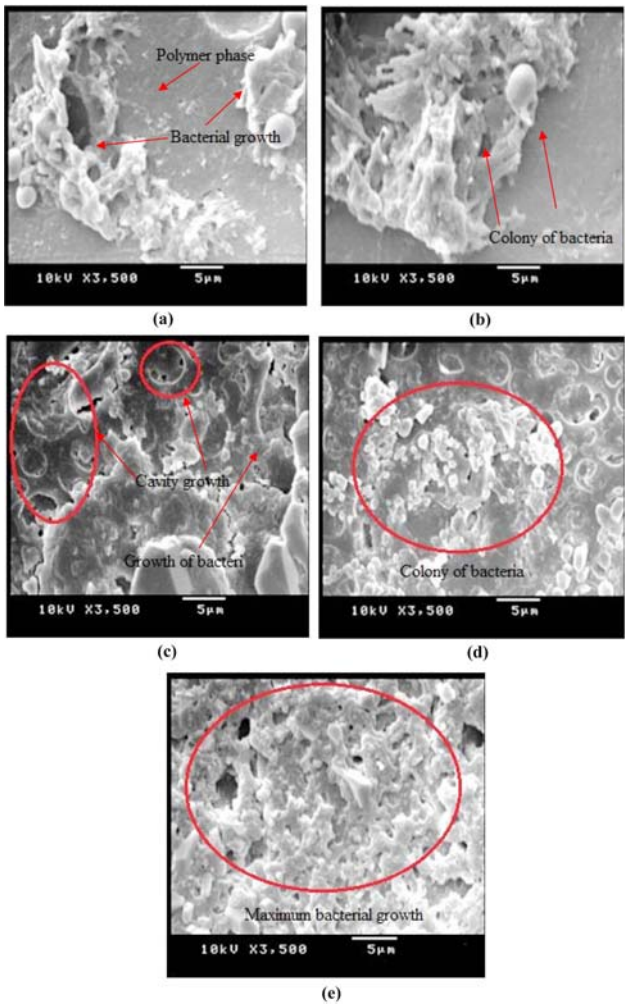

Figure 10. SEM of PS: PLA: OMMT polymer nanocomposites after (28 days) degradation (a) 0.5, (b) 0.75, (c) 1, (d) 2, and (e) 5 phr.

days of incubation with microbial strain. The composites shows somewhere rough surface even before degradation. 15% and 25% PS: PLA composites show smooth surface as compared to other compositions (10%, 20%, and 30%) while SEM pictures of PS: PLA: OMMT nanocomposites (Figure 8) seems to be very smooth and glossy due to soft nature of OMMT and uniform mixing of OMMT and PLA in PS before degradation. Figures 7 and 8 is clearly shows the difference between both polymer composites, upon comparing 2 and 5 phr PS: PLA: OMMT nanocomposites shows smooth surface as compared to 25% and 30% PS: PLA composites. Figure 8 shows SEM of PS: PLA composites after microbial degradation (28 day). Due to microbial attack up to (28 days) composites shows the rough surface with growth of bacterial culture. At 25% and 30% of PS: PLA composites, was bacterial growth maximum with hierarchical structure. Whereas Figure 10 shows SEM of PS: PLA: OMMT nanocomposites after microbial degradation (28 days). The rate of degradation was found to be more effective in case of PS: PLA: OMMT nanocomposites as compared to PS: PLA composites. Figure 10(b)-(e) shows the maximum degradation due to OMMT loading, which enhance the rate of degradation along with PLA.

Conclusions

The biodegradation of PS: PLA and PS: PLA: OMMT nanocomposite was studied using *P. aeruginosa* with various compositions. It was found that all composition supported to the degradation nature properly. The bacterial turbidity and a visual degradation measure in test assay tube were supported by bacterial growth, extracellular protein excretion, percent change in degradation and optical microscopic images. The bacterial growth and extracellular protein concentration varies with various composition. 10% PS: PLA and 2 phr PS: PLA: OMMT nanocomposite showed maximum degradation efficiency. More addition of some

bio accessible material in composition might be showed increase in degradability of polymer. This degradation of PS: PLA: OMMT (2 phr) and PS: PLA (10%) might be due to secreation amount of protein in PS: PLA (10%) and this secreation amount was well protect by OMMT, which initiating the fast degradation of PS: PLA: OMMT (2 phr) nanocomposites. The increase in degradation of PS: PLA: OMMT was due to OMMT and PLA, and degradation rate was more due to loading of OMMT filler, this is because of improved *d*-spacing of OMMT facilitates secretion of bacteria within the polymer chains. Moreover, due to increased *d*-spacing the MMT becomes hydrophilic and it permits easy absorption of water into the polymer chains and activates the rate of degradation with bacterial growth inside the polymer matrix.

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