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HYDROXYETHYLMETHACRYLATE-GRAFT-CARBOXYMETHYL CHITOSAN-GRAFT PHA (HEMA-g-CMCH-g-PHA): SYNTHESIS, CHARACTERIZATION AND BIODEGRADATION

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ABSTRACT

Chemical modifications of polyhydroxyalkanoates are important to improve the properties and increase the applicability of PHAs in different fields. In the present study using grafting method, the graft of PHA_{MCL} produced by *Comamonas testosteroni* was synthesized using acrylic acid and O- carboxymethylchitosan as a backbone. Successful synthesis of HEMA-g-CMCH-g-PHA was confirmed by FTIR analysis. Thermal properties of the synthesized graft were studied by Thermo Gravimetric Analysis and Differential Scanning Calorimetry. Degradation studies of PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA were carried out using bacterial isolate *Burkholderia cepacia* 202 and fungal isolate *Aspergillus fumigatus* 202 for 15 days. Different parameters such as extracellular protein concentration, growth of organism and % weight loss of polymer were studied to correlate it to its degradation. *Aspergillus fumigatus* 202 was found to degrade 94% of CMCH and 84% of HEMA-g-CMCH-g-PHA in 15 days at static condition.

Key words: acrylic acid, biodegradation, chemical modification, HEMA-g-CMCH-g-PHA, PHA_{MCL}

INTRODUCTION

The increased sensitivity to ecological problems will have a major impact in the future on the disposal of plastic articles. Polymer and plastic industries may be forced to explore the production of biodegradable polymers as an alternative to traditional plastics. Poly(3-hydroxybutyrate) was identified as an intriguing bacterial inclusion body more than seven decades ago [1] and is now classified as one of the many different types of bacterial polyesters with common name polyhydroxyalkanoates (PHA). These polymers are accumulated intracellularly to the level as high as 90% of cell dry weight under condition of nutrient stress and act as a carbon and energy reserve material [2]. Polyhydroxyalkanoates are a kind of polymer having similar mechanical properties to those of polypropylene with the additional advantage of being completely biodegradable and biocompatible [3]. The monomer composition of the PHA depends on the bacterial strain as well as the carbon sources supplied [4]. PHA_{MCL} are elastomeric but have very low mechanical strength which limit the application of these PHAs. The physical and mechanical properties of these PHAs need to be diversified and improved for packaging materials, biomedical applications, tissue engineering and other specific applications [5]. Both biological and chemical modifications are carried out to improve polymer properties. Chemical modifications include blending, grafting, cross linking, epoxidation etc. Insertion of an additional different polymer segment into an existing polymer backbone or as the side chain of an existing polymer yields blocks or graft copolymer respectively [6].

The natural polymers that have attracted great attention recently are polyhydroxyalkanoates and chitosan. Chitosan is a liner polymer composed of β-1,4-linked glucosamine residues [7]. It is prepared commercially by alkaline deacetylation of chitin. Chitosan has some advantages due to its nontoxicity, biodegradability and it is environmental friendly. It is a biocompatible material that breaks down slowly to harmless products that are absorbed completely in body [8]. Carboxymethylation of chitosan provides attractive sites for further chemical modifications such as grafting, hydrolysis and oxidation. Hybridization of natural polymers with synthetic polymers is of great interest because of their application to biomedical and biodegradable materials [9]. Acrylic acid is a synthetic monomer which undergoes reactions characteristics of both unsaturated acids and aliphatic carbolic acids or esters. The β carbon atom of acrylic acid polarized by carbonyl group, behaves as an electrophile; and favours the addition of large variety of nucleophiles and active hydrogen compounds to the vinyl group. Acrylic acid and its esters are used in the production of coatings, adhesives, elastomers, super absorbent polymers and flocculants. Considering these facts the present study has been deal with the synthesis of graft with chitosan, PHA and acrylic acid as these materials having applications in different fields.

The ability to degrade PHA is widely distributed among bacteria as well as fungi, and depends on the secretion of specific PHA depolymerase and also on the physiochemical nature of the PHA itself [10]. In the cell, PHA forms amorphous granules which can be degraded by the PHA-accumulating organism itself. After PHA is extracted from the cell, it can be enzymatically degraded by extracellular PHA depolymerase [11].

MATERIALS AND METHODS

Microorganisms and culture media

Comamonas testosteroni as a PHA_{MCL} producer in this study, was reported to accumulate PHB and PHA_{MCL} [12,13]. *C. testosteroni* produced PHA_{MCL} utilizing coconut oil as sole carbon source. The medium used for PHA_{MCL} production was Bushnell Haas mineral salt medium (Hi-Media,India), which contained (g/l of distilled water) $MgSO_4$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1 , $FeCl_3$ 0.05 and 1% w/v coconut oil purchased from the local market.

For degradation studies, a bacterial culture *Burkholderia cepacia* 202 and a fungal culture *Aspergillus fumigatus* were used. Both these organisms are reported to be degraders of PHAs [14]. The medium used for the degradation studies was Bushnell Haas mineral salt medium with synthesized polymer as a carbon source [14].

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PHA_{MCL} production and extraction

Inoculum was prepared by growing *C. testosteroni* cells in Nutrient Broth at 30 ºC overnight on a rotary shaker at 150 rpm. The overnight grown culture was centrifuged and the pellet was washed twice with sterile normal saline. The cells were then transferred to 50 ml of production media containing 1% w/v coconut oil and incubated overnight at 30 ºC on a rotary shaker at 150 rpm. Five ml broth was centrifuged and cell pellet was suspended in 5 ml normal saline to measure the OD_{660} for monitoring the growth. This cell suspension was used as an inoculum for PHA_{MCL} production.

After the 48 h of incubation on a rotary shaker at 150 rpm at 30 ºC, the cells were harvested from the production media by centrifugation at 8000 rpm for 10 min. The pellet was washed with acetone thrice and dried overnight at room temperature. PHA was extracted from dried cell mass by overnight chloroform extraction at 30 ºC at 200 rpm on rotary shaker and then cell mass was removed by filtration through Whatman No. 1 filter paper. Chloroform extract was concentrated by evaporating chloroform. PHA was precipitated by adding cold methanol drop wise and purified PHA was obtained by repeating this step three times.

Synthesis of HEMA-g-CMCH-g-PHA

O-carboxymethylchitosan (CMCH) was prepared according to the method described by Chen and Park [15], where chitosan: sodium hydroxide: isopropanol were mixed in the ratio 10 g:13.5 g:100 ml in the flask. Chitosan was allowed to swell and alkalize at room temperature for 1 h. The monochloroacetic acid dissolved in isopropanol was added drop wise into the reaction mixture for 30 min and then allowed it to react at 55 ºC for 4 h. The reaction was stopped with acetic acid and carboxymethylchitosan obtained as a solid product which was separated by filtration. Desalting was done by rinsing the CMCH with 80-90% ethanol.

For preparation of HEMA-g-CMCH-g-PHA graft, first grafting was carried out between CMCH and PHA in the ratio of 2 g:0.5 g. For this CMCH was stirred in 120 ml double distilled water with slow stream of nitrogen for 30 min at room temperature to remove dissolved oxygen and then 2M ceric ammonium nitrate prepared in 0.3M HNO_3 was slowly added over a period of 20 min. PHA was dissolved in dichloromethane and half of PHA added drop wise for 20 min. In the next step, after the incubation of 30 min, remaining half PHA solution was added with 1.5 ml of acrylic acid. Grafting reaction was allowed to occur at constant stirring at 40 ºC for 5 h and product was dried under vacuum.

Characterization of PHA_{MCL}, CMCH, and HEMA-g-CMCH-g-PHA

Infrared spectra of PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA were recorded with a Perkin Elmer, Fourier-transform infrared spectrometer. Thermal stability of all three polymers was determined by Thermo Gravimetric Analysis (TGA) (Universal V2.6D, TA instrument) with heating rate of 10 ºC. The synthesized polymers were also characterized by Differential Scanning Calorimetry (DSC).

Biodegradation of HEMA-g-CMCH-g-PHA

Biodegradation studies of all three polymers PHA_{MCL}, CMCH, HEMA-g-CMCH-g-PHA were carried out using one bacterial isolate *B. cepacia* 202 and one fungal isolate *A. fumigatus* 202. Graft (0.5g) was kept in a 250 ml Erlenmeyer flask containing 100 ml BHM. The flasks were inoculated with *B. cepacia* 202 (1.0 initial OD_{660}) and incubated at 37 ºC under static condition as well as shaking conditions (150 rpm). Similarly flasks were inoculated with 10^6 spores/ml of *A. fumigatus* 202. Samples of 2 ml each were removed at an interval of two days for a period of 15 days and analyzed for growth (OD_{660}) and extracellular protein concentration [16]. % weight loss of the polymer in each flask was determined after 15 days [14,17].

RESULTS AND DISCUSSION

It is known from the earlier reports [13] that *C. testosteroni* accumulates PHA_{MCL} when grown it on vegetable oil [13]. The present study reports PHA_{MCL} accumulation upto 83% (w/w) of dry cell mass when *C. testosteroni* was grown on coconut oil (1% w/v) as a sole carbon sorce. The PHA_{MCL} thus produced has been used for further grafting reactions with an aim of getting a chemically modified product.

Various PHA_{MCL} bearing different functional groups in the side chain has been synthesized by some organisms, including *Pseudomonas oleovorans* and *Pseudomonas putida*, when they are grown with substrates containing the corresponding chemical structure [18]. PHA_{MCL} with functional groups are of great interest, because the functional groups can improve the physical properties. Moreover, some functional groups can be modified by chemical reactions to obtain more useful polymer and extend the potential application of PHA_{MCL} as environmentally biodegradable polymers as well as functional biomaterials for biomedical uses. Therefore, attempts to modify the properties of PHA_{MCL} by chemical and physical methods such as blending, crosslinking, and graft copolymerization, have attracted a great deal of interest. As chitosn has reactive amino and hydroxyl group, acrylic acid also has two functional groups [19]. The carbonyl group of β carbon atom of acrylic acid behaves as an electrophile; favouring addition of large variety of nucleophiles and active hydrogen compounds. Therefore it has been used in this study for grafting on to a PHA chain.

In the present study grafting of PHA_{MCL} with chitosan and acrylic acid was carried out and PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA were analysed for their thermal stability and biodegradability.

Characterization of PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA

Very few studies on grafting of PHA with chitosan and acrylic acid have been carried out. Yalpani *et al.* [20] and Arslan *et al*. [8] developed graft of PHA onto chitosan and cellulose. PHA_{MCL} was graft polymerized with various polymers like polyisoprene, acrylic acid, methyl methacrylate [21] and vinyl monomer. PHB-*g*-PI has much better ductility and tenacity than homo-PHB [22]. Mainly the acrylic acid was grafted with the synthetic polymers such as polystyrene, polyethylene [23].

Infrared Spectroscopic analysis of PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA polymers were analyzed from 400 to 4000 cm^{-1} by Perkin-Elmer Spectrum GX. Analysis of IR spectra of PHA_{MCL} (Fig. 1) and HEMA-g-CMCH-g-PHA (Fig. 3) showed the presence of some of the additional peaks at 3427.86 cm^{-1}, 1738.24 cm^{-1} and 517.48 cm^{-1}. When IR spectrum of HEMA-g-CMCH-g-PHA (Fig. 3) was compared with that of CMCH (Fig. 2), the presence of additional peak at 1159.44 cm^{-1} and loss of peak at 2103.32cm^{-1} was observed. The predominant peak at 1738.24 cm^{-1} was observed in the IR spectrum of graft (Fig. 3), which shows C=O stretching, indicating the insertion of acrylic acid into the graft. This confirms the grafting has occurred successfully with PHA_{MCL}, CMCH and acrylic acid. Presence of additional functional group provides the attractive site for further modification and increase the graft applicability. Grondahl *et al.* [24] reported the graft copolymerization of acrylic acid (AAc) onto PHBV by

gamma-irradiation to induce surface hydrophilicity of PHBV for biomedical applications. Graft copolymers of acrylic acid on cellulosic materials [25] and PHBV [26] have been studied. For the foregoing account it is clear that, this is the first report on grafting of PHA with both chitosan and acrylic acid.

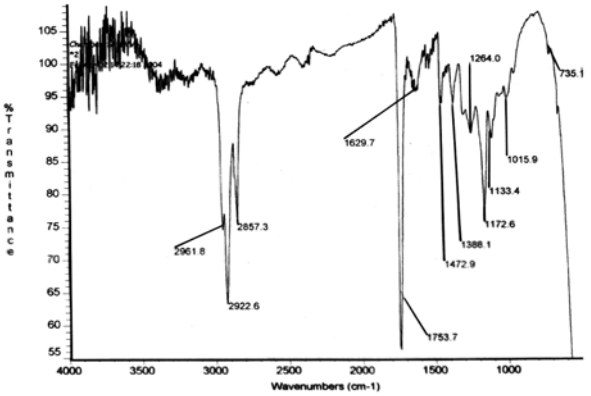


Fig. 1 FTIR of PHA_{MCL} produced by *C. testosteroni*.

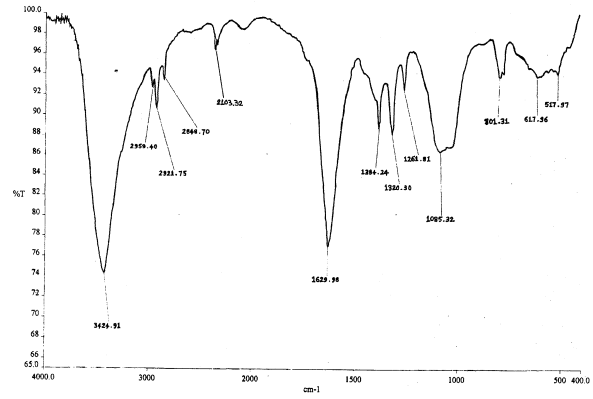


Fig. 2 FTIR of CMCH

Thermo gravimetric analysis of HEMA-g-CMCH-g-PHA was carried out by Perkin-Elmer TGA-7 DSC-PYRIS-1 DTA-7 instrument with the heating rate of 10 °C. The change in the thermal properties of PHA_{MCL} after grafting can be observed from the thermal analysis of both. Thermo gravimetric analysis (TGA) of the PHA_{MCL} (Fig. 4) obtained from *C. testosteroni* revealed that the polymer was stable up to 233.15 °C with weight loss of 4.39%; thereafter the thermal decomposition started which showed weight loss of 74.28% at 299.37 °C, whereas the polymeric graft showed about 29% weight loss at same temperature and 50% weight loss was observed at 280.57 °C for PHA_{MCL}.

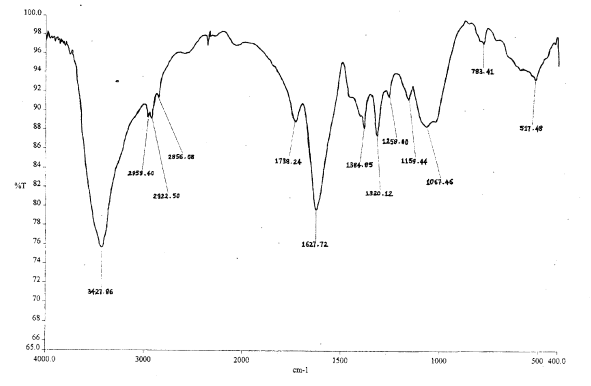


Fig. 3 FTIR of HEMA-g-CMCH-g-PHA

The 3.07% weight loss of HEMA-g-CMCH-g-PHA (Fig. 6) at 100 °C was due to the moisture content present in the sample. 50% weight loss was observed at 425 °C and at 550 °C, the observed weight loss was 62.94%. Average % weight loss observed at every 50 °C rise in temperature was 6.77. Decreased thermal stability was reported by Arslan *et al*. [8] while grafting polyhydroxyoctanoate (PHO) onto chitosan. In contrast, due to grafting of PHA_{MCL} with CMCH and acrylic acid the thermal stability of PHA_{MCL} (Fig. 4) and CMCH (Fig. 5) increased for HEMA-g-CMCH-g-PHA (Fig. 6). The increased thermal stability helps in applying the polymer for preparation of articles which require higher temperatures for proper molding.

Differential scanning calorimetric (DSC) thermo gram of PHA_{MCL} (Fig. 7) depicted the thermal stability of native PHA_{MCL} between 230-240 °C which confirmed the results of TGA analysis. The melting point of PHA_{MCL} was 50 °C which was similar to the reported T_m range between 39 °C to 61°C [27]. DSC thermogram of HEMA-g-CMCH-g-PHA (Fig. 8) didn't show any sharp Glass Transition (Tg). Endotherm up to the temperature 180 °C was observed. At around 205 °C peak shows high temperature decomposition or reaction which may have resulted in two extra endotherm at 220 °C and 264 °C. This high temperature decomposition might be attributed by acrylic acid present in the graft. Integral Procedural Decomposition Temperature (IPDT) was 250 °C. Single endotherm from 50 °C to 74.90 °c for CMCH-g-PHA was reported by Bhatt *et al* [14].

Biodegradation of PHA_{MCL}, CMCH and HEMA-g-CMCH-g-PHA

Many bacteria as well as fungi have the ability to degrade PHA [28]. Biodegradation of polymers by microorganisms is catalysed by extracellular, degradative enzymes that produce water soluble, low molecular weight products from the macromolecular substrate. The extracellular PHB depolymerase of *A. fumigatus* was found to have a broad hydrolytic activity towards bacterial and synthetic aliphatic polyesters. Bacterial poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) were found to be readily degraded by microorganisms in marine, sewage sludge, soil and compost ecosystems [29]. CMCH is also a biodegradable polymer derived from chitin through chitosan [30]. CMCH and acrylic acid both possess versatile groups responsible for the biodegradation. Dave *et al.* reported the degradation of acrylic acid by fungi [31]. Biodegradability and antibacterial activity of PHB and PHBN membranes grafted with acrylic acid, chitosan and chitooligosaccharide were assessed by Hu *et al*. [32].

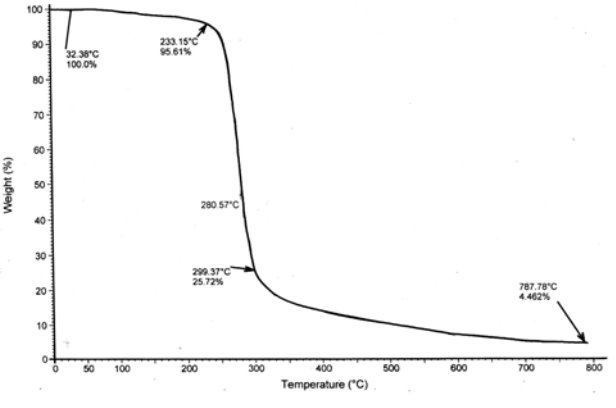


Fig. 4 Thermo gravimetric analysis of PHA_{MCL} produced by *C. testosteroni*.

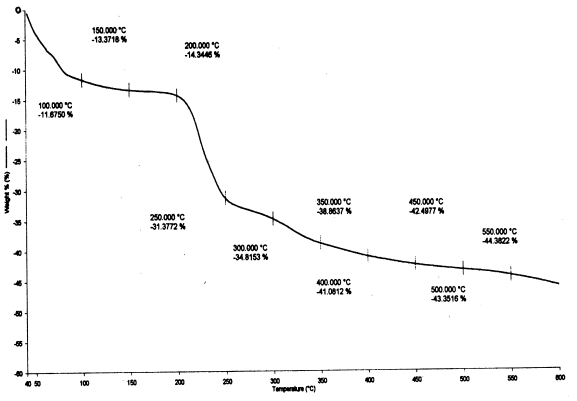


Fig. 5 Thermo gravimetric analysis of CMCH

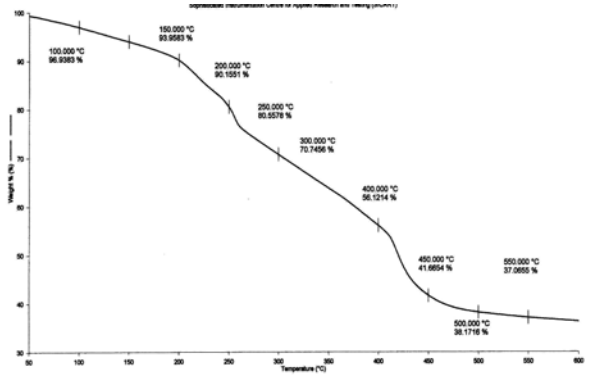


Fig. 6 Thermo gravimetric analysis of HEMA-g-CMCH-g-PHA

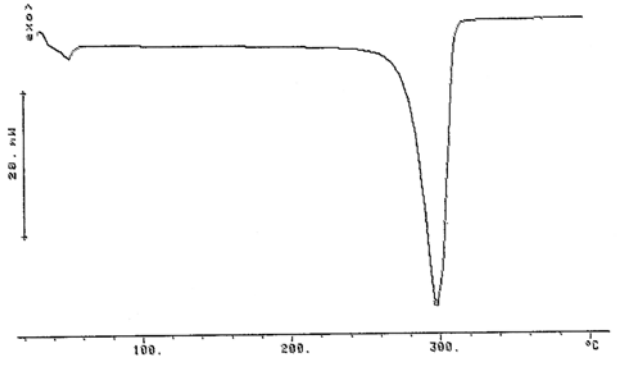


Fig. 7 DSC thermo gram of PHA_{MCL} produced by *C. testosteroni*.

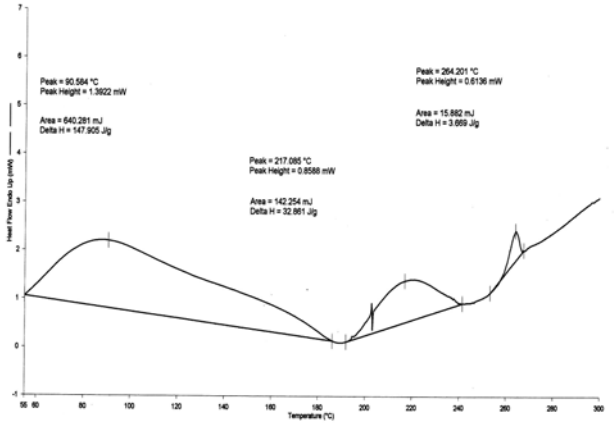
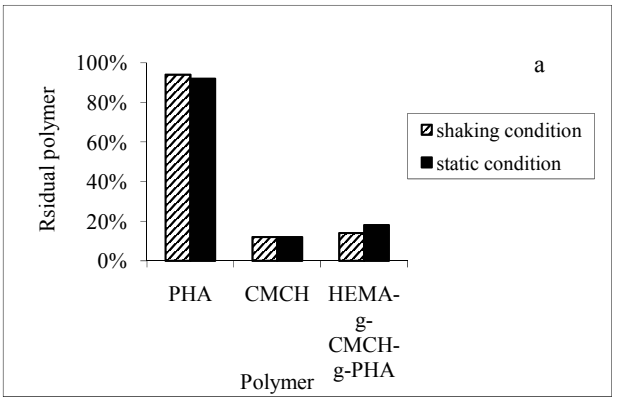


Fig. 8 DSC thermo gram of HEMA-g-CMCH-g-PHA



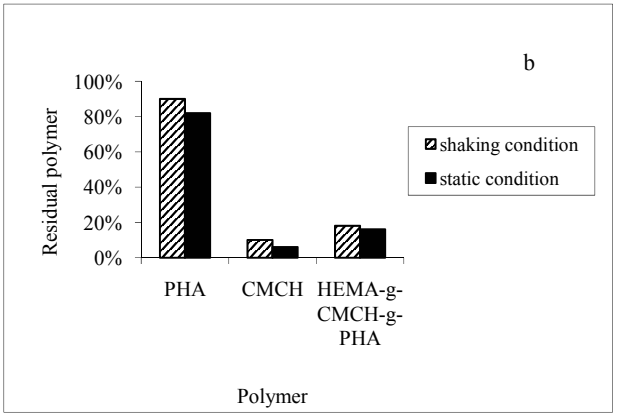


Fig. 9 Degradation of HEMA-g-CMCH-g-PHA graft polymer after fifteen days **(a)** *B. Cepacia* 202; **(b)** *A. fumigatus* 202.

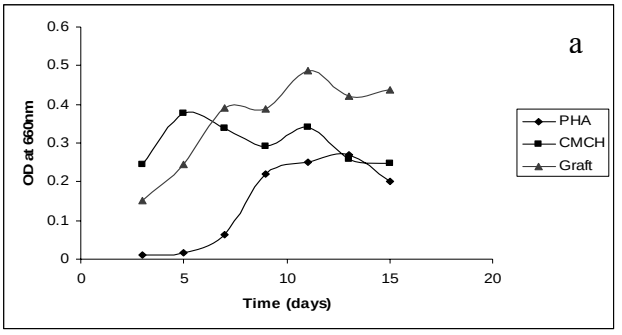


Fig. 10 (a) Growth of *B. cepacia* 202 on polymer under shaking condition

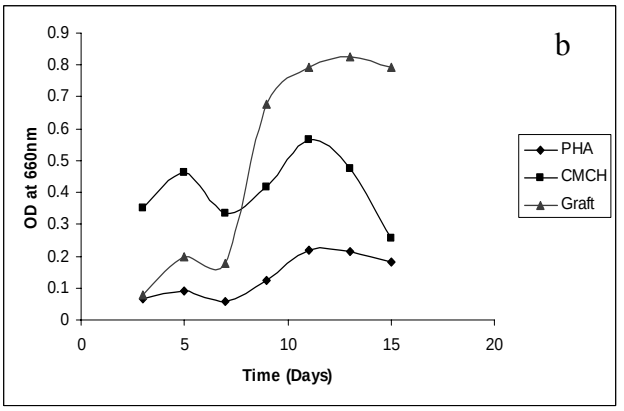


Fig. 10 (b) Growth of *B. cepacia* 202 on polymer under static condition

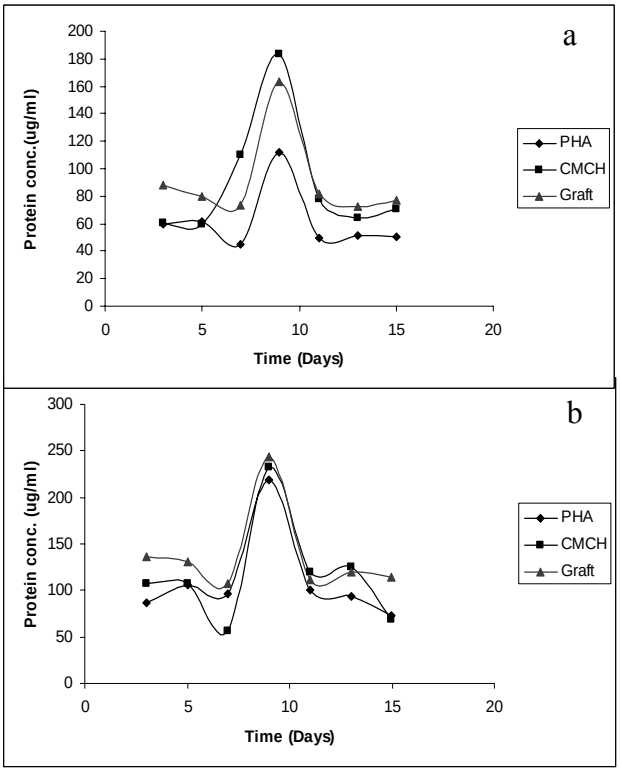


Fig. 11 Extracellular protein profile of *B. cepacia* 202 under **(a)** shaking condition; **(b)** static condition

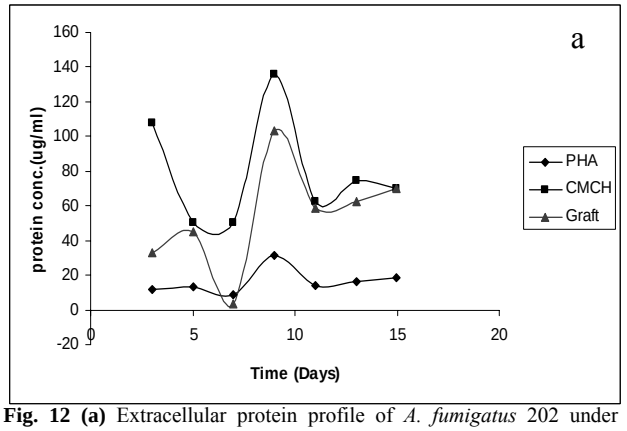


Fig. 12 (a) Extracellular protein profile of *A. fumigatus* 202 under shaking condition

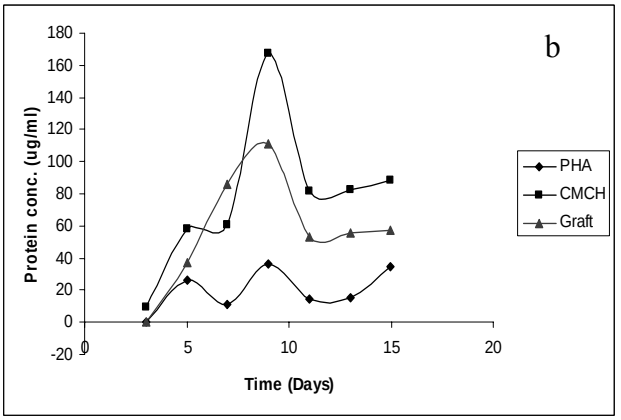


Fig. 12 (b) Extracellular protein profile of *A. fumigatus* 202 under static condition

The graft synthesized from biodegradable material may also be biodegradable and eco-friendly. Moreover CMCH-*g*-PHA_{MCL} was insoluble in various solvents [14] and had high thermal stability which proved it to be an industrially viable polymer. Among all the three polymers; CMCH, PHA_{MCL} and HEMA-g-CMCH-*g*-PHA; weight loss of CMCH was maximum followed by that of graft and least weight loss was observed in pure PHA_{MCL} polymeric samples (Fig. 9 a,b).

Carboxymethylchitosan, derived from chitosan, showed the highest degradation (94% weight loss) by both fungal and bacterial cultures. HEMA-g-CMCH-*g*-PHA_{MCL} was also degraded by both fungi as well as bacteria, but the extent of degradation was less as compared to pure CMCH. This might be attributed to the insertion of PHA_{MCL} and acrylic acid onto CMCH which resulted into blockage of few carboxyl groups where PHA_{MCL} and acrylic acid might be grafted. Least degradation, ranging from 6% - 18 % weight loss was observed in case of PHA_{MCL}, this might be due to presence of long aliphatic chains in the structure of PHA_{MCL}. From the results of % weight loss it can be concluded that static conditions may be more suitable for degradation studies as it would be economically more feasible. We obtained increase in % weight loss in static condition than the % weight loss reported in biodegradation studies earlier by Bhatt *et al.* [14].

The increase in growth of the *B. cepacia* 202 (Fig. 10 a,b) using PHA_{MCL}, CMCH and HEMA-g-CMCH-*g*- PHA as a sole source of carbon, checked in terms of OD_{660} showed that the polymers supported the growth of *B. cepacia* 202 during its degradation. Increase in growth proved to be an indirect indication for the biodegradation of the graft.

Along with the weight loss, extracellular protein content was also determined, considering that the concentration of protein can be attributed to the concentration of enzymes which are responsible for the degradation of the polymer. *B. cepacia* 202 showed increase in extra cellular protein concentration up to 243.6 µg/ml under static and 183.6 µg/ml under shaking condition and *A. fumigatus* 202 showed highest protein concentration up to 135.6 µg/ml under shaking and up to 167.4 µg/ml under static conditions. This support the results of % weight loss experiment. Increase in the growth as well as extracellular protein concentration (Fig. 11 a,b and 12 a,b) in media containing PHA_{MCL}, CMCH and HEMA-g-CMCH-*g*-PHA proved their degradation by *B. cepacia* 202 and *A. Fumigatus* 202.

CONCLUSION

Grafting of acrylic acid and chitosan to PHA_{MCL} increase the avaibility of functional group on graft and widen the application range of medium chain length polyhydroxyalkanoates by altering its thermal properties and also lead to synthesis of biodegradable material.

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