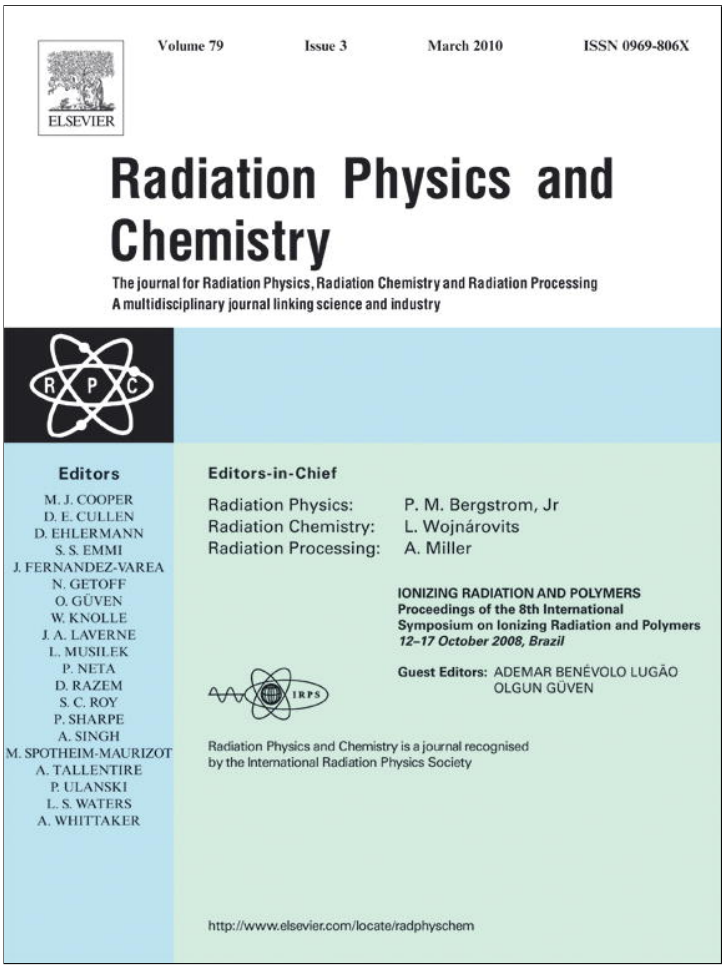

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Immobilization of bacteria in microgel grafted onto macroporous polyethylene

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ABSTRACT

The development of "*Green Chemistry*" requires new materials to replace the conventional organic chemistry by biological catalysts, to produce fine chemicals in an environmentally friendly manner. Microbial whole cells can be directly used as biocatalysts, providing a simple and cheap methodology since enzyme isolation and purification are avoided.

High-density polyethylene (HDPE) is a very stable polymer though it can be activated by gamma radiation to induce grafting. Glycidyl methacrylate was grafted onto macroporous HDPE and PP in the range of 1–6%, proportional to the initial monomer concentration. Grafted polymers were further chemically modified with ethylenediamine to generate a cationic hydrogel of micron-size thickness onto the internal polymer surfaces. Modified polymers were able to immobilize Gram-positive and Gram-negative bacteria that can catalyze a chemical reaction as efficient as free cells do.

1. Introduction

The development of "*Green Chemistry*" requires new materials to replace the conventional organic chemistry by novel catalyzed-driven reactions, such as biological catalysts (microorganisms, organelles, and enzymes), to produce fine chemicals in an environmentally friendly manner. Microbial whole cells can be directly used as biocatalysts, providing a simple and cheap methodology since enzyme isolation and purification are avoided (Prasad et al., 1999; Utagawa, 1999). Up to now most of these reactions are carried on using free cells. Cell immobilization can improve the process economy by increasing reusability and/or feasibility. Entrapment techniques into hydrogels are the most used way to immobilize microbial cells; however, mechanical resistance is their major drawback.

Recent advances in macromolecular biomaterial technology combine the efforts of scientists in various fields to obtain polymers with well-defined structures and specific chemical, physicochemical and mechanical properties (Angelova and Hunkeler, 1999). In this way our laboratory proposes to combine the macroscopic mechanical strength and the low chemical reactivity of the high-density polyethylene (HDPE) with hydrophilicity and biocompatibility properties of hydrogels based on polymethacrylates. Due to the fact that microbial cells have predominantly negative charges on their surfaces, they can be efficiently adsorbed onto positively charged polymer surfaces (Terada et al., 2006).

HDPE and PP are very chemically stable polymers, however they can be activated by gamma radiation to induce grafting polymerization (Grasselli et al., 1999). In this work, we synthesize and characterize a methacrylate-based microhydrogel onto macroporous HDPE and PP to use them as an inert and rigid support of a bacteria-based biocatalyst.

2. Materials and methods

Macroporous sheets of HDPE and PP were kindly donated by Porex Technology (Fairburn, USA). HDPE sheets (1.5 mm wide) have a pore volume ranging from 40% to 50% and pore sizes between 45 and 90 μm. PP sheets have a similar pore volume and pore sizes between 125 and 175 μm.

Glycidyl methacrylate (GMA), iminodiacetic acid (IDA) and ethylenediamine (EDA) for synthesis were from Sigma Chemical Co., USA, and were used without further purification. Uridine and uracil were purchased from Sigma or ICN. Culture media chemicals were from Merck, and HPLC grade methanol was from Fischer. All other chemicals employed were of analytical grade.

Small pieces of HDPE and PP were immersed in nitrogen flushed monomer solutions and irradiated at room temperature in a ^{60}Co radiation source (PISI, CNEA-Eze, Argentine). After irradiation, they were thrice washed with 50% ethanol, and then water. Washed polymers were dried for 24 h in a vacuum oven at 60 °C

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and weighed. The degree of grafting was calculated as the percentage of increase in dry weight.

GMA-grafted materials were reacted with EDA by soaking the grafted material in EDA: water (1:2, v/v) at 45 °C for 16 h. The remaining epoxy group was hydrolyzed to a diol group by incubation in 0.5 mol/l sulfuric acid for 2 h at 80 °C.

The EDA-modified materials were soaked in 0.01 mol/L cupric sulfate solution for 15 min. After washing with deionized water, copper was released from the materials shaking them with 0.1 mol/L EDTA, pH 7.0, at room temperature for 2 h. Copper content was determined by spectrophotometric measurement of the EDTA–Cu^{2+} at 715 nm.

2.1. Cell growth and immobilization conditions

E. coli BL21 (ATCC 47092) and *Bacillus cereus* were grown at 30 °C for 16 h, with shaking, in 250 ml Erlenmeyer flasks containing 50 ml of LB culture medium: 1% (w/v) meat extract, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in deionized water adjusted to pH 7 with KOH. Cells were harvested by centrifugation for 10 min at 12,000*g*, washed once with a 30 mM pH 7 potassium phosphate buffer and re-centrifuged. The wet cell paste suspended in buffer was directly incubated with the polymer during 24 h at 35 °C and shaking at 200 rpm. The biocatalyst (polymer plus cells) was washed and stored in buffer at 4 °C until use (catalyst load $50{,}000 \times 10^6$ cells/g).

Biocatalysts were dyed with methylene blue solution for 2 min, washed with distilled water and rinsed. Thin slices were observed in optical microscope *Olympus CH-2* at 1000× magnification.

All chemical and biochemical steps were followed by FT-IR ATR spectroscopy.

2.2. Biocatalyzed chemical reaction

The standard chemical reaction mixture comprising $5 \times 5\ mm^2$ biocatalyst prepared as above, 5 mM uridine, 10 mM adenine, and 1 ml 30 mM potassium phosphate buffer (pH 7), was stirred at 250 rpm at 50 °C for 3 h. Samples were centrifuged at 10,000*g* for 30 s, and the supernatants were analyzed by HPLC.

For the quantitative analysis of the reaction products an isocratic HPLC equipped with an UV detector (254 nm) and a Kromasil 100 C-18, 5 μm, 25×0.4 mm column was used. Production of uracil was determined using HPLC with the mobile phase water/methanol (12:88, v/v) and a flow rate 0.9 ml/min.

3. Results

HDPE and PP are very inert polymers to be used in biological environment; however they lack suitable surface properties to interact with living organisms. In addition to chemical inertness, mechanical properties and physical shape are important issues to be considered for the final application. Porous open-cell HDPE and PP in sheet shapes were chosen for grafting modification. The main topics in the material selection were that they have good mechanical properties to the share stress coming from the agitation of stirred tank and/or good liquid filtration properties, and no compression stress in liquid liter-scale tubular reactors in the biotech industry.

Macroporous PE and PP sheets were irradiated at 10 kGy soaked in 3% v/v GMA solutions under N_2 atmosphere at a dose rate of 1 kGy/h. The epoxy reactive pendant group of GMA monomer becomes a very interesting methacrylic monomer to modify non-reactive polymers such as HDPE. Under these experimental conditions most of the grafting polymerization is reached at the internal polymer surface, which was accessible to the monomer solution (Grasselli et al., 1999). Homopolymerization side reaction is the main drawback of this technique, particularly working with reactive methacrylates where harsh cleanup procedure cannot be applied to avoid chemical deactivation of reactive pendant groups.

Thus, in order to find out a suitable solvent to improve the yield of the grafting polymerization and reduce the homopolymer, we performed a preliminary screening of several polar solvents in a standard grafting condition. The results of the grafting yield performed in different alcohols as solvent are summarized in Fig. 1. Water was excluded as a consequence of monomer insolubility. The highest grafting yield was performed in methanol. The other alcohols gave similar grafting values, with slightly lower yields when alcohols with longer carbon chains were used.

In addition, grafting yield, homopolymer aspect and grafting distribution were taken into account. Homopolymer yielded in methanol was sticky and hard to remove under mild conditions (washing steps using alcohol and water at room temperature), which was also observed in the high standard deviation of grafting yield (Fig. 1). Also, inhomogeneous grafting distribution was revealed using a IDA/Cu reaction onto the grafted sample. Samples grafted in ethanol and isopropanol were also slightly inhomogeneous. Then, the ethanol/water mixture was chosen for further experimental work because it is a less harmful solvent and homopolymer is easily removed.

Fig. 2 shows the grafting yield of HDPE and PP macroporous sheets using different initial GMA concentrations in ethanol/water. Solutions of GMA from 1% to 5% v/v were employed. A linear relationship can be obtained at these very low monomer concentrations, as previously demonstrated onto porous polysulfone membranes (Carbajal et al., 2003). Grafting yield for a given solvent is proportional to the monomer concentration when available at low concentrations. At these conditions, monomer is the limiting reagent and polymerization stopped when it is exhausted. Additionally, yield reproducibility is higher than when other radiation-induced graft polymerization techniques are used. Differences between HDPE and PP in grafting yield were assigned mainly to the different polymer densities and internal surfaces of both materials (around 1 m^2/g).

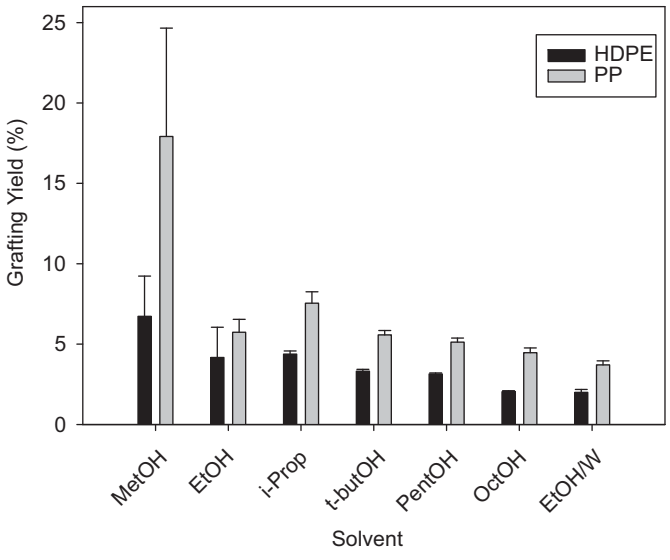

Fig. 1. Grafting yield of GMA grafted HDPE and PP using different solvents. Total dose: 10 kGy at 1 kGy/h.

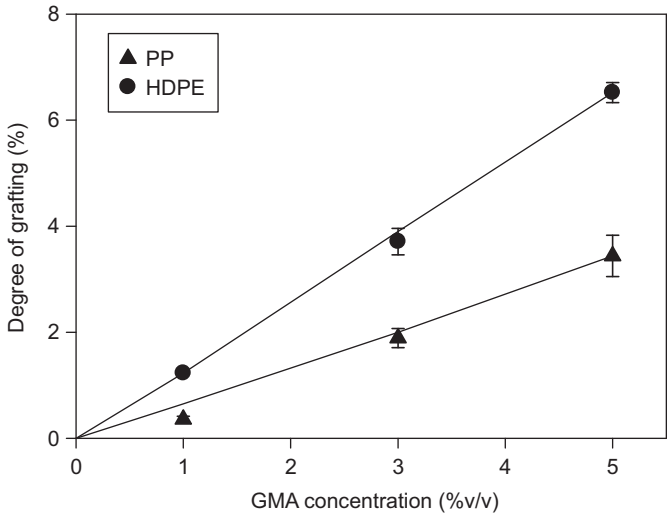

Fig. 2. Degree of grafting of GMA grafted HDPE and PP using different initial monomer concentrations in ethanol/water. Total dose: 10 kGy at 1 kGy/h.

PP sheets have slightly lower internal surface and bigger pores than the HDPE sheets used in this work.

Grafting degree was substantially lower than in previous reports using the same monomer, solvent and other conditions (Carbajal et al., 2003; Wolman et al., 2000). We assign such difference to the significant reduction in the specific surface area of the grafted materials. Macroporous HDPE and PP sheets used in this study have around ten-fold lower internal surface area than membranes (Carbajal et al., 2003; Wolman et al., 2000).

Fig. 3 shows SEM pictures of pristine and grafted HDPE. A different coating layer onto the polymer surface of grafted HDPE is clearly seen.

In order to functionalise and increase the hydrophilicity of grafted polyGMA material a primary and a secondary amino group were introduced by an add-on reaction with EDA onto the epoxy pendant groups, giving HDPE-gGMA-EDA and PP-gGMA-EDA.

Different monomer concentration reaches proportional grafted polymer, which is further chemically modified by the ring-opening reaction onto the epoxy groups. The chemical reaction of EDA creates a cationic hydrogel with micron-size thickness onto the polymer surface. The surface modification was also revealed by the complexation of copper ions.

After neutralization, EDA-modified materials were tested for loading microorganisms. A Gram-positive (*B. cereus*) and a Gram-negative (*E. coli*) bacteria were immobilized onto the HDPE/PP-gGMA-EDA materials. Immobilization step was as straightforward as incubation of macroporous materials into the microbial suspension for 16 h shaking. Washing steps using aqueous buffer solutions were performed to remove non-adsorbed bacteria. Different quantities of cells were immobilized, observing that similar catalytically yields were obtained when 50,000 or $100{,}000 \times 10^6$ cells/g were used (Trelles et al., 2008). The shallow area for cell immobilization was 0.1 m^2/g, calculated from nitrogen adsorption/desorption isotherms using a BET-sorptometer onto HDPE. Considering that the microbial area is 2 μm^2, in theory $50{,}000 \times 10^6$ cells/g could be immobilized.

An optical microscopy was used to visualize bacteria immobilization. Wet polymer samples with the immobilized bacteria were sliced and dyed using methylene blue. This cationic dye is usually applied to microbial dyeing and it was easily removed from the grafted polymer. In Fig. 4 bacteria are recognized as rods at 1000× magnification. GRAM tinting to recognize Gram-positive and Gram-negative bacteria could not be applied due to interferences with the chemical composition of the hydrogel. In a

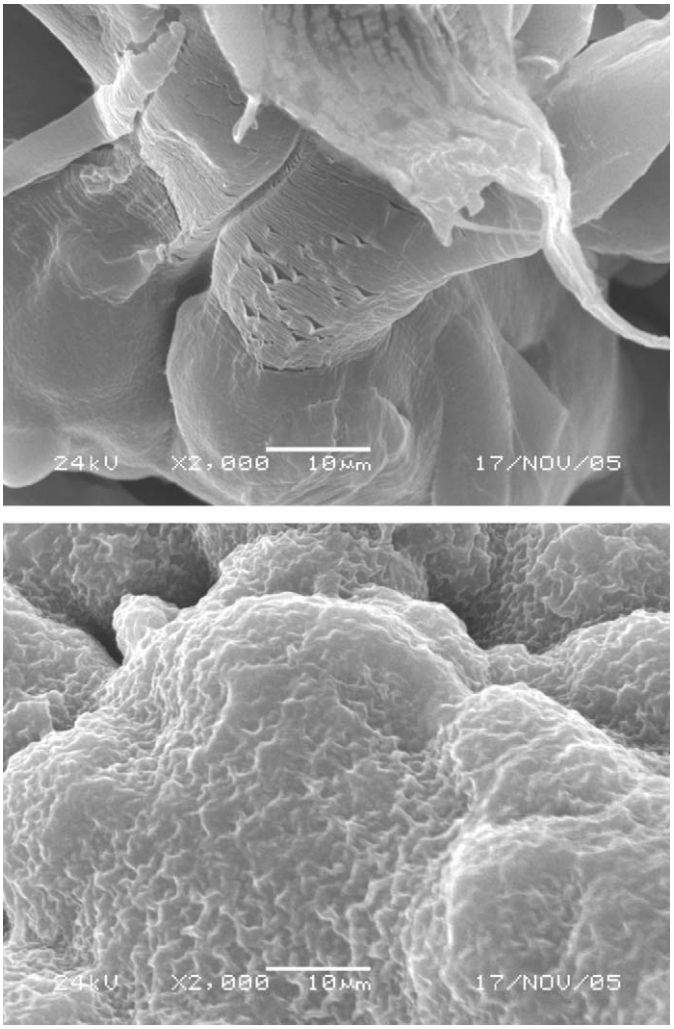

Fig. 3. SEM pictures of HDPE (upper) and GMA-g-HDPE (lower) internal surfaces.

SEM microscopy of air dried biocatalyst, sample shows phantoms of microbial cells onto the polymer surface (Fig. 4). These results suggest that the external part of polymethacrylates behaves as a hydrogel where the cells are entrapped.

ATR-Infrared spectroscopy was performed onto the plain HDPE to the full biocatalyst (Fig. 5). The IR spectra of grafted polyGMA (Fig. 5b) introduces many signals to the PE spectrum: the typical 1720 cm^{-1} corresponding to the methacrylate carbonyl moiety and 907 cm^{-1} corresponding to the epoxy functional group. In the 3000–4000 cm^{-1} region a broad peek corresponding to amine and hydroxyl groups is present. The characteristic IR frequencies of primary and secondary amine groups stretching bands appear in the same spectral region as hydroxyl stretching bands. Thus, their signals should be masked for the corresponding hydroxyl of grafted polyGMA polymer. Finally, the presence of bacterial biomass into the polymer (Fig. 5d) add the characteristic absorption bands corresponding to carbohydrates at 1080 cm^{-1}, nucleic acids at 1250 cm^{-1}, amide I and II of the proteins at 1550 and 1660 cm^{-1}, and 2830–2930 cm^{-1} for lipids. In addition to a broad peak centered at 3300 cm^{-1} of hydroxyl moiety from biological compounds (Grube et al., 2002).

A biochemical conversion was carried on in order to check the new biocatalysts. In a reaction tube, uridine in the presence of free or immobilized bacteria is converted to uracil and ribose-1-phosphate. This phosphorolysis reaction is catalyzed by the

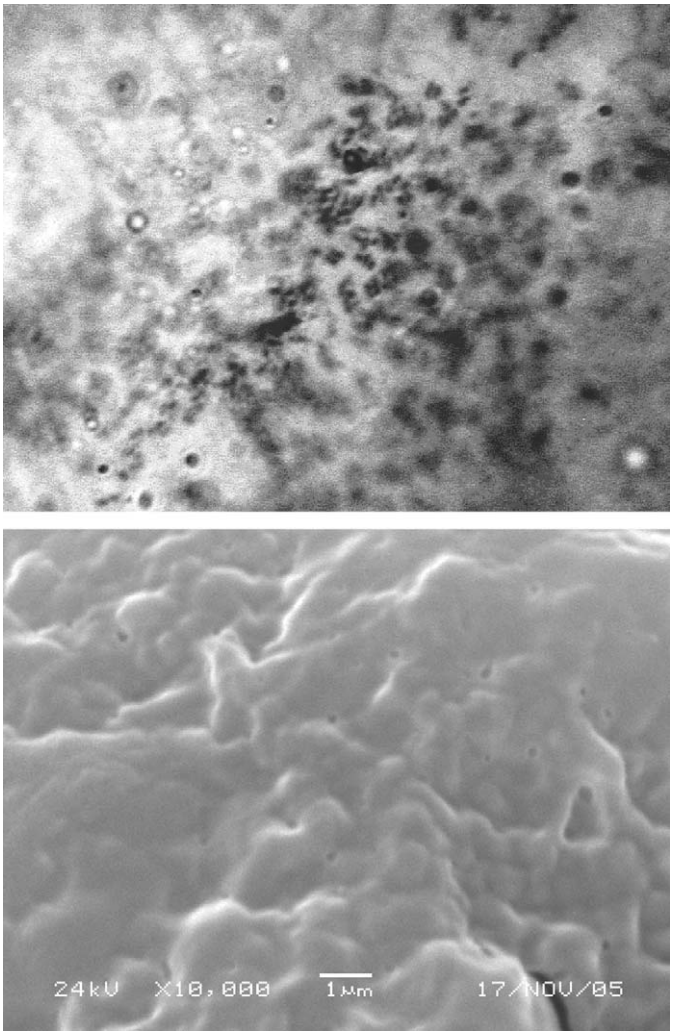

Fig. 4. Optical microscope picture (upper) at 1000 × magnification of biocatalyst. SEM picture (lower) of air-dried surface of the biocatalyst.

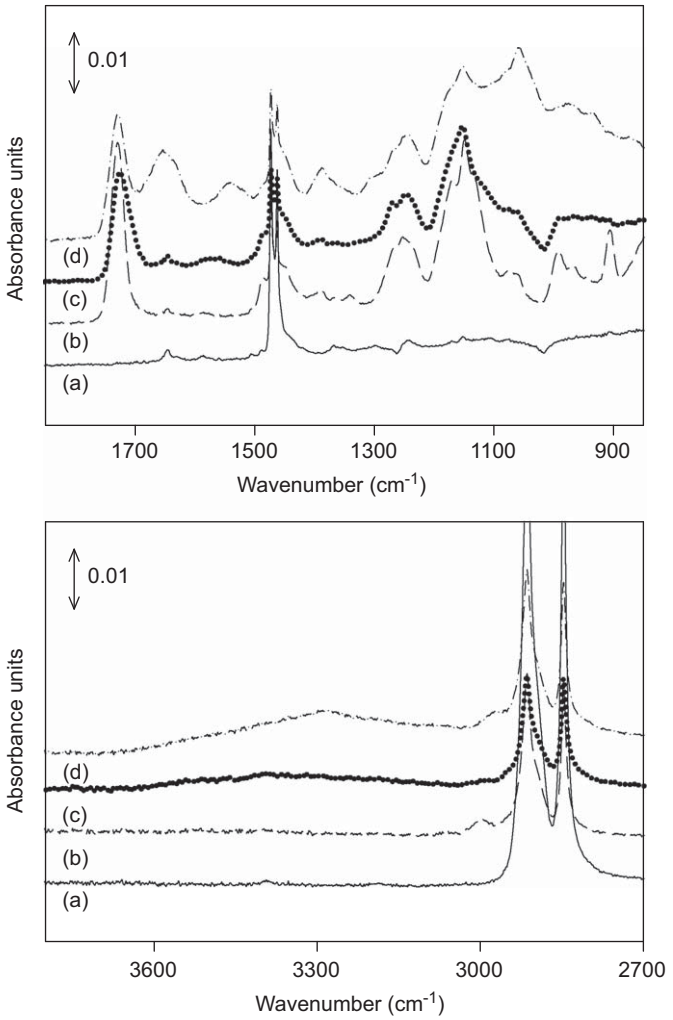

Fig. 5. ATR-IR spectra of HDPE (a); HDPE-g-GMA (b); HDPE-g-GMA-EDA (c) and full biocatalyst (d).

Table 1
Percentage of uracile yield (%) after 3 h incubation of uridine solution with free or immobilized cells onto modified HDPE and PP.

Microorganism	Free cells	HDPE-gGMA-EDA	PP-gGMA-EDA	HDPE-gGMA-EDA-cells	PP-gGMA-EDA-cells
E. coli	95	18	10	95	91
B. cereus	100	19	12	100	65

uridine-nucleoside phosphorilase, a constitutive enzyme of these bacteria. Table 1 shows the uracil conversion after 3 h of reaction using modified polymers, modified polymers with immobilized bacteria, and an equivalent quantity of free bacteria. The yield of uracil using HDPE-gGMA-EDA-cells was the same as the one obtained with free cells for both cells. Only in the case of *B. cereus* immobilization onto PP-gGMA-EDA the reaction yield was low. These results agree with the assumption of cells immobilized onto an ultra thin hydrogel structure.

4. Conclusions

Simultaneous grafting of HDPE and PP using low initial monomer concentration can be performed without major inconvenient. Homopolymer yield will also be low and easily removed. This operational condition can be selected when very low grafting modification is desired or the initial polymeric sample has surface area higher than 1 m^2/g. Another possibility is to find a suitable solvent with high grafting yield and/or the homopolymer is highly soluble.

Cationic hydrogel based on the grafted polymethacrylate was able to immobilize Gram-positive and Gram-negative bacteria by a simple adsortive method. Surface modification of HDPE and PP, and the immobilization of bacteria onto the grafted microhydrogel were demonstrated by optical and electronic microscopes and by IR spectroscopy. Immobilized bacterial viability was verified by bioconversion of uridine to uracil.

We envisioned the possibility of a great improvement in biocatalytical process efficiency by the use of immobilized bacteria onto microhydrogel grafted macroporous polymers.

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