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Enhanced biodegradation of polyethylene by development of a consortium

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ABSTRACT

Mixed cultures may be more useful than individual cultures for biodegradation studies since they grow in symbiosis and enhance the degradation process. The potential strains of six microorganisms, able to utilize polyethylene (PE) as the sole carbon source were selected for the development of a consortium. In vitro biodegradation efficiency of the consortium for degradation of PE was tested by scanning electron microscope (SEM)-Energy Dispersive X-ray Analyzer (EDAX), to analyze the growth of the microbial cultures on the PE films and Fourier Transform Infrared Spectrophotometer (FTIR) to reveal the shifting of existing chemical bonds and formation of new bonds as a result of microbial activity. The degraded products of PE were determined by Gas Chromatography- Mass Spectrometer (GC-MS).

Key words: Consortium, Polyethylene

INTRODUCTION

The most common polymer is polyethylene, which is made from ethylene monomers (CH2=CH2). The first polyethylene was made in 1934. Today, we call it low-density polyethylene (LDPE) because it will float in a mixture of alcohol and water. In LDPE, the polymer strands are entangled and loosely organized, so it is soft and flexible. It was first used to insulate electrical wires, but today it is used in films, wraps, bottles, disposable gloves and garbage bags. In the 1950s, Karl Ziegler polymerized ethylene in the presence of various m*et al*s. The resulting polyethylene polymer was composed of mostly linear polymers. This linear form produced tighter, denser, more organized structures and is now called high-density polyethylene (HDPE). HDPE is a harder plastic with a higher melting point than LDPE, and it sinks in an alcohol-water mixture. HDPE was first introduced in the hula hoop, but today it is mostly used in containers. Polyethylene (PE) is one of the synthetic polymers of high hydrophobic level and high molecular weight. In natural form, it is not biodegradable. Thus, their use in the production of disposal or packing materials causes dangerous environmental problems (Kwpp and Jewell, 1992). To make PE biodegradable requires modifying its crystalline level, molecular weight and mechanical properties that are responsible for PE resistance towards degradation (Albertsson et al., 1994). This can be achieved by improving PE hydrophilic level and/or reducing its polymer chain length by oxidation to be accessible for microbial degradation (Bikiaris et al., 1999). The present study was focused on analyzing the enhanced degradation of PE by developing consortia of microbial cultures.

Aim & objectives:

1. Screening of microbial cultures for PE degradation.
2. Identification and characterization of the cultures.
3. Development of a consortium.
4. Invitro biodegradation assay by SEM-EDAX, FTIR and GC-MS.

MATERIALS AND METHODS

ISOLATION AND SCREENING OF MICROBIAL CULTURES:

Soil samples were collected from a plastic dumpsite inside Madras Christian college campus, Chennai. A total of 1g of the soil sample was suspended in 10ml of sterile Milli-Q water and vortexed for 15 minutes. Nearly 100 µl of suspension was used as inoculum. Erlenmeyer flasks, containing 100 ml of mineral salt medium, strips of untreated polyethylene, 0.01 %(w/v) glucose and 1 ml of inoculum were used for maintaining the first preculture.

The later subcultures did not contain glucose but only the polymer as the sole carbon source. After three successive subcultures, microorganisms grew in the presence of PE and without glucose. Pure cultures were isolated on Nutrient agar plates (Himedia Limited, Mumbai, India) for bacterial isolation and potato dextrose agar plates containing 50 mg of chloramphenicol to avoid bacterial contamination for fungal isolation(Artham and Doble ., 2009).

Polyethylene powder was added into mineral salt medium for a final concentration of 0.1% (w/v) and the mixture was sonicated for 1hour at 120 rpm in shaker. After sonication the medium was sterilized at 121°C and pressure for 15 lbs/inch2 for 20 minutes. About 15ml sterilized medium was poured before cooling in each plate. The isolated organisms were inoculated on polymer containing agar plates and then incubated at 25-30°C for 2-4 weeks. The organisms, producing zone of clearance around their colonies were selected for further analysis (Augusta *et al.*, 1993).

IDENTIFICATION AND CHARACTERIZATION OF PE DEGRADING STRAINS

Bacterial strains were identified based on colony morphology and gram staining. Biochemical tests were also performed for the identification of the isolates (Bergey's manual of determinative bacteriology, 1994). Confirmation of the isolates was based on molecular characterization.

Fungal strains were identified by the morphological features of their colony and conidia using microscopic examination.

DEVELOPMENT OF CONSORTIUM

A single colony from each culture of Bacillus megaterium, Pseudomonas mediterranea, Aspergillus sp, Penicillium sp, and the standard strains, Pseudomonas putida- MTCC-1192 and Phanerochaete chrysogenum-MTCC-787 was inoculated in 20ml test tubes containing 5ml nutrient broth (pH 7.0± 0.2) and the tubes were incubated at 37°c for 10 hours with continuous shaking at 120rpm(Soni,et al., 2009).

INVITRO BIODEGRADATION ASSAY

For biodegradation assay, 100ml deci-strength minimal salt broth was taken in 250ml Erlenmeyer flask containing PE film strips. The flasks were inoculated with 90 µl of active consortium and incubated at 37°c with continuous shaking (120rpm) for 30 days. (Soni.R, et al.,2009). The plastic films were recovered from the broth and subjected to physiochemical analysis viz. SEM-EDAX, FITR for surface and chemical changes and for degraded products by Gas chromatography-Mass Spectrometer.

Physicochemical analysis

1. Surface changes-SEM-EDAX analysis

Surface morphology and composition of the polymer was investigated with a scanning electron microscope (SEM)-Energy Dispersive X-ray Analyzer (EDAX; FEI Quanta 200). A 10x10mm piece was cut from the polymer sample and placed on the sample holder and was scanned within an area of 100 μm^2 at a magnification of 500X . Chemical analysis (microanalysis) of the polymer surface was performed by measuring the wavelength and intensity distribution of X-ray signals generated by a focused electron beam on the specimen with the Energy Dispersive Spectrometer (EDS). The sample preparation for this analysis was the same used for SEM analyses. The voltage was set at 20 kV, and the magnification was set at 200X (Artham and Doble., 2009).

2. Chemical changes :

A PERKIN ELMER spectrum one Fourier Transform Infrared Spectrophotometer (FTIR) was used to detect the formation of new functional groups or changes in the amount of existing functional groups. The Perkin Elmer spectrum one FT-IR instrument consists of Nernst glower as source, an interferometer chamber comprising of KBr

(potassium bromide) beam splitters followed by a sample chamber and detector. Entire region of 4000-450 cm-1 is covered by this instrument. The spectrometer works under purged conditions. Solid samples were dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 4.0 cm-1. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible. In KBr method, the solid sample was grounded using an agate mortar and pestle to give a very fine powder.

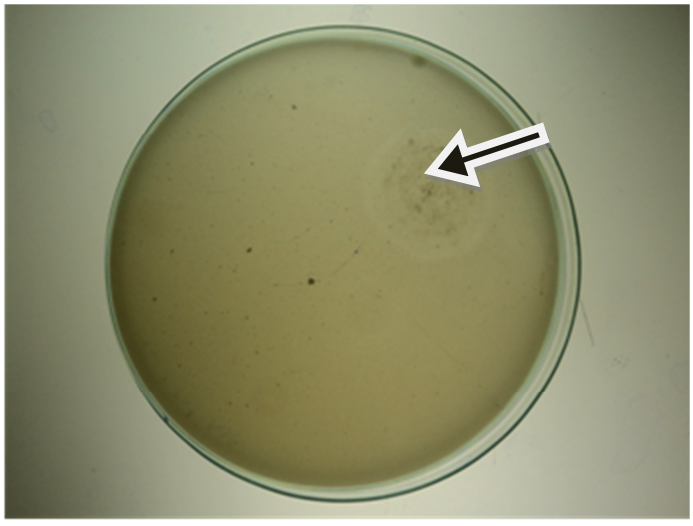
The fine powder sample was then mixed with 100mg dried KBr salt. The mixture was then pressed under hydraulic press using a dye to yield a transparent disc measuring about 13mm diameter and 0.3mm in thickness. Then the sample was placed in the instrument sample holder for scanning.

The procedure for recording the %T or %A is as follows:
Air was first scanned for the reference and stored. The sample was then recorded and finally the ratio of the sample and reference data was computed to give required %T or %A at various frequencies (Indian Institute of Technology, Chennai).

1. ANALYSIS OF DEGRADED PRODUCTS BY GAS CHROMATOGRAPHY
After 2 months of incubation period, the mycelial pellet (in case of fungal culture) or the bacterial pellet (in case of bacterial culture) was removed by filtration, and the filtrates were extracted with distilled ether. The degraded products of PE were determined by Gas chromatography-mass spectrometer (JEOL GCMATE II GC-MASS SPECTROMETER, Indian Institute of Technology, Chennai.) using HP5 column. Helium gas was programmed to raise the oven temperature from 70°C to 200°C (maximum temperature - 250°C at 15°C/min, Injection liquid 1 micro liter). Mass spectrometer with tungsten filament as electron source works with 70eV, a double focusing analyzer and photo multiplier tube as detector with resolution of maximum 5000. Using PerFluoro Kerosene (PFK) as standard, mass spectrometer was calibrated (Wen Chai, et al. 2008)

RESULTS

SCREENING OF PE DEGRADING MICROORGANISMS
The formation of a clear halo around the colony indicated that microorganisms were able to depolymerize the PE by utilizing it as a carbon source, since that was the only nutrient available in the medium.



CHARACTERIZATION AND IDENTIFICATION OF PE DEGRADING STRAINS
Based on molecular characterization the bacterial strain BS1 was identified as Bacillus megaterium strain B1, Accession number(Gen bank) - JQ904750 and the bacterial strain BS2 was identified as Pseudomonas mediterranea and strain M2, Accession number (Gen bank) - JQ904748.

After staining with cotton blue following the keys Raper and Fennell (Raper, *et al*.,1987), the fungus was finally identified. Based on colony morphology and Lacto phenol cotton blue staining the fungal strain(FS1)was identified as Penicillium species and the fungal strain FS2 as Aspergillus species.

SEM Analysis of consortium

Within 30 days of incubation with the microbial consortia, the PE film appeared corroded. This may be due to the combined effort of individual cultures in the consortium to effectively degrade PE. SEM images of degraded films showed the presence of different types of bacterial cells and hyphal structures. This therefore, confirmed the utilization of PE as a carbon source by these microbes. Further incubation would have yielded better results. (Figure 1).

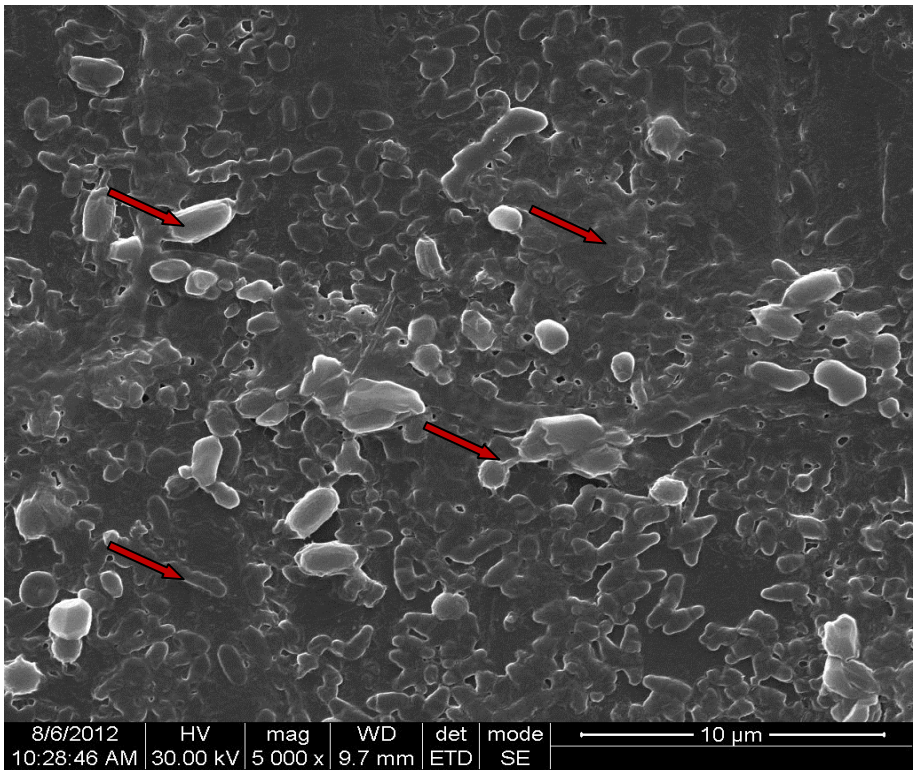


Figure 1- SEM image of degraded PE film inoculated with the microbial consortia

EDAX Analysis of consortium

EDAX analysis further confirms the effective use PE as a carbon source by the consortium when compared to the control (Table 1).

Table 1 - Elemental analysis of degraded PE film by consortium compared with control

Element Wt %	Control	Consortium
Carbon	80.13	72.55
Oxygen	5.63	4.62

FTIR Analysis of consortium

The FTIR spectrum of degraded PE film by consortium showed considerable changes in the absortion intensities when compared to control. The peak at wavelength 1019 cm^{-1} in control increased to 1051 cm^{-1} in the consortium. Newer peaks with wavelength 2331 cm^{-1}, 1641 cm^{-1} and 1368 cm^{-1} were also observed in the consortia sample as compared to control. This showed the shifting of bonds in the PE structure whereby the chemical structure of the film was altered, which is a prerequisite for biodegradation. (Figure 2).

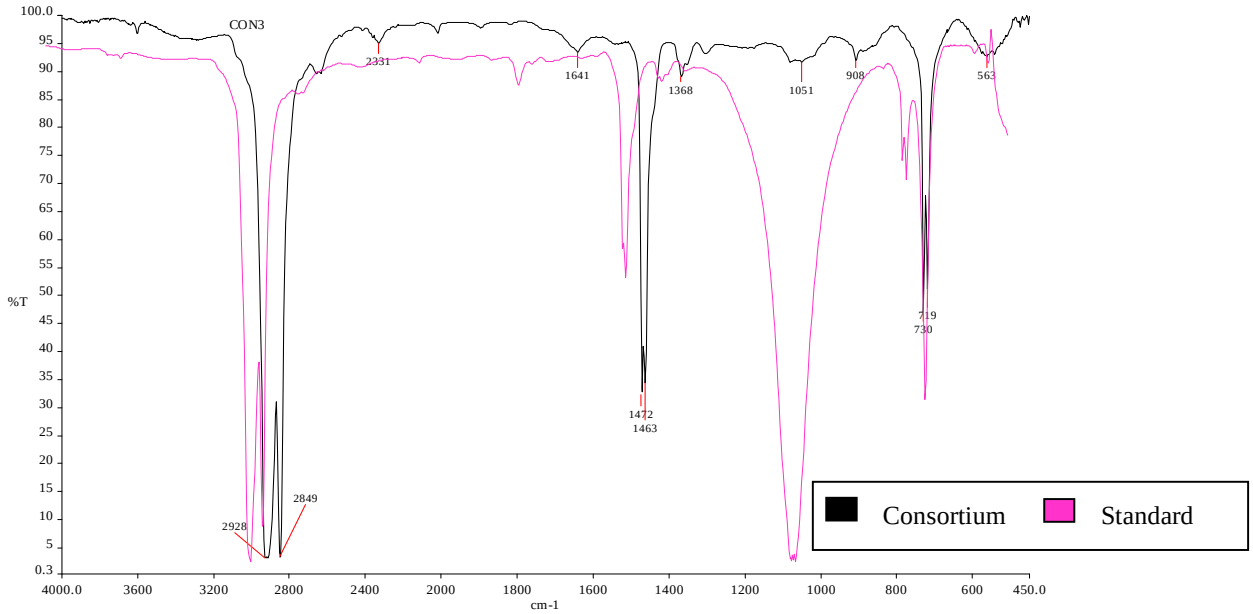
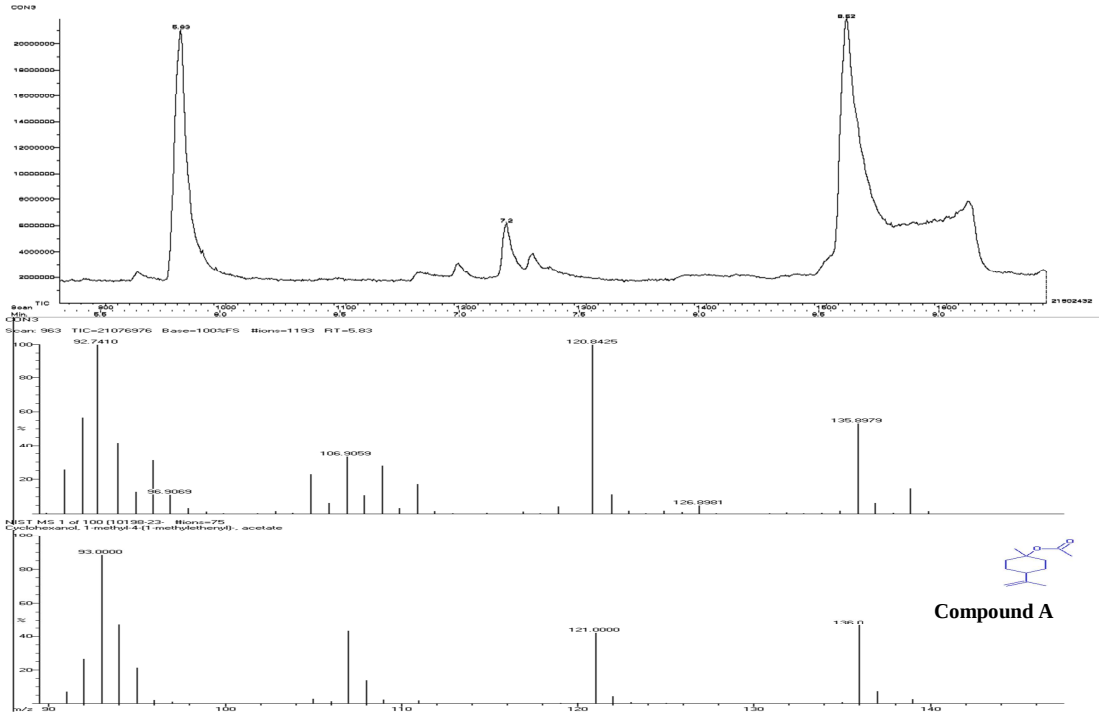


Figure 2 - FTIR spectrum of degraded PE film by consortium compared with control

GC-MS Analysis of consortium

The consortia of six cultures namely the four isolates and two standard strains were found to degrade PE effectively within an incubation period of 30 days and the decomposed products were analysed by GC-MS (Figure 3). GC-MS spectra of degraded products by consortium (a-c) were as follows: Compound a. m/z : 136 (45)[M]+ to 93 (90)[M]+, Compound b. m/z : 136 (85)[M]+ to 67 (15)[M]+ and Compound c. m/z : 162 (100)[M]+ to 77 (45)[M]+. The spectra of compound a was found to be Cyclohexanol, 1-methyl-4-{1-methylethenyl}-acetate, compound b to be Cyclohexene, 1-methyl1-3-{1-methylethenyl}-[n] and compound c to be Benzene,1,2-[methylenedioxy]-4-propenyl-,[E].



Compound A

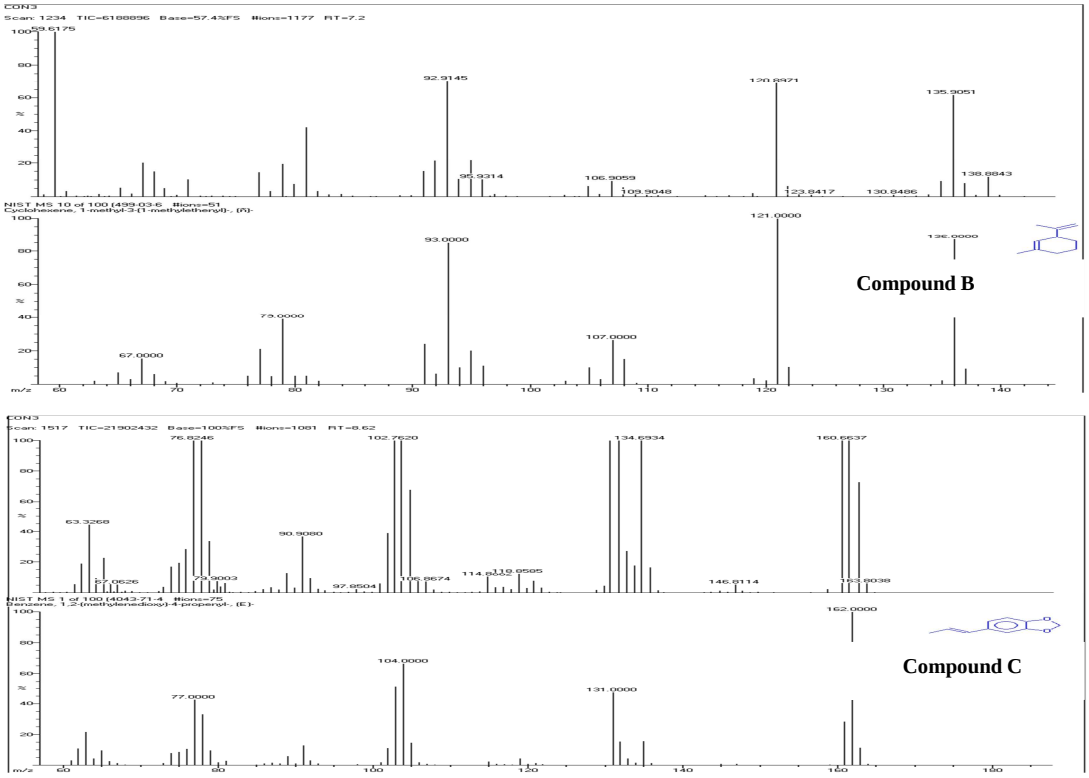


Figure 3 - The spectrum of products (A-C) using Consortium analyzed by GC-MS

DISCUSSION

Individual cultures, both bacterial and fungal have been used for the biodegradation of polyethylene and other polymers (Hadad D, *et al*., 2005; Goel R, *et al*., 2008; Manzur A, *et al*., 1997; Soni R, *et al*., 2009) but consortia have been found to be more efficient biodegraders (Orhan Y, *et al*., 2000; Kapri A,*et al*., 2009). The suggestive reason behind this behavior is that the degradation of a polymer compound might involve multiple steps and for catalyzing the formation and catabolism of intermediate compounds, different enzymes/co-factors are needed, which is carried out by suitable microbial 'consortia' (Soni R, *et al*., 2009). FTIR analysis further substantiated that the chemical bonds in the polymer backbone of LDPE were acted upon by the consortium, causing breakage of some native bonds and formation of newer ones. Significant shifts in CH2 and C-O stretching frequencies were recorded in the biodegraded supernatants of both non-poronized as well as poronized LDPE (Soni R, *et al*., 2009).

Mixed culture can be more useful than single culture, and when they grow in symbiosis they may enhance the growth of the biofilm formed. It also increases the hydropillicity of the polymer surface when compared to growth of individual organism which may ultimately make the polymer more susceptible to degradation. Microbial culture like Klebsiella pneumonia and

Pseudomonas aeruginosa are reported to form a biofilm on the surface of steel. The later colonises the surface faster while the former grows faster on the surface layer of this biofilm [Stewart , 1997]. The mixed culture reduced the weight of the polymer by 7% whereas single culture of the same microorganism showed ~ 0.50% reduction in weight, indicating a synergy between the two microorganisms leading to 14 – times increase in the biodegradability of the polymer [Seneviratne, 2006]. Effective degradation of PE films were observed in the microbial consortia of *Bacillus megaterium, Pseudomomas mediterranea, Aspergillus sp, Penicillium sp, Pseudomonas putida* and Phanerochaete within 45 days of incubation compared to 90 days of incubation with individual microbial cultures. SEM micrograph showed a mixed growth of cultures on the film surface and EDAX results substantiated the use of PE film as carbon source. FTIR analysis also confirmed the formation of newer bonds with wavelength 2,331 cm-1, 1,641 cm-1 and 1,368 cm-1 as when compared to control due to effective degradation of PE by the consortia. The spectra of compounds as analysed by GC-MS for the microbial consortia were found to be Cyclohexanol, 1-

methyl-4-{1-methylethenyl}-acetate, Cyclohexene,1-methyl1-3-{1-methylethenyl}-[n] and Benzene,1,2-[methylene dioxy]-4-propenyl-,[E].

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