9

Possible Uses of Micro-organisms in the Manufacture of Plastics and Synthetic Fibres

MARTIN GRIFFIN AND ANN M. MAGOR

Department of Life Sciences, Trent Polytechnic, Clifton Lane, Nottingham NG11 8NS, UK

Introduction

In historical terms, man's attempts to replace natural materials with synthetic plastics and fibres are very recent. At the Great International Exhibition of 1862 held in London the English inventor Alexander Parkes was awarded the bronze medallion for his exhibit Parkesine. This was a cellulose polymer which was processed by dissolving in the minimum amount of solvent, heated on a rolling machine and then shaped by dies or pressure. Later, in 1869, John Wesley Hyatt and his brother Isaiah, in seeking a suitable substitute for ivory, realized the importance of camphor as a plasticizer for cellulose nitrate. They patented a process (US Patent 105338) for producing a horn-like material which was later termed celluloid. Thirty years after this, Adolf Spitteler treated casein with formaldehyde to make commercial plastic materials and shortly after, in 1909, Leo Baekeland developed a practical process for making 'Baekelite' from phenol–formaldehyde resins (Jones and Simon, 1983).

It was not until the 1920s, however, that it was realized that plastics owe their most significant properties to their polymeric nature. This concept, together with the increased availability of petrochemicals, has led to the production, or in some cases the rediscovery, of many thousands of polymers, such that the world production of plastics had reached 30 million tonnes per annum in 1970 and 45 million tonnes in 1975. The output for the 1990s is predicted to be in the region of 90 million tonnes. Similarly, the world production of synthetic fibres had grown to over 8 million tonnes in 1975 and reached approximately 13 million tonnes in 1985. With an estimated growth rate to 20 million tonnes by the year 2000 it is expected that some 63% of the world's fibres will, by then, be of synthetic origin.

Biotechnology and Genetic Engineering Reviews—Vol. 4, September 1986

Table 1. W. European consumption of various plastics (million tonnes) (data after Brydson, 1982)

Product	1970	1974	1978	1985
Polyethylene (LD)	2·00	3·10	3·6	4·00
Polyethylene (HD)	0·61	1·03	1·33	1·80
Polypropylene	0·3	0·6	1·08	2·00
PVC	2·3	3·5	3·5	—
Polystyrene	0·86	1·15	1·30	—
Nylons	0·09	0·15	0·17	—
Polyurethanes	0·42	0·77	0·94	—
Silicones	0·03	0·05	0·09	—

Synthetic plastics and fibres—the majority of which are now dependent on petrochemical feedstocks—have therefore become an everyday part of society and our dependence on them will continue to increase (*Table 1*); the plastics and fibres industry, which is now practically inseparable from the petrochemical industry, has grown accordingly and, over one hundred years from the discovery of Parkesine, it can now be termed a relatively mature industry.

This growth has not, however, been achieved without large-scale investment in research and development by those concerned. The steep rise in oil prices during the 1970s demanded innovation of new products and processes if producers were to hope to stay one step ahead of competition. Manufacturing needed to be less energy consuming and less dependent on oil-based feedstocks. This requirement was heightened further by an increasing awareness and concern for the state of the environment, the conservation of fossil fuels and the pollution created by chemical manufacturing processes.

During the late 1970s another technology concerned with new concepts and techniques in genetic manipulation and large-scale fermentation was undergoing rapid development and, as a consequence, new and exciting possibilities for the application of micro-organisms in the manufacturing industries emerged. The use of micro-organisms in the manufacture of plastics and synthetic fibres was therefore considered by the petrochemical industries to be one possible means of alleviating some of their problems. Although, at present, no large-tonnage plastics or their polymerization monomers are produced commercially by micro-organisms, a considerable amount of original research has been undertaken in this area; these investigations are the basis of this review.

The different roles of micro-organisms in the manufacture of plastics and synthetic fibres

The use of micro-organisms in the manufacture of plastics and synthetic fibres may be divided into two categories. The first involves the microbial biosynthesis of polymers which, by virtue of their chemical and physical properties, resemble plastics derived by other means and so may be further

processed, by similar methods, into moulded items, films or fibres. The second employs micro-organisms as biocatalysts in the formation of chemical intermediates or monomers, which may then be polymerized and processed by traditional chemical routes to form plastics and synthetic fibres.

Biosynthesis of polymers by micro-organisms

Several microbial polymers, often referred to as biopolymers, are known to be produced by micro-organisms, for example dextran, xanthan, gellan gum, Zanflo and Polytran. Several excellent reviews have covered both the properties and applications of these (Sandford, 1979; Sutherland and Ellwood, 1979; Sutherland, 1982; Baird, Sandford and Cottrell, 1983). It is therefore not the purpose of this chapter to reiterate this information as only two of these polymers—the polysaccharide pullulan and the aliphatic polyester poly-β-hydroxybutyrate—have the properties necessary to allow them to be moulded into plastics or extruded into films or fibres.

PULLULAN

Pullulan, discovered in 1939 by R. Bauer, is a viscous exopolysaccharide produced by a fungus-like yeast. This yeast is commonly known as 'black yeast' and named *Aureobasidium pullulans*, formerly designated as *Pullularia pullulans* (Yuen, 1974). The polysaccharide was originally produced by the Hayashibara Biochemical Laboratories of Japan as a substrate to determine the activity of the enzymes isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) which are required for production of a series of starch hydrolysates. However, after further investigations into the properties of pullulan, in 1974 the same company hailed pullulan as a new biodegradable transparent plastic which could be produced independently of petroleum feedstocks. Pilot plant production of pullulan was reported by Hayashibara Biochemical laboratories in 1974 to be in the region of 12 tonnes per annum. Their expectation, depending on the marketing acceptability of pullulan, is to increase this output by about 10 000 tonnes annually (Lawson and Sutherland, 1978).

The accepted structure of pullulan ($(C_6H_{10}O_5)_n.H_2O$) is that of a linear polymer of maltotriose units or occasionally maltotetraose units connected by α(1–6) linkages (*Figure 1*). The degree of polymerization, from 100 to 5000, is variable according to the micro-organism used, culture conditions and isolation procedure.

Any starch syrup containing oligosaccharides such as those derived from cereal and potato starches may be used as a carbon source in the fermentation for pullulan production. In fact, using these cheaper carbon sources at a concentration range of 5–15%, instead of mono- or disaccharides, was found to increase the yields of pullulan by up to 70% (*Table 2*). Since these original findings, the optimization of the fermentation conditions for pullulan production has been studied by both industrial and academic workers (Sugimoto, 1981; Lacroix *et al.*, 1985).

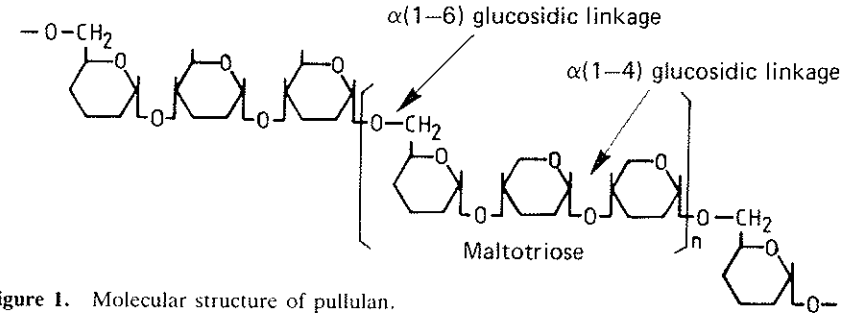

Figure 1. Molecular structure of pullulan.

Table 2. Comparison of pullulan yields obtained from different saccharide growth substrates (data from Yuen, 1974)

Carbon source	*Pullularia pullulans* AHU 9553 (% yield)	*Pullularia pullulans* IFO 6353 (% yield)	*Dematium pullulans* IFO 4464 (% yield)
Conventional			
Glucose	35	31	43
Sucrose	51	35	54
Partial starch hydrolysate			
Maltose (90%)	52	51	61
Acidic conversion syrup DE45	65	76	75
Enzymatic conversion syrup DE35	63	63	72

For industrial use the exopolysaccharide is isolated from the fermentation broth by alcohol precipitation. In a continuous operation this may involve the spraying of an aqueous pullulan solution into alcohol and then fractionating and drying the precipitate (Sugimoto, 1981). The powdered product, after addition of a small amount of water, can be formed into compression mouldings, films or fibres. Pullulan is highly water soluble but its degree of solubility can be adjusted either by partial esterification or by etherification of the polymer chain. Hayashibara claim that, as a moulding plastic, pullulan has physical properties that are close to those of polystyrene with regard to strength and toughness. When used as a film, it is oxygen impermeable and has the advantage of being non-toxic. Some of the physical properties of pullulan film are shown in *Table 3*.

Pullulan may also be employed for use as a fibre for fabric production, as fibres can be made from concentrated aqueous solutions of pullulan of the appropriate degree of polymerization. The fibres have a shiny gloss resembling that of rayon but their tensile strength after stretching is comparable to that of nylon (Yuen, 1974). As pullulan is a polysaccharide it offers many

Table 3. Some physical properties of pullulan film (data from Yuen, 1974)

Physical property	Pullulan film (0·1 mm thick)
Transparency (solar-ray transmittance)	95%
Gloss	Excellent
Tensile strength (kg/mm^2)	7–8
Elongation percentage	8–20
Schopper double-fold no. (folding endurance)	800–900

other advantages in its present and projected uses: for example, as a moulded item it is reported to require neither plasticizers nor stabilizers, and is biodegradable even after further chemical modification.

POLY-β-HYDROXYBUTYRATE

Poly-β-hydroxybutyrate is an aliphatic polyester which serves as an intracellular reserve material in many prokaryotic micro-organisms during unbalanced growth conditions. In common with other storage polymers, it may be used as an endogenous carbon source when exogenous supplies are limiting (Dawes and Ribbons, 1962).

The potential of poly-β-hydroxybutyrate as a thermoplastic was first heralded by W.R. Grace & Co., Clarksville, Maryland in 1963. The chemists of that company devised a method for extraction of the polymer from a number of bacterial species, using organic solvents such as chloroform or pyridine; the polymer could then be isolated by its precipitation with ether (Baptist, 1962). Although poly-β-hydroxybutyrate was recognized in bacteria as early as 1926 by the French scientist M. Lemoigne (Dawes and Senior, 1973), its potential use as a thermoplastic had been thwarted by earlier isolation procedures using sodium hypochlorite. These procedures resulted in chain breakage and a decrease in the molecular weight of the polymer (Anonymous, 1963).

Poly-β-hydroxybutyrate is a straight-chain homopolymer of D(−)-3-hydroxybutyrate, the formula of which is shown in *Figure 2* (Dawes and Senior, 1973). Conventional chemical synthesis of such a polyester is extremely difficult as β-hydroxy acids do not readily yield polyesters because of their high reactivity leading to internal dehydration. Other methods involving the polymerization of β-butyrolactone have also proved relatively unsuccessful.

Although considerable work was carried out on poly-β-hydroxybutyrate by W.R. Grace & Co. (Baptist, 1962) it was thought that the severe thermodegradability of the polymer at temperatures slightly above its melting point would hinder industrial use of this polymer. However, other parties also had a keen interest in this novel polymer which, like pullulan, has the advantage of being obtainable from a non-petroleum feedstock. In 1981, the work of Imperial Chemical Industries (ICI) on poly-β-hydroxybutyrate was made public at the Eastbourne conference on Biotechnology (Howells, 1982).

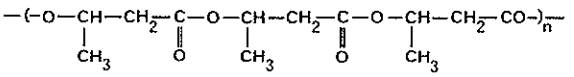

Figure 2. Structure of poly-β-hydroxybutyrate.

The screening of various micro-organisms by ICI had led to the identification of a bacterium, *Alcaligenes eutrophus*, which can synthesize the polymer in such a way that poly-β-hydroxybutyrate accounts for up to 80% of the cell dry weight (King, 1982). These high concentrations of the polymer are obtained by growing the organism on a variety of carbon sources, including that of hydrolysed starch; this growth period is then followed by a period of nutrient depletion. During this period, which involves limitation of a nutrient required for growth but not for polymer formation, the polymer is accumulated (Holmes, Wright and Collins, 1982). To be of any practical use as plastics materials, these polymers must have a molecular weight above 10 000 (Holmes, Wright and Collins, 1983; Holmes, Collins and Wright, 1984). Research undertaken at ICI has indicated that the crystallization of poly-β-hydroxybutyrate can be modified by the incorporation of other β-hydroxy-acids into the polymer chain. This production of copolymers is achieved by cultivating the micro-organism during its period of nutrient depletion in the presence of specified organic acids which may include propionic, isobutyric and acrylic acids. Modification of the final polymer in this way can reduce the crystallinity of the polymer and so permit its use as a moulding material or in the production of a film by melt extrusion (Holmes, Wright and Collins, 1982).

Various efforts to increase the yields of poly-β-hydroxybutyrate further have involved the use of mutant strains defective in some aspect of the regulation of poly-β-hydroxybutyrate synthesis. Using this strategy, mutant strains of the original organism have now been produced which accumulate poly-β-hydroxybutyrate to the extent of 92% of the cell dry weight (Lafferty *et al.*, 1980).

Poly-β-hydroxybutyrate, in common with pullulan, is biodegradable; furthermore it may be plasticized and also mixed with glass fibre for extra reinforcement. Its mechanical properties (*Table 4*) do not, however, offer any advantages over large-tonnage plastics such as polypropylene and polyethylene (Howells, 1982). For this reason, ICI have not developed poly-β-hydroxybutyrate as a general large-scale plastic, although numerous patents have been produced on its properties, fermentation and extraction (Holmes and Jones, 1982; Hughes and Richardson, 1982; Barham and Selwood, 1982; Walker, Whitton and Alderson, 1982; Holmes, 1984; Richardson, 1984; Stageman, 1984). Production of poly-β-hydroxybutyrate is still only on the 100 kg scale (King, 1982).

Nevertheless, poly-β-hydroxybutyrate does have some properties which justify special high-added-value applications and it is for this reason that ICI still intend to purify and process the polymer to a saleable commodity (Senior, 1984). For example, like polyvinylidene fluoride (PVDF) and a

Table 4. Properties of poly-β-hydroxybutyrate (PHB) compared with the homopolymer polypropylene (PP) (data from Howells, 1982)

Property	PHB	PP
Crystalline melting point (°C)	175	176
Crystallinity (%)	80	70
Molecular weight (daltons)	5×10^5	2×10^5
Glass transition temperature (°C)	15	−10
Density (g/cm^3)	1·250	0·905
Flexural modulus (GPa)	4·0	1·7
Tensile strength (MPa)	40	38
Extension to break (%)	6	400
Ultraviolet resistance	good	poor
Solvent resistance	poor	good

small number of other polymers, it has piezoelectric properties and may therefore find applications in the microelectronics industry, which already uses orientated PVDF film (Howells, 1982). Numerous potential uses are suggested, many of which depend on biodegradability, lack of toxicity and mechanical properties, such as the manufacture of surgical pins and sutures.

The use of micro-organisms as biocatalysts

Other possible commercial applications of micro-organisms in the manufacture of plastics and synthetic fibres relate to their ability to act as biocatalysts (*see* Neidleman, 1984; Powell, 1984; Mattiasson and Larsson, 1985). Micro-organisms, by virtue of the numerous types of enzymes they contain, have the capacity to carry out, at ambient temperatures and normal pressures, complex chemical reactions relevant to the chemical industries. These reactions, when carried out by conventional chemical means, would involve energy-consuming mechanisms and/or would be extremely difficult because of their lack of selectivity.

PRODUCTION OF INTERMEDIATES IMPORTANT TO THE MANUFACTURE OF POLYPHENYLENE

The production of 5,6-dihydroxycyclohexa-1,3-diene (or benzene *cis*-glycol) (*Figure 3a*) for use as a polymerization monomer in the manufacture of poly(*p*-phenylene) (*Figure 3b*), is a good example of the use of micro-organisms to carry out reactions which are difficult using conventional chemistry.

The published non-enzymic route for the synthesis of this molecule (Nakajima, Tomida and Takei, 1959) involves a partial chlorination of benzene followed by oxidation and dechlorination (*Figure 4*). The problems inherent in this three-stage process are the difficulties associated with separating the mixed reaction products and the very low overall yield. Furthermore, it is not applicable to aromatics other than benzene.

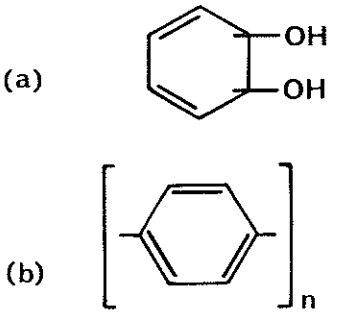

Figure 3. Structure of benzene *cis*-glycol (a) used for the production of poly(*p*-phenylene) (b).

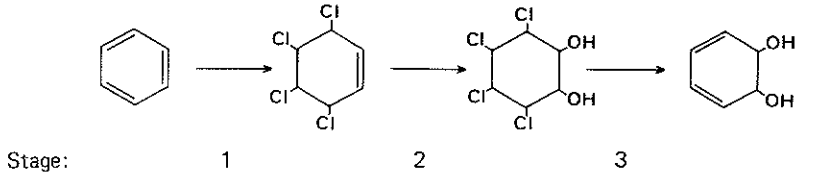

Figure 4. Chemical route for the synthesis of 5,6-dihydroxycyclohexa-1,3-diene.

A microbial route for the production of benzene *cis*-glycol has recently been developed by Taylor and co-workers (Ballard *et al.*, 1983) at ICI. This route uses a mutant strain of *Pseudomonas putida*. The wild type of this organism grows naturally on benzene (Axcell and Geary, 1975) and produces benzene *cis*-glycol in the initial metabolic step of benzene degradation (Gibson, Koch and Kallio, 1968). The enzyme responsible for this reaction, benzene 1,2-dioxygenase (EC 1.14.12.3), requires NADH and O_2 (*Figure 5*); its purification and characteristics have been described recently by Geary *et al.* (1984). The mutant strains of *P. putida* obtained by Taylor and co-workers (Ballard *et al.*, 1983) are unable to metabolize the glycol, which

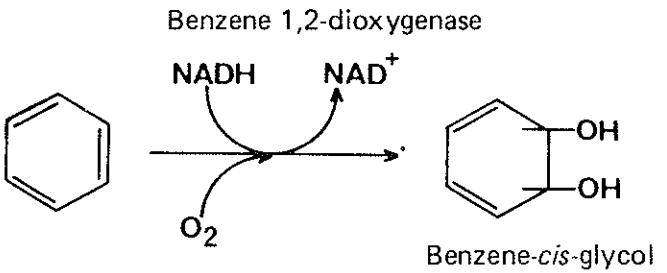

Figure 5. Microbial route for the production of benzene *cis*-glycol (5,6-dihydroxycyclohexa-1,3-diene).

therefore accumulates in the medium when the organism is supplied with a source of benzene. Conventional mutation and strain selection techniques have yielded strains showing tolerance to both benzene and its oxidation product so that kilogram quantities of benzene *cis*-glycol can be produced.

This biotransformation of benzene is performed with resting cells, i.e. cells unable to divide because of some nutrient limitation, in the presence of a co-substrate such as ethanol or acetate. Utilizing intact cells in this way overcomes the problem of cofactor regeneration as the complete oxidation of a co-substrate, such as ethanol, can serve to regenerate the NADH consumed in the oxygenase reaction (*Figure 6*) (Taylor, 1985). The benzene *cis*-glycol formed in this way may then be isolated by solvent extraction and further purified by crystallization.

The industrial interest in benzene *cis*-glycol production stems from the ease with which derivatives of this compound are polymerized by radical initiators to form high-molecular-weight polymers which are readily soluble in organic solvents (*Figure 7*). Solutions of these polymers may be cast into films, used to coat substrates or to spin fibres. Precursor polymers of this type when heated (140–240°C) then undergo a smooth conversion to polyphenylene. Production of polyphenylene using this microbiological route

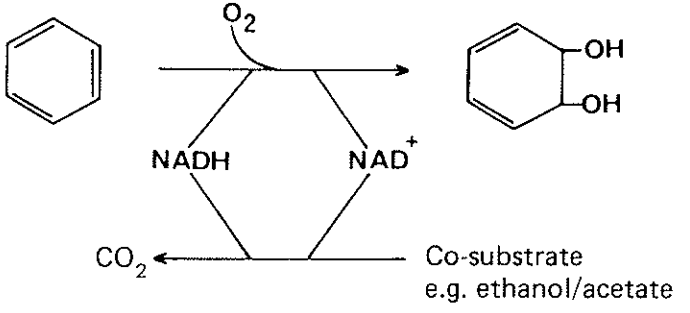

Figure 6. Cofactor regeneration by co-substrate utilization during biotransformation of benzene to benzene *cis*-glycol.

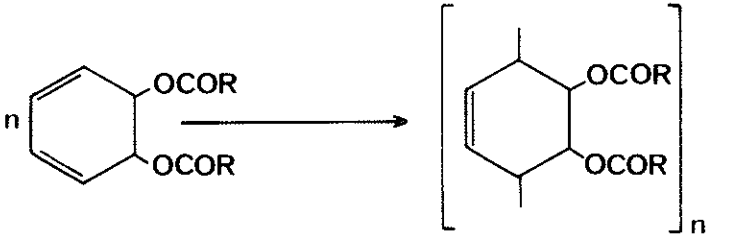

Figure 7. Polymerization of derivatives of benzene *cis*-glycol (5,6-dihydroxycyclohexa-1,3-diene) to precursor high-molecular-weight polymers of polyphenylene, soluble in organic solvents.

overcomes many of the difficulties normally associated with both the chemical synthesis (Marvel and Hartzell, 1959) and further fabrication of this thermally stable plastic (Yamamoto, Hayashi and Yamamoto, 1978). The use of this microbiological route therefore opens up new applications for this interesting polymer.

Although the polymer is still undergoing evaluation at ICI, S.C. Taylor and co-workers (personal communication) predict that its first application may be as a speciality polymer in the electronics industry.

ACRYLIC ACID PRODUCTION

Although still very much in its infancy, the microbial production of acrylate from renewable carbon sources has been investigated by one group (Dalal *et al.*, 1980) as a potential future source of this petroleum-derived monomer.

Acrylic acid and its esters are industrial chemical feedstocks of considerable value owing to their ability to partake in a variety of reactions to produce a large number of polymers, which are used in fibres, adhesives, films and resins. The normal materials for commercial synthesis of the acrylates include acetylene, propylene and ethylene (Waddams, 1978). An example of such a route is:

$$C_2H_2 + CO + H_2O \xrightarrow[\text{Temp.}\sim 225^\circ C,\ \text{Pressure}\sim 100\ \text{MPa}]{\text{Catalyst, e.g. nickel, \& halogen}} CH_2 = CHCOOH$$

which involves both high temperatures and high pressures.

It is now known that acrylate, in the form of acrylyl CoA, occurs as an intermediate in a number of microbial metabolic pathways. These include the catabolic metabolism of propionate by *E. coli* (Wegener, Reeves and Ajl, 1967) or fermentation pathways in which the major end product is propionate. Examples of the latter include the 'direct reductive pathway' or the 'acrylate pathway' in which lactate is reduced to propionate without the involvement of succinate as an intermediate. Micro-organisms exhibiting this pathway include the anaerobic bacteria *Clostridium propionicum* (Johns, 1952) and *Megasphaera elsdenii* (Ladd and Walker, 1965).

Attempts to produce acrylate by exploitation of this pathway have so far concentrated on the micro-organism *Clostridium propionicum* (Dalal *et al.*, 1980). Initial investigations with this organism indicated that resting cell suspensions of cells grown on β-alanine were able transiently to accumulate acrylate from β-alanine under anaerobic conditions. In the metabolic sequence involving the formation of propionate from acrylate by this anaerobic micro-organism, acrylyl-CoA acts as a terminal electron acceptor so that a reduction–oxidation balance can be maintained in the cell (*Figure 8*). To circumvent the reduction of acrylyl-CoA so that acrylate may be allowed to accumulate, various alternative electron acceptors were investigated for their effects on acrylate formation by resting cells. Of those tested, it was found that both oxygen and the artificial electron acceptor methylene blue,

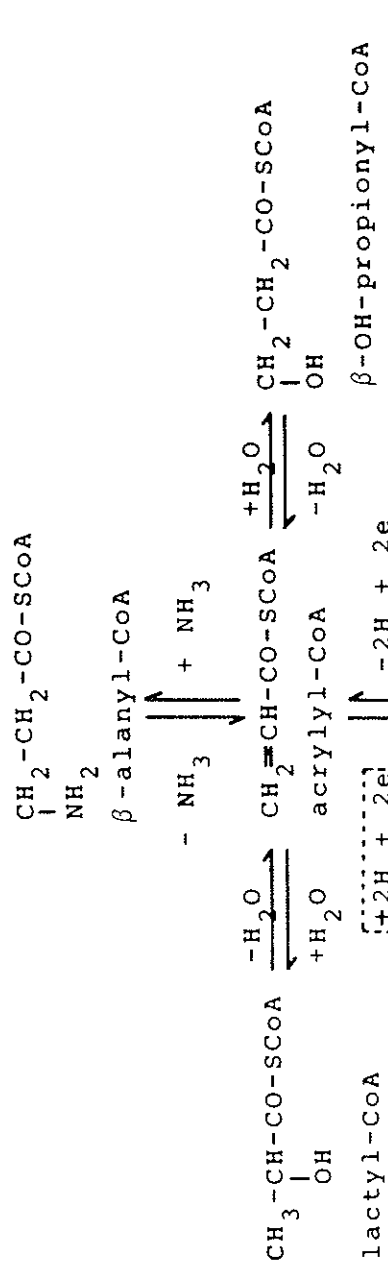

Figure 8. The importance of acrylyl–CoA as a metabolic intermediate. The use of alternative electron acceptors to promote acrylate formation in a bioconversion system is indicated (after Sinskey, Akedo and Cooney, 1981).

used either separately or together, could promote acrylate accumulation using either β-alanine or propionate as the starting substrate. This resting cell bioconversion system was also found to be capable of accumulating methacrylate from isobutyrate (Dalal *et al.*, 1980).

Although it is still in its early stages, this work represents the first attempt, using micro-organisms, to produce acrylate from a non-petroleum feedstock.

PRODUCTION OF 4,4'-DIHYDROXYBIPHENYL

Work by Smith and Rosazza (1974) and later by Dodge, Cerniglia and Gibson (1979) indicated that certain species of fungi convert biphenyl to 4,4'-dihydroxybiphenyl and other metabolites similar to those formed by mammalian systems, thus differing from most bacteria, which metabolize biphenyl to form *cis*-dihydrodiol-2,3-dihydro-2,3-dihydroxybiphenyl as the major oxidation product (Schwartz, Williams and Hutchinson, 1980).

The sequential microbial hydroxylation of relatively inexpensive chemicals such as biphenyl to 4-hydroxybiphenyl and then to 4',4-dihydroxybiphenyl (*Figure 9a*) represents an alternative to the chemical synthesis of this biphenol and has recently been investigated by Schwarz and his group at the Union

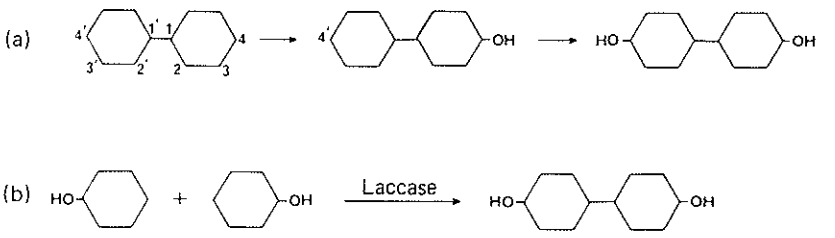

Figure 9. (a) Sequential microbial hydroxylation of biphenyl to 4,4'-dihydroxybiphenyl; (b) biological coupling of phenol.

Carbide Corporation (Schwartz, Williams and Hutchinson, 1980; Schwartz, 1981), because 4,4'-dihydroxybiphenyl is one of the monomers used in the synthesis of the sulphonebiphenyl engineering polymer, Radel.

Since these earlier reports, Schwartz and colleagues have screened various species of fungi for their ability to hydroxylate biphenyl. Ten of the fungi screened produced 4-hydroxybiphenyl and seven of the ten produced 4,4'-dihydroxybiphenyl (Schwartz, Williams and Hutchinson, 1980). Closer examination of the most efficient strains, *Absidia pseudocylindrospora* NRRL 2770 and *Absidia* sp. MRRL 1341, indicated that one of these strains, *Absidia* sp. 1341, when growing in simple defined media was capable of transforming biphenyl to 4,4'-dihydroxybiphenyl with yields of up to 5·6 mg of 4,4'-dihydroxybiphenyl per gram of biomass. Further analysis of the rate of conversion by these fungi indicated that the relatively low yields were a result of the toxicity of the intermediate product 4-hydroxybiphenyl. To

overcome this problem, the group are currently investigating the production of mutant strains that are resistant to 4-hydroxybiphenyl. Another approach involves the continuous removal of the 4-hydroxybiphenyl by conducting fermentations in the presence of a water-insoluble solvent that extracts the 4-hydroxybiphenyl without harming the cells.

A further microbial approach to the production of 4,4′-dihydroxybiphenyl is based on phenol coupling. The biological coupling of phenol was first reported by Sjoblad, Minard and Bollag (1976) using an extracellular laccase (EC 1.10.3.2) from the fungus *Rhizoctonia practicola* (*Figure 9b*). More recent work carried out at Union Carbide (Schwartz and Hutchinson, 1981) has indicated that the mycelial fungi *Absidia pseudocylindrospora* 2770 and *Absidia* sp. 1341—the two strains selected for the most efficient hydroxylation of biphenyl—are also capable of coupling phenol to produce 4,4′-dihydroxybiphenyl. Although initial yields were low, (1·5 mg/ℓ over a 8–15-day period) this route offers a possible alternative to the sequential hydroxylation of biphenyl that may be brought about by micro-organisms.

THE SYNTHESIS OF EPOXIDES FROM GASEOUS ALKENES

The commercial application of micro-organisms for the synthesis of epoxides from gaseous alkenes has received considerable interest over the last decade, resulting in the publication of a large number of original papers and patents (Hou *et al.*, 1980; Furuhashi *et al.*, 1981; Neidleman, Amon and Geigert, 1981a,b; Hou, Patel and Laskin, 1983; Neidleman and Geigert, 1983; Habets-Crutzen *et al.*, 1985).

The two epoxides which are currently produced on the large-tonnage scale using conventional chemistry, are ethylene oxide and propylene oxide. Hydration of these two epoxides leads to the production of ethylene glycol and propylene glycol respectively. Apart from its well-known use in the production of car antifreeze, ethylene glycol is used extensively in the synthesis of polyester fibres, including brands such as Terylene, Dacron and Trevira. Propylene glycol is a major component for the synthesis of unsaturated polyester resins (epoxyresins) which are produced by the esterification of propylene glycol with unsaturated dibasic acids. Such resins, when used in conjunction with glass fibre to produce reinforced plastics, find wide application in the mass production of boats, tanks, vessels and other equipment. In addition, propylene glycol is used as a component of polymeric plasticizers (Brydson, 1982).

To form the epoxides three major approaches have been used in the chemical industries (*Figure 10*). One involves the direct oxidation of the alkene. The second approach is to form an intermediate halohydrin and to convert this with a suitable base to the epoxide. The third, commonly referred to as the oxirane process and which is a less major route, involves the oxidation of the gaseous alkene with a hydroperoxide. In this route the hydroperoxide is preformed by air oxidation of a suitable cosubstrate.

For the production of ethylene oxide, the currently favoured route is the direct oxidation of ethylene. In this process, pure oxygen is generally used

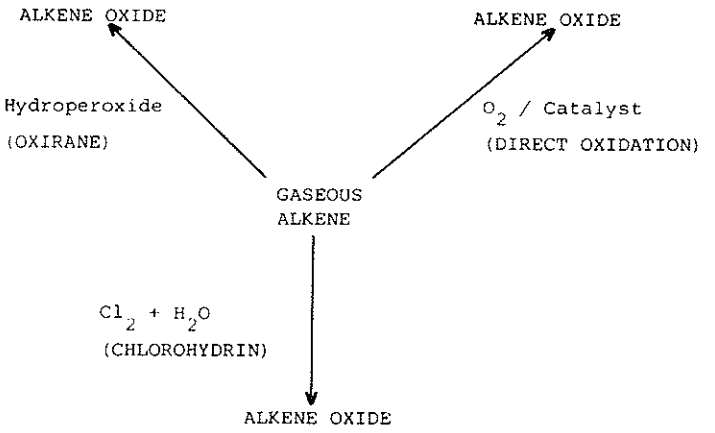

Figure 10. Chemical processes for epoxide production.

in conjunction with a silver oxide catalyst and temperatures of about 270°C. The process, therefore, entails expensive safety procedures and high costs with regard to both the catalyst and energy requirements. Furthermore, the reaction yields are decreased by the production of large quantities of carbon dioxide and water as by-products (Neidleman, Amon and Geigert, 1981a).

The inability to find a suitable catalyst has necessitated the industrial production of propylene oxide either via a halohydrin intermediate or via the oxirane process. The current processes for the production of halohydrins from propylene typically involve the addition of alkene, halogen and water in a reactor under controlled conditions:

$$\text{Propylene} + Cl_2 + H_2O \xrightarrow{\Delta} \text{Propylene chlorohydrin} + HCl$$

$$\text{Propylene chlorohydrin} \xrightarrow{\text{lime}} \text{Propylene oxide} + CaCl_2$$

Such a process often results in the formation of by-products, e.g. hydrochloric acid, haloalkanes and bis-(haloalkyl) ethers, while the use of free halogen requires control measures to prevent loss of this toxic agent (Neidleman, 1980).

The more recently developed oxirane process involves the conversion of a substrate hydrocarbon, e.g. ethylbenzene or isobutane, to its hydroperoxide by air oxidation in the presence of an initiator (*Figure 11*). The hydroperoxide is then reacted with propylene to form propylene oxide and the corresponding alcohol co-product, i.e. isobutanol or phenyl methyl carbinol. Reaction conditions require both elevated temperatures (90°C) and increased pressures (1·6–6·5 MPa). The main disadvantage of this process is that the yield of co-product may be considerably greater than that of propylene oxide (Waddams, 1978).

The use of micro-organisms, or enzymes derived from them, in the production of epoxides offers processes which are potentially safer, less expens-

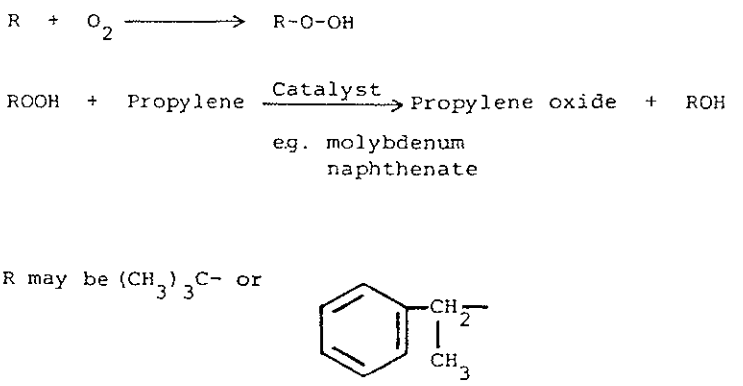

Figure 11. Oxirane process for production of propylene oxide.

ive, and lower in energy requirements than present methods. Two major pathways have been investigated.

The first of these routes, developed by Neidleman and colleagues for the Cetus Corporation (Neidleman, 1980), uses whole cells or enzymes derived from them in a group of reactions which bear similarities to both the chlorohydrin and oxirane processes normally used for the production of propylene oxide. The series of enzymic reactions employed for propylene oxide production are illustrated in *Figure 12*. The key enzyme in this biological route is the haloperoxidase or halogenating enzyme (Geigert *et al.*, 1983a). The enzyme preferred by Cetus is chloride peroxidase (EC 1.11.1.10) found in the micro-organism *Caldariomyces fumago*; this enzyme may be used either in solution or in the intact cell. Other microbial sources of haloperoxidases include a series of bromoperoxidases isolated from over 50 algae (Geigert *et al.*, 1983c).

The enzymic process differs from the chemical halohydrin process in that halide ions rather than free halogen are used, thus reducing cost and toxicity problems. In addition to the halogenating enzyme and a source of inorganic halide, an oxidizing agent is required in the reaction mixture: Cetus use

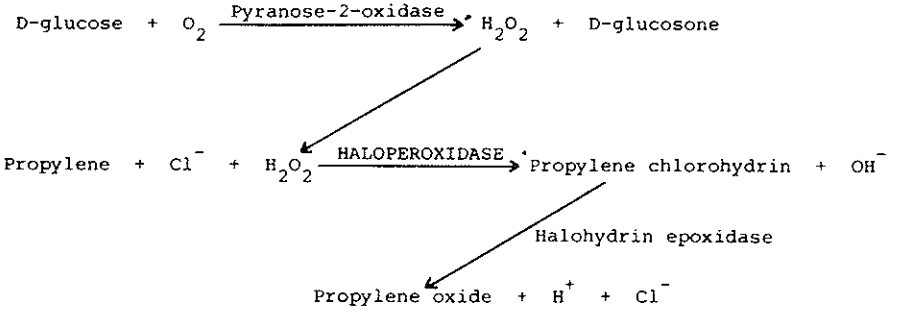

Figure 12. Production of propylene oxide using the enzymic route (after Neidleman, 1980).

hydrogen peroxide, which may be added directly or may alternatively be generated by a hydrogen peroxide-producing enzyme such as pyranose oxidase (EC 1.1.3.10). The alkene halohydrin thus formed is then converted by a further enzyme, a halohydrin epoxidase (Geigert *et al.*, 1983a), to the alkene epoxide and free halide; the latter can then be recycled. This type of enzyme has been found in *Flavobacterium* (Geigert *et al.*, 1983b).

The ultimate commercial viability of this process is dependent upon a number of factors, including the cost of generating H_2O_2 and the nature of any co-product which might also be manufactured in the process. One such product considered by Cetus is the synthesis of D-fructose from D-glucose using the H_2O_2-producing enzyme pyranose oxidase which converts D-glucose to D-glucosone, which may then be further reduced to D-fructose using catalytic hydrogenation. Other enzymes which have been studied by Cetus for the generation of H_2O_2, together with their co-products, are shown in *Table 5*.

The other microbial route to alkene epoxides involves direct oxidation of the alkene and hence in chemical terms may be compared to the direct oxidation process used in the commercial production of ethylene epoxide. The ability of micro-organisms to utilize gaseous alkenes such as ethylene and propylene as growth substrates has been detected in only a few cases to date. The majority of the earlier studies involving the oxidation of gaseous

Table 5. Biogeneration of hydrogen peroxide with concomitant formation of useful co-products (Substrate + $O_2 \xrightarrow{\text{enzyme}} H_2O_2$ + co-product). (Data from Neidleman, 1980)

Substrate	Enzyme (source)	Co-product
Methanol	Alcohol oxidase (yeast)	FORMALDEHYDE
D-Glucose	Pyranose oxidase (fungus)	D-Glucosone ↓ Catalytic hydrogenation D-FRUCTOSE
D-Glucose	Glucose-1-oxidase (fungus)	D-Glucono-δ-lactone ↓ in aqueous solution D-GLUCONIC ACID
D-Glucosone	Glucose-1-oxidase (fungus)	D-2-Ketogluconic acid ↓ heat + HCl FURFURAL

alkenes to their corresponding epoxides was, therefore, mainly undertaken with organisms capable of growth on alkanes. Although such alkane-utilizing organisms are generally not able to grow on alkenes, they are often able to cause epoxidation of the double bond. The enzymes which carry out this reaction (*Figure 13*) are a complex series of external monooxygenase enzymes normally involved in the terminal oxidation of alkanes to their corresponding alkanols (May, 1979).

Perhaps the most well-studied micro-organisms capable of forming epoxides are the methylotrophs. Although the capacity of oxidation reactions carried out by these organisms varies with species, they are in general capable of oxidizing a range of compounds as well as oxidizing gaseous alkenes to their corresponding epoxides (Colby, Stirling and Dalton, 1977; Higgins, Best and Hammond, 1980; Hou, Patel and Laskin, 1980; Hou *et al.*, 1981; Patel *et al.*, 1982; Higgins, Best and Scott, 1981). Various research groups and industrial concerns, including Exxon Research and Engineering Company and ICI, have therefore pursued this property and many patents have

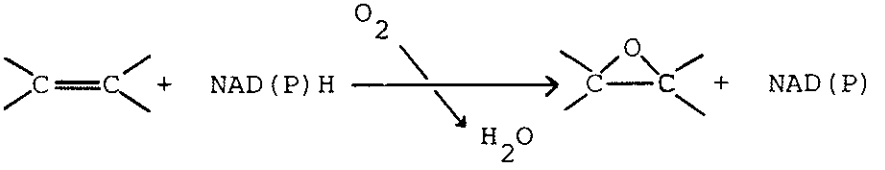

Figure 13. Microbial oxidation of an alkene by an alkane monooxygenase.

been filed on this subject (Higgins, 1980; Hou, Patel and Laskin, 1982; Hill and Higgins, 1982; Hou, Patel and Laskin, 1983; Higgins, 1984).

Although relatively few in number, organisms capable of growth on gaseous alkenes have also been studied for their potential to produce ethylene and propylene oxides. The alkene monooxygenases which carry out this reaction tend to be much more specific than their alkane counterparts. For example, neither the propylene monooxygenase (EC 1.13.12.-) of *Mycobacterium* Py1 nor the ethylene monooxygenase (EC 1.13.12.-) of *Mycobacterium* E_{20} are capable of oxidizing alkanes (de Bont *et al.*, 1983) yet each is capable of oxidizing various alkenes. Further specificity is also shown by these micro-organisms in their ability to utilize alkene epoxides, which makes them ideal biocatalysts for alkene epoxide production. For example, the propylene growing *Mycobacterium* Py1, which can use propylene as the carbon source, is capable of oxidizing ethylene to ethylene epoxide but the enzymes metabolizing propylene epoxide are not capable of degrading ethylene epoxide and hence allow accumulation of this product.

One of the major problems associated with epoxide formation when employing a monooxygenase-catalysed reaction is the need to regenerate the pyridine nucleotide cofactor NAD(P)H which is required by this group of enzymes. Early attempts to utilize enzymes such as methane monooxygenase

as biocatalysts involved the use of cell-free preparations (Higgins, Best and Hammond, 1980). Using such preparations, attempts were made to provide reducing equivalents electrochemically, thereby eliminating any requirement for expensive pyridine nucleotide coenzymes; however, the use of whole cells rather than isolated enzymes has overcome this difficulty, as well as obviating expensive enzyme isolation procedures. The intact cell can regenerate cofactor by utilization of a suitable co-substrate which is fully oxidized to CO_2 and H_2O. For example, Hou (1984) showed that intact cells of *Methylosinus* sp. CRL 31, when immobilized by adsorption on glass beads, were capable of oxidizing propylene to propylene oxide for a limited period, i.e. until depletion of endogenous reduced NAD^+ cofactor. The oxidative capacity of the cells subsequently could be restored by addition of methanol which led to cofactor regeneration by reaction with an appropriate dehydrogenase (*see also* Chapter 6 of this volume).

A further problem associated with the microbiological production of alkene epoxides is the toxicity of the reaction product to the biocatalyst. Propylene oxide is often used as a sterilizing agent because of its powerful alkylating capacity. It is therefore important to avoid accumulation of epoxide in the microenvironment of the micro-organism and hence its rapid removal is essential. To overcome this problem a variety of bioreactors have been designed including those of the gas/solid and multiliquid/gas/solid types. In the gas/solid reactor designed by Hou (1984), epoxide accumulation was avoided by maintaining the temperature of the reactor above 40°C, whereas in the gas/solid reactor designed by de Bont *et al.* (1983), epoxides were extracted into water acidified with sulphuric acid, resulting in their concomitant hydrolysis to glycols.

Microbial production of intermediates for polyamide synthesis

The interest in the use of micro-organisms in the production of intermediates in polyamide synthesis may have been further stimulated by the explosion which occurred at Flixborough in 1974 in a process involving the oxidation of cyclohexane. This demonstrated, certainly to the public, that the conventional thermochemical routes involved in the manufacture of nylon monomers are potentially dangerous.

The commercial importance of polyamides or nylons lies primarily in the manufacture of synthetic fibres, although they are also used as thermoplastics for injection moulding and extrusion (approximately 10% of polyamide production).

Chemically, polyamides are –CONH– units in alternating sequence with $-(CH_2)_n-$ chains and are synthesized either by the polycondensation of diamines with dicarboxylic acids or by polymerization of monomers based on lactams (Weissermal and Arpe, 1978). The polyamides of commercial significance, together with their starting monomers and their designated code, are shown in *Table 6*. Commercial interest in the microbial production of polyamide monomers has centred principally around the production of dicarboxylic acids, particularly adipic acid because it is one of the major

Table 6. Commercially significant polyamides

Polyamide code	Starting material	Polyamide structure
6	ϵ-Caprolactam	$-(-NH(CH_2)_5CO)_n-$
6.6	HMDA/adipic acid	$-(-NH(CH_2)_6NHOC(CH_2)_4CO)_n-$
6.10	HMDA/sebacic acid	$-(-NH(CH_2)_6NHOC(CH_2)_8CO)_n-$
6.12	HMDA/1,12-dodecanedioic acid	$-(-NH(CH_2)_6NHOC(CH_2)_{10}CO)_n-$
11.	ϵ-Aminoundecanoic acid	$-(-NH(CH_2)_{10}CO)_n-$
12.	Lauryl lactam	$-(-NH(CH_2)_{11}CO)_n-$

HMDA = Hexamethylene diamine

starting materials for the synthesis of Nylon 6.6, one of the most important polyamides. Hexamethylenediamine, the other starting material for Nylon 6.6, may also be synthesized from adipic acid and various commercial routes are available.

The most favoured thermochemical route to adipic acid is via the oxidative cleavage of cyclohexane. The most direct route involves a one stage-oxidation process with either nitric acid or air in combination with a suitable catalyst. This route has not, however, found commercial success owing to small yields and the production of high amounts of valueless by-products. Furthermore, large amounts of nitric acid are consumed in this process (*Figure 14*). The principal chemical route for adipic acid production is therefore based on a

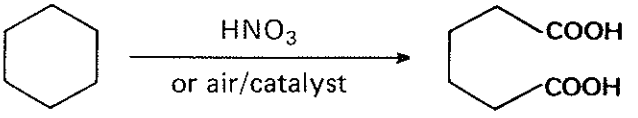

Figure 14. Direct route for the one-stage oxidation of cyclohexane to adipic acid.

two-stage process. In the first stage, cyclohexane is oxidized by air to a mixture of cyclohexanol and cyclohexanone (KA: ketone/alcohol mixture) using both elevated temperatures (125–165°C) and pressures (1·5 MPa), and a suitable catalyst. Because further oxidation products may occur in this process, cyclohexane conversion is limited to 10–15% in order to increase the selectivity for the KA mixture. The unused cyclohexane is separated by distillation and recycled (Waddams, 1978). The second stage for further oxidation of the cyclohexanone/cyclohexanol mixture to adipic acid commonly uses nitric acid. The process requires temperatures of 60–80°C, slightly elevated pressures and a suitable copper–vanadium complex catalyst (*Figure 15*). This process consumes considerable amounts of energy; in addition, the first stage is potentially explosive, while the second uses large quantities of concentrated nitric acid.

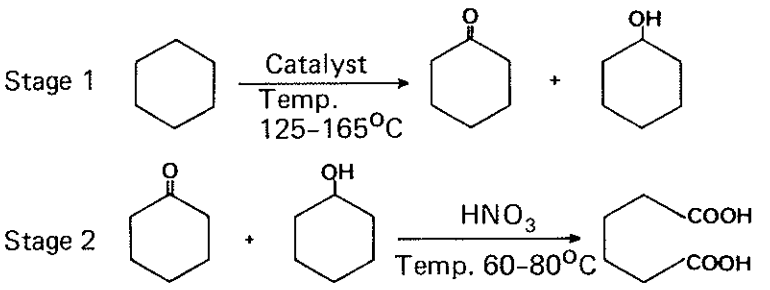

Figure 15. Principal industrial chemical route for adipic acid production using a two-stage process.

A one-stage oxidation process using micro-organisms could offer advantages if economic yields and large-scale production prove feasible. Three major microbial routes to adipic acid have been investigated.

The first microbial route utilizes toluene as the feedstock and was first proposed by Williams and Franklin (1980) using the micro-organism *Pseudomonas putida* mt-2. This particular micro-organism, which is capable of growth on toluene, carries on the TOL plasmid the ability to cleave toluene by the *meta*-cleavage pathway (*Figure 16*). The chemically distinct ability to use the *ortho* pathway is also carried by this micro-organism but is chromosomally encoded. The *ortho* pathway is normally used by the micro-organism for the degradation of benzoate and catechol when the plasmid is absent, as induction of the *ortho* pathway requires an accumulation of catechol which cannot occur when the plasmid is present. If the plasmid is absent or if production of catechol 2,3-oxygenase (EC 1.13.11.2) by the plasmid is blocked by mutation, then the catechol formed from toluene (*Figure 16*) accumulates and is further metabolized by the chromosomal *ortho* pathway (Worsey, Franklin and Williams, 1978). One of the prime intermediates in this hybrid plasmid/chromosomal pathway is *cis,cis*-muconic acid. Williams and Franklin (1980) proposed that if a mutational blockage was introduced to inactivate muconate cycloisomerase (EC 5.5.1.1), the enzyme responsible for the further metabolism of *cis,cis*-muconate, then *cis, cis*-muconate should accumulate. This compound, if extracted, could then be chemically reduced to adipic acid. A patent was granted to the Celanese corporation in 1984 for the continuous fermentation and bioconversion of toluene to *cis,cis*-muconic acid (Hsieh, 1984). The micro-organism used in this process is a *Pseudomonas putida* Biotype A strain ATCC 31,916 and has the desired mutations for the accumulation of *cis,cis*-muconate as originally proposed by Williams and Franklin (1980). According to the patent (Hsieh, 1984) the micro-organism is kept in continuous culture under nutrient limitation, using acetate as the preferred carbon source. Toluene, which can no longer act as a growth substrate for the organism, is fed to the reactor in the vapour phase. The novelty of the reactor is that the withdrawn fermentation broth is continuously passed through a cross-flow membrane

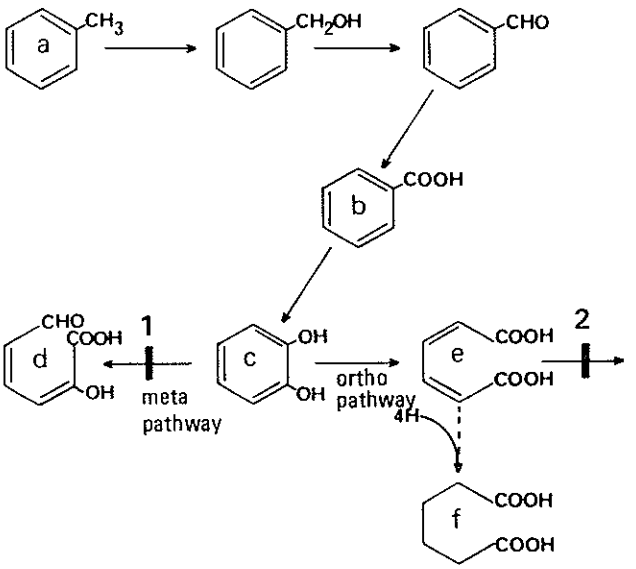

Figure 16. Proposed procedure for the formation of adipic acid from toluene, using *Pseudomonas putida* mt-2 carrying the TOL plasmid. Compounds: (a) toluene; (b) benzoic acid; (c) catechol; (d) 2-hydroxymuconic semialdehyde; (e) *cis, cis*-muconic acid; (f) adipic acid. Enzymes: (1) catechol 2,3-oxygenase; (2) muconate isomerase. Routes: – – – →, extraction and chemical reduction of *cis, cis*-muconic acid; →, enzymic route.

filtration zone. The retained cells are then recycled back to the original fermentation. Similarly, the permeate stream passing through the filter is processed for the removal of *cis,cis*-muconate and the medium then is also recycled to the original fermentation zone. Operating the reactor in this way leads to a *cis,cis*-muconate productivity of 1·4 g/ℓ/h with a specific productivity of 0·8–1·2 g/g dry weight cells/h. Recycling of the cells and media minimizes the amount of growth carbon and other nutrients required for cell maintenance. The small amount of growth of the micro-organism under these conditions is, however, important to the process as it acts as an energy sink to remove the excess energy generated during the oxidation of toluene by the organism. The *cis,cis*-muconate which is recovered by precipitation from the aqueous permeate is finally hydrogenated to adipic acid in the presence of a catalyst such as Raney nickel.

The second potential microbial route for the production of adipic acid involves the direct oxidative cleavage of cyclohexane and in chemical terms is analogous to the conventional chemical pathway. Micro-organisms have been isolated which are capable of growth on cyclohexane (Stirling, Watkinson and Higgins, 1977; Anderson, Hall and Griffin, 1980; Trower *et al.*, 1985). In each case the route of metabolism, as defined by studies with both whole cells and cell-free extracts, is that illustrated in *Figure 17*. The key reaction in this sequence of events is the initial oxidation of the cyclohexane

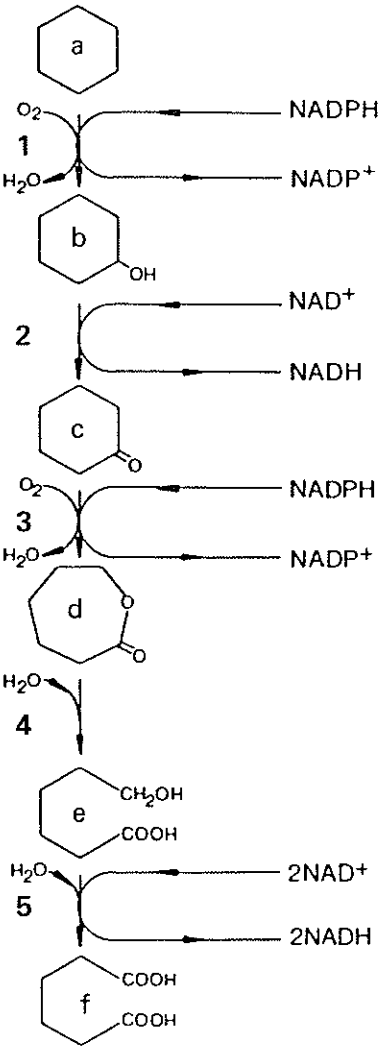

Figure 17. Reaction sequence for the microbial oxidation of cyclohexane to adipic acid. Cofactors shown are those for *Xanthobacter* sp. (Trower *et al.*, 1985). Compounds: (a) cyclohexane; (b) cyclohexanol; (c) cyclohexanone; (d) ε-caprolactone; (e) 6-hydroxyhexanoate; (f) adipic acid. Enzymes: (1) cyclohexane hydroxylase; (2) cyclohexanol dehydrogenase; (3) cyclohexanone monooxygenase; (4) ε-caprolactone hydrolase; (5) 6-hydroxyhexanoate dehydrogenase.

ring to cyclohexanol by cyclohexane hydroxylase (Trower, Buckland and Griffin, 1984). Further oxidation of cyclohexanol to adipic acid then follows the classical route, typical of those micro-organisms capable of growth on cycloalkanols (Donoghue *et al.*, 1976).

Preliminary studies with *Xanthobacter* sp. grown with cyclohexane have indicated that adipic acid accumulates in the medium when resting-cell

cultures of this micro-organism are incubated under oxygen-limiting conditions if cyclohexane is fed to the reactor. The specific productivity of adipic acid synthesized in this way is about 6·5 mg adipate/g dry weight organism/h. This productivity is significantly less than that from toluene using mutant strains of *Pseudomonas putida*, but optimization of yields is still under investigation. An advantage of using *Xanthobacter* sp. is that it offers the potential of producing other dicarboxylic acids useful in polyamide synthesis, as it is capable of oxidizing other cycloalkanes closely related to cyclohexane.

The third method which uses micro-organisms for the synthesis of dicarboxylic acids such as adipic and sebacic acids, utilizes the ability of certain micro-organisms to cause diterminal oxidation of long-chain alkanes without chain shortening (Kester and Foster, 1963). Under normal growth conditions the dicarboxylic acids produced would be further oxidized by the process of β-oxidation (*Figure 18*). Various patents have been filed for this process including those by the large Japanese companies Mitsui Petrochemical Industries Ltd and Nippon Mining Company Ltd. A recent report (Anonymous, 1983) mentioned that Mitsui Petroleum Industries Ltd have now successfully used this method for the synthesis of both adipic and sebacic acids using an unnamed organism in culture tanks of 3000 litres. The report indicates that the company has already reached the pilot test stage and is confident of future commercial production.

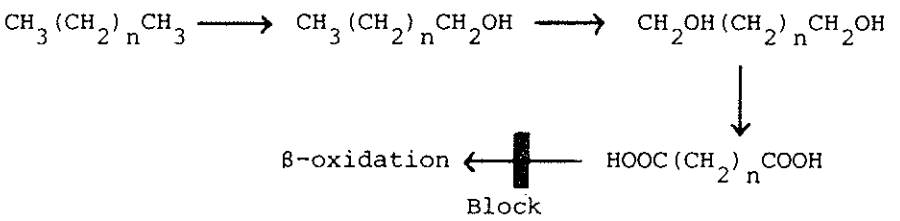

Figure 18. Diterminal oxidation process for long-chain alkanes by micro-organisms.

Conclusions

This chapter has attempted to review some of the useful roles of micro-organisms in the manufacture of plastics and synthetic fibres. The fact that only a few of these have reached commercial production does not necessarily indicate that the others are unacceptable but is a reflection of the highly competitive industry for which they have been developed. The dire predictions for the future of plastic materials during the onset of the oil crisis in the early 1970s have, to date, not been realized. Oil prices have stabilized to a large extent and the petrochemical industry has, of necessity, absorbed the increased costs incurred by energy requirements and raw materials. The result is that the prices of plastics and synthetic fibres have continued to increase at a lower rate than those of competitive alternative materials. Some

of the pressures exerted on the petrochemical industry to develop new or alternative processes have therefore been relaxed in the short term.

Nevertheless, any increase in production costs must be absorbed at the expense of other items within the petrochemical industry. It is unfortunate that in many instances this has been at the expense of long-term (10–20 years) research projects. As a result, a greater degree of selectivity has been exercised by the chemical industry concerning the future role of micro-organisms in large-scale biocatalysis. This has arisen at a particularly unfortunate time, as the continuing development of new techniques and concepts in both genetic manipulation and large-scale fermentation processes offers the possibility of further improving the performance of micro-organisms as biocatalysts. In the present economic climate (for the short or medium term) it therefore seems unlikely that micro-organisms, in the role of biocatalysts, will replace the conventional chemical routes for the production of bulk chemicals required for large-tonnage plastics. The many advantages of using biocatalytic processes, including those of specificity, controllability, decreased energy requirements and environmental acceptability, are frequently offset by the considerable capital investment required to introduce this new technology on a very large scale. In the short and medium term it therefore seems that a successful role for micro-organisms within the petrochemical industry will depend on identifying processes where they can be used to their maximum advantage, e.g. in the production of high-added-value chemicals: the partial oxidation of benzene to *cis*-benzene glycol, which was developed for the manufacture of polyphenylene, serves as a good example. Similarly, the future role of micro-organisms in the production of biopolymers which have application in the production of plastics and fibres depends on these products having special properties which cannot easily be obtained in their chemically produced counterparts: thus, both pullulan and poly-β-hydroxybutyrate possess unique properties that have merited their continued development for specialized applications.

A successful outcome for the role of micro-organisms in these novel processes is vitally important as the lessons learnt will have a profound effect on the continued development of this technology in the petrochemical industries.

Acknowledgements

We would like to thank the following companies for answering our requests for helpful information which has been used in this article: ICI Petrochemicals and Plastics Division, ICI Heavy Chemicals Business Area, New Science Group, Cetus Corporation and Hayashibara Biochemical Laboratories. We would also like to thank the respective authors and publishers for permission to use the data shown in *Tables 1–5* and *Figures 7* and *12*. These are respectively: *Table 1*, from *Plastics Materials*, Butterworth Scientific, author J.A. Brydson; *Tables 2* and *3*, from *Process Biochemistry*, author S. Yuen; *Table 4*, from *The Society of Chemical Industry*, author E.R. Howells; *Table 5* and *Figure 12*, from *Hydrocarbon Processing*, author S.L. Neidleman; and *Figure 7*, from *Trends in the Biology of Fermentations for Fuels and Chemi-*

cals, Plenum Press, authors A.J. Sinskey, M. Akedo and C.L. Cooney. Our thanks are due to Rozanne Pare for her patience shown typing this manuscript. Ann M. Magor is a Post-doctoral Research Fellow funded by the SERC Biotechnology Directorate.

References

ANDERSON, M.S., HALL, R.A. AND GRIFFIN, M. (1980). Microbial metabolism of alicyclic hydrocarbons: Cyclohexane catabolism by a pure strain of *Pseudomonas* sp. *Journal of General Microbiology* **120**, 119–125.

ANONYMOUS (1963). Bacteria produce polyester thermoplastic. *Chemistry and Engineering* **18**, 40–41.

ANONYMOUS (1983). Synthesis of new nylon raw material. *Chemical Economy and Engineering Review* **15**, 42–43.

AXCELL, B.C. AND GEARY, P.J. (1975). Purification and some properties of a soluble benzene-oxidizing system from a strain of *Pseudomonas*. *Biochemical Journal* **146**, 173–183.

BAIRD, J.K., SANDFORD, P.A. AND COTTRELL, I.W. (1983). Industrial applications of some new microbial polysaccharides. *Bio/Technology* **1**, 778–783.

BALLARD, D.G.H., COURTIS, A., SHIRLEY, I.M. AND TAYLOR, S.C. (1983). A biotech route to polyphenylene. *Journal of the Chemical Society, Chemical Communications* **17**, 954–955.

BAPTIST, J.N. (1962). *Process for Preparing Poly-β-hydroxybutyric Acid*. US Patent 3,036,959.

BARHAM, P.J. AND SELWOOD, A. (1982). *Extraction of Poly (beta-hydroxybutyric Acid)*. European Patent 0,058,480.

BONT, J.A.M. DE, VAN GINKEL, G.C., TRAMPER, J. AND LUYBEN, K.C.A.M. (1983). Ethylene oxide production by immobilized *Mycobacterium* Pyl in a gas-solid bioreactor. *Enzyme and Microbial Technology* **5**, 55–59.

BRYDSON, J.A. (1982). *Plastics Materials*, pp. 1–17. Butterworth Scientific, London.

COLBY, J., STIRLING, D.I. AND DALTON, H. (1977). The soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers and alicyclic, aromatic and heterocyclic compounds. *Biochemical Journal* **165**, 395–402.

DALAL, R.K., AKEDO, M., COONEY, C.L. AND SINSKEY, A.J. (1980). A microbial route for acrylic acid production. *Biosources Digest* **2**, 89–97.

DAWES, E.A. AND RIBBONS, D.W. (1962). The endogenous metabolism of microorganisms. *Annual Review of Microbiology* **16**, 241–246.

DAWES, E.A. AND SENIOR, P.J. (1973). The role and regulation of energy reserve polymers in microorganisms. *Advances in Microbial Physiology* **10**, 135–266.

DODGE, R.H., CERNIGLIA, C.E. AND GIBSON, D.T. (1979). Fungal metabolism of biphenyl. *Biochemical Journal* **178**, 223–230.

DONOGHUE, N.A., GRIFFIN, M., NORRIS, D.B. AND TRUDGILL, P.W. (1976). The microbial metabolism of cyclohexane and related compounds. In *Proceedings of the Third International Biodegradation Symposium* (J.M. Sharpley and A.M. Kaplan, Eds), pp. 45–56. Applied Science, Essex.

FURUHASHI, K., TAOKA, A., UCHIDA, S., KARUBE, I. AND SUZUKI, S. (1981). Production of 1,2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B-276. *Applied Microbiology and Biotechnology* **12**, 39–45.

GEARY, P.J., SABOOWALLA, F., PATIL, D. AND CAMMACK, R. (1984). An investigation of the iron–sulphur proteins of benzene dioxygenase from *Pseudomonas putida* by electron-spin resonance spectroscopy. *Biochemical Journal* **217**, 667–673.

GEIGERT, J., NEIDLEMAN, S.L., DALIETOS, D.J. AND DEWITT, S.K. (1983a). Haloperoxidases: Enzymatic synthesis of α,β-halohydrins from gaseous alkenes. *Applied and Environmental Microbiology* **45**, 366–374.

GEIGERT, J., NEIDLEMAN, S.L., LIU, T-N.E., DEWITT, S.K., PANSCHAR, B.M., DALI-

ETOS, D.J. AND SIEGEL, E.R. (1983b). Production of epoxides from α,β-halohydrins by *Flavobacterium* sp. *Applied and Environmental Microbiology* **45**, 1148–1149.

GEIGERT, J., NEIDLEMAN, S.L., DALIETOS, D.J. AND DEWITT, S.K. (1983c). Novel haloperoxidase reaction: synthesis of dihalogenated products. *Applied and Environmental Microbiology* **45**, 1575–1581.

GIBSON, D.T., KOCH, J.R. AND KALLIO, R.E. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymic formation of catechol from benzene. *Biochemistry* **7**, 2653–2662.

HABETS-CRUTZEN, A.Q.H., CARLIER, S.J.N., BONT, J.A.M. DE, WISTUBA, D., SCHURIG, V., HARTMANS, S. AND TRAMPER, J. (1985). Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. *Enzyme and Microbial Technology* **7**, 17–21.

HIGGINS, I.J. (1980). *Biotransformations*. UK Patent 2,024,205 A.

HIGGINS, I.J. (1984). *Microbiological Oxidations*. UK Patent 2,081,306 B.

HIGGINS, I.J., BEST, D.J. AND HAMMOND, R.C. (1980). New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. *Nature* **286**, 561–564.

HIGGINS, I.J., BEST, D.J. AND SCOTT, D. (1981). Generation of products by methanotrophs. In *Genetic Engineering of Microorganisms for Chemicals* (A.H. Hollaender, R.D. DeMoss, S. Kaplan, J. Konisky, D. Savage and R.S. Wolfe, Eds), pp. 383–402. Plenum Press, New York.

HILL, H.A.O. AND HIGGINS, I.J. (1982). *Enzymatic Processes*. UK Patent 2,033,428 B.

HOLMES, P.A. (1984). *3-Hydroxybutyrate Polymers*. European Patent 0,104,731.

HOLMES, P.A. AND JONES, E. (1982). *Extraction of poly (beta-Hydroxybutyric Acid)*. European Patent 0,046,335.

HOLMES, P.A., COLLINS, S.H. AND WRIGHT, L.F. (1984). *3-Hydroxybutyrate Polymers*. US Patent 4,477,654.

HOLMES, P.A., WRIGHT, L.F. AND COLLINS, S.H. (1982). *Beta-Hydroxybutyrate Polymers*. European Patent 0,052,459.

HOLMES, P.A., WRIGHT, L.F. AND COLLINS, S.H. (1983). *3-Hydroxybutyrate Polymers*. European Patent 0,069,497.

HOU, C.T. (1984). Propylene oxide production from propylene by immobilized whole cells of *Methylosinus* sp. CRL31 in a gas–solid bioreactor. *Applied Microbiology and Biotechnology* **19**, 1–4.

HOU, C.T., PATEL, R.N. AND LASKIN, A.I. (1980). Epoxidation and ketone formation by C1-utilizing microbes. *Advances in Applied Microbiology* **26**, 41–69.

HOU, C.T., PATEL, R.N. AND LASKIN, A.I. (1982). *Epoxidation of C2 to C4-Olefins and C4 Dienes*. UK Patent 2,019,390 C.

HOU, C.T., PATEL, R.N. AND LASKIN, A.I. (1983). *Method for Producing Microbial Cells and Use Thereof to Produce Oxidation Products (C-865)*. UK Patent 2,018,822 B.

HOU, C.T., PATEL, R.N., LASKIN, A.I. AND BARNABE, N. (1980). Microbial oxidation of gaseous hydrocarbons: oxidation of lower *n*-alkenes and *n*-alkanes by resting cell suspensions of various methylotrophic bacteria, and the effect of methane metabolites. *FEMS Microbiology Letters* **9**, 267–270.

HOU, C.T., PATEL, R.N., LASKIN, A.I., MARCZAK, I. AND BARNABE, N. (1981). Microbial oxidation of gaseous hydrocarbons: production of alcohols and methyl ketones from their corresponding *n*-alkanes by methylotrophic bacteria. *Canadian Journal of Microbiology* **27**, 107–115.

HOWELLS, E.R. (1982). Opportunities in biotechnology for the chemical industry. *Chemistry and Industry* **15**, 508–511.

HSIEH, J-H. (1984). *Continuous Fermentation Process and Bioconversion—Product Recovery*. US Patent 4,480,034.

HUGHES, L. AND RICHARDSON, K.R. (1982). *Fermentation Process*. European Patent 0,046,344.

JOHNS, A.T. (1952). The mechanism of propionic acid formation by *Clostridium*

propionicum. *Journal of General Microbiology* **6**, 123–127.

JONES, R.W. AND SIMON, R.H.M. (1983). Synthetic plastics. In *Riegels Handbook of Industrial Chemistry* (J.A. Kent, Ed.), pp. 311–377. Van Nostrand Reinhold, NY.

KESTER, A.S. AND FOSTER, J.W. (1963). Diterminal oxidation of long-chain alkanes by bacteria. *Journal of Bacteriology* **85**, 859–869.

KING, P.P. (1982). Biotechnology. An industrial view. *Journal of Chemical Technology and Biotechnology* **32**, 2–8.

LACROIX, C., LEDUY, A., NOEL, G. AND CHOPLIN, L. (1985). Effect of pH on the batch fermentation of pullulan from sucrose medium. *Biotechnology and Bioengineering* **27**, 202–207.

LADD, J.N. AND WALKER, D.J. (1965). Fermentation of lactic acid by the rumen microorganism *Peptostreptococcus elsdenii*. *Annals of the New York Academy of Sciences* **119**, 1038–1045.

LAFFERTY, R.M., BRAUNEGG, G., HEINZLE, E., KATZOJANNOS, E., SONNLEITNER, B. AND SRIENC, F. (1980). Kinetics of the biotechnological production of poly-β-hydroxybutyric acid. In *Proceedings of the Fifth International Fermentation Symposium, London, Canada, 20 July 1980*, pp. 481–486.

LAWSON, C.J. AND SUTHERLAND, I.W. (1978). Polysaccharides. In *Primary Products of Metabolism, Economic Microbiology* (A.H. Rose, Ed.), volume 2, pp. 328–392. Academic Press, London.

MARVEL, C.S. AND HARTZELL, G.E. (1959). Preparation and aromatization of poly-1,3-cyclohexadiene. *Journal of the American Chemical Society* **81**, 448–452.

MATTIASSON, B. AND LARSSON, M. (1985). Extractive bioconversions with emphasis on solvent production. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 3, pp. 137-174. Intercept, Ponteland, Newcastle upon Tyne.

MAY, S.W. (1979). Enzyme epoxidation reactions. *Enzyme and Microbial Technology* **1**, 15–22.

NAKAJIMA, M., TOMIDA, I. AND TAKEI, S. (1959). Darstellung von *cis*-benzolglyhol, stereoisomeren mucondialdehyden und kondurit. *Chemische Berichte* **92**, 163–172.

NEIDLEMAN, S.L. (1980). Use of enzymes as catalysts for alkene oxide production. *Hydrocarbon Processing* **59**, 135–138.

NEIDLEMAN, S. L. (1984). Applications of biocatalysis to biotechnology. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 1, pp. 1–38. Intercept, Ponteland, Newcastle upon Tyne.

NEIDLEMAN, S.L. AND GEIGERT, J. (1983). Biological halogenation and epoxidation. In *Biotechnology: Biochemical Society Symposium 48* (C.F. Phelps and P.H. Clarke, Eds), pp. 39–52. Cambridge University Press, Cambridge.

NEIDLEMAN, S.L., AMON, W.F., JR AND GEIGERT, J. (1981a). *Method for Producing Epoxides and Glycols from Alkenes*. US Patent 4,247,641.

NEIDLEMAN, S.L., AMON, W.F., JR AND GEIGERT, J. (1981b). *Preparation of Epoxides and Glycols from Gaseous Alkenes*. US Patent 4,284,723.

PATEL, R.N., HOU, C.T., LASKIN, A.I. AND FELIX, A. (1982). Epoxidation of alkenes and hydroxylation of alkanes by soluble methane monooxygenase: regeneration of cofactor $NADH_2$. *Journal of Applied Biochemistry* **4**, 175–184.

POWELL, L. W. (1984). Developments in immobilized-enzyme technology. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 2, pp. 409–438. Intercept, Ponteland, Newcastle upon Tyne.

RICHARDSON, K.R. (1984). *Production of beta-Hydroxybutyrate Polymers*. European Patent 0,114,086.

SANDFORD, P.A. (1979). Exocellular, microbial polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry* **36**, 265–313.

SCHWARTZ, R.D. (1981). A novel reaction: meta hydroxylation of biphenyl by an actinomycete. *Enzyme and Microbial Technology* **3**, 158–159.

SCHWARTZ, R.D. AND HUTCHINSON, D.B. (1981). Microbial and enzymatic production of 4,4′-dihydroxybiphenyl via phenol coupling. *Enzyme and Microbial Tech-*

nology **3**, 361–363.

SCHWARTZ, R.D., WILLIAMS, A.L. AND HUTCHINSON, D.B. (1980). Microbial production of 4,4'-dihydroxybiphenyl: Biphenyl hydroxylation by fungi. *Applied and Environmental Microbiology* **39**, 702–708.

SENIOR, P.J. (1984). Polyhydroxybutyrate, a speciality polymer of microbial origin. In *Continuous Culture* (A.C.R. Dean, D.C. Ellwood and C.G.T. Evans, Eds), volume 8, pp. 266–271. Ellis Horwood, Chichester.

SINSKEY, A.J., AKEDO, M. AND COONEY, C.L. (1981). Acrylate fermentations. In *Basic Life Sciences, Volume 18: Trends in the Biology of Fermentations for Fuels and Chemicals* (A. Hollaender, Ed.), pp. 473–492. Plenum Press, New York.

SJOBLAD, R.D., MINARD, R.D. AND BOLLAG, J.M. (1976). Polymerization of 1-naphthol and related phenolic compounds by an extracellular fungal enzyme. *Pesticide Biochemistry and Physiology* **6**, 457–463.

SMITH, R.V. AND ROSAZZA, J.P. (1974). Microbial models of mammalian metabolism. Aromatic hydroxylation. *Archives of Biochemistry and Biophysics* **161**, 551–558.

STAGEMAN, J.F. (1984). *Extraction Process*. European Patent 0,124,309.

STIRLING, L.A., WATKINSON, R.J. AND HIGGINS, I.J. (1977). Microbial metabolism of alicyclic hydrocarbons: Isolation and properties of a cyclohexane-degrading bacterium. *Journal of General Microbiology* **99**, 119–125.

SUGIMOTO, K. (1981). Pullulan—production and applications. *Journal of the Fermentation Association, Japan* **36**, 98–108.

SUTHERLAND, I.W. (1982). Biosynthesis of microbial exopolysaccharides. *Advances in Microbial Physiology* **23**, 79–150.

SUTHERLAND, I.W. AND ELLWOOD, D.C. (1979). Microbial exopolysaccharides—industrial polymers of current and future potential. In *Microbial Technology—Current State and Future Prospects, SGM Symposium 29*, (A.T. Bull, D.C. Ellwood and C. Ratledge, Eds), pp. 107–150. University Press, Cambridge.

TAYLOR, S.C. (1985). Enzymic synthesis of 5,6-dihydroxycyclohexa-1,3-diene. In *Enzymes in Organic Synthesis, Ciba Foundation Symposium 111* (R. Porter and S. Clark, Eds), pp. 71–75. Pitman, London.

TROWER, M.K., BUCKLAND, R.M. AND GRIFFIN, M. (1984). Characterisation of a cyclohexane hydroxylase and cyclohexanone monooxygenase from a cyclohexane-degrading *Xanthobacter* sp. *Biochemical Society Transactions* **13**, 463–464.

TROWER, M.K., BUCKLAND, R.M., HIGGINS, R. AND GRIFFIN, M. (1985). Isolation and characterisation of a cyclohexane-metabolising *Xanthobacter* sp. *Applied and Environmental Microbiology* **49**, 1282–1289.

WADDAMS, A.L. (1978). *Chemicals from Petroleum*, pp. 283–296. Murray, London.

WALKER, J., WHITTON, J.R. AND ALDERSON, B. (1982). *Extraction of Poly (beta-Hydroxybutyric acid)*. European Patent 0,046,017.

WEGENER, W.S., REEVES, H.C. AND AJL, S.J. (1967). Propionate oxidation in *Escherichia coli*. *Archives of Biochemistry and Biophysics* **121**, 440–442.

WEISSERMAL, K. AND ARPE, H.J. (1978). Components for polyamides. In *Industrial Organic Chemistry—Important Raw Materials and Intermediates* (A. Mullen, Ed.), pp. 209–233. Verlag Chemie, New York.

WILLIAMS, P.A. AND FRANKLIN, F.C.H. (1980). Genetic possibilities for extending the range of microbial transformations. In *Hydrocarbons in Biotechnology* (D.E.F. Harrison, I.J. Higgins and R. Watkinson, Eds), pp. 155–168. Heyden, for Institute of Petroleum, London.

WORSEY, M.J., FRANKLIN, F.C.H. AND WILLIAMS, P.A. (1978). Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida*. *Journal of Bacteriology* **134**, 757–764.

YAMAMOTO, T., HAYASHI, Y. AND YAMAMOTO, A. (1978). A novel type of polycondensation utilizing transition metal-catalysed C–C coupling. I. Preparation of thermostable polyphenylene type polymers. *Bulletin of Chemical Society of Japan* **51**, 2091–2097.

YUEN, S. (1974). Pullulan and its applications. *Process Biochemistry* **9**, 7–9.