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Interrelations of Chemistry and Biotechnology—VI†

MICROBIAL PRODUCTION OF COMMODITY CHEMICALS

(Technical Report)

Prepared for publication by

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†The Commission has chosen this series with a view to intensifying the interrelations of chemistry and biotechnology. By improving the knowledge of chemists in the field of biotechnology it is hoped to initiate more ideas for applying biological methods in chemistry, inspire more use of chemical knowledge in the biological sciences and help scientists in both fields to work closer together. In the articles in the series, to be published in this journal, outstanding experts in their respective fields will give (a) an overview on topics related to the practical use of biotechnological methods in organic chemistry, and (b) an outlook on upcoming research topics, their impact on existing areas and their potential for future developments.

The Commission solicits comments as well as suggestions for future topics, and will aim to help in providing answers to any questions in this field.

Interrelations of chemistry and biotechnology—VI† Microbial production of commodity chemicals (Technical report)

Synopsis

Microbial processes for industrial production of commodity chemicals are rapidly gaining practical significance for preparation of high purity products, in an environmentally acceptable manner while realizing energy savings. The use of bacterial nitrile hydratase for industrial production of the important chemical, acrylamide, was recently pioneered in Japan. We review here the enzymatic production of acrylamide and recent progress in the production of other commodity chemicals through microbial processes.

INTRODUCTION

The bioindustry originated from the brewing industry and then developed into the early chemical industry aimed at the utility of penicillin was established, the bioindustry moved into production of fine chemicals such as antibiotics, amino acids and nucleotides. Microbial synthetic processes often competed with the chemical synthetic processes. With the development of petrochemical industry after 1950, microorganisms lost their valuable position because of the reduced importance for production of bulk chemicals. However, microbial production of industrial chemicals is again attracting attention, because of the possibility of the preparation of high purity products in an environmentally acceptable, energy efficient manner. We outline here the application of the biological processes for the production of commodity chemicals, focusing in particular on a recent successful example, the production of acrylamide by nitrile hydratase.

METABOLIC END PRODUCTS AS COMMODITY CHEMICALS

Microorganisms are used to produce a wide variety of low molecular weight compounds. For example, antibiotics, pharmacologically active agents (cyclosporin and FK506, immunosupressant; mevalochin, inhibitor for cholesterol synthesis; ergot alkaloids, etc.,), vitamins, amino acids and steroids. These compounds can be subdivided into those whose production is associated with growth (primary metabolites) and those whose synthesis occurs after growth has ceased (secondary metabolites). Primary metabolites can be further subdivided into those such vitamins and amino acids, which normally are produced in quantities sufficient only for cell growth, and those such as ethanol and lactic acid, which are produced in larger quantities because they are normal metabolic end-products.

Ethanol, acetone, butanol, lactic acid and glycerol are the traditional products of the fermentation industry. They are formed as a result of the anaerobic metabolism of sugars. In the absence of oxygen as the terminal electron acceptor, different cells regenerate NAD^+ from NADH by alternative means, some of which lead to the formation of commercially important chemicals. Ethanol is a commodity chemicals like others, and its production is also the basis of alcoholic beverage industry. In the immediate post-war period many organic chemicals, including acetone and butanol, became readily available as by-products of the petroleum industry and the fermentation processes were discontinued. The fermentative production of these metabolic end-products is subject to economic and political pressures. As the price of oil has risen, there has been a move back to fermentation processes as a means of production. Thus, the percentage of industrial alcohol production which is based on fermentation has risen in the past ten years from less than 10% of total world demand to more than 20%. Ethanol produced from sugar cane is mixed with gasoline and used as motor fuel in Brazil. The acetone-butanol fermentation might be of benefit to many Third World countries which cannot afford to spend vast sums of money on either petroleum itself or on petrochemical-based products. Since these

countries often have an abundance of necessary cheap raw materials such sugars or starch, this fermentation process may make a comeback.
The world-wide production of citric acid through fermentation amounts to approximately 100 000 tons per year. Citric acid has various applications, such as food additive, plasticizer, detergent, and abrasive. Gluconic acid, 2-ketogluconic acid, lactic acid, itaconic acid and L-malic acid are also produced on an industrial scale through fermentation. Dihydroxyacetone as a cosmetic material is produced from glycerol through fermentation by *Acetobacter* sp.
About 700 000 tons/year of amino acids, mostly, L-glutamic acid, L-lysine and L-methionine are produced per year in the world. Above, all, the fermentative production of L-glutamic acid reaches 340 000 tons per year in the world and its price almost ranks with those of other commodity chemicals. It is predominantly used as a food additive and it is the starting material for the synthesis of polyglutamic acid.

APPLICATION OF BIOTRANSFORMATION FOR THE PRODUCTION OF COMMODITY CHEMICALS

Microbes are also used to effect chemical transformations; that is, the desired product is not a normal metabolite of the cell but is produced as a result of enzymatic conversion of an unusual substrate added to the culture medium. Often such substrates do not support growth, they simply undergo a chemical transformation. Such BIOTRANSFORMATION comprise only one, or a small number of enzymatic reactions, as opposed to the multi-reaction sequences of fermentative metabolite production.
Biotransformation is used commercially when conventional chemical approaches are not feasible or too costly. In contrast to fine chemicals, the commodity chemicals are inexpensive, have larger demands, and are produced and sold in bulk. Most of them are intermediates for further syntheses.
Commodity chemicals are characterized by relatively high raw materials costs as compared to the cost of production. However, usually the preparation of the catalyst is quite costly. Therefore, to surpass the corresponding chemical processes utilizing the same raw materials, microbial processes need enormously high productivity (high accumulation of product per unit volume of reactor). In addition, the repeated use of the biocatalyst is beneficial.
A prominent feature by which biocatalysts are favorably distinguished from common chemical catalysts is their high specificity, not only with respect to the reaction they catalyze, but also with respect to the structure and even the stereo chemistry of the substrate they accept and the product they form. Moreover, the high specificity of biocatalysts does not require a highly purified substrate.
A further characteristic of biocatalysts is its mild reaction conditions. Enzymes display high catalytic activities even under mild reaction conditions, i.e. in aqueous media, at temperatures below 40 °C, at pH values near neutrality and at ambient pressure. Thus, the severe and energy intensive reaction conditions needed for chemical catalysis can be avoided and even labile molecules can be converted without undesired decomposition or their side reactions. The demands of the energy efficiency, conservation of natural resources and reduction of environmental pollution, which is currently being faced by the chemical industry, may be better handled through the introduction of microbial processes into production.
The application of bioconversion processes has been generally restricted to the production of fine chemicals which are difficult to make by chemical syntheses. In 1979, a novel production process for propene oxide, the 'Cetus Process' was proposed. Thus, for the first time, an enzymatic process was incorporated into a petrochemical process for production of a bulk chemical. Next, in 1985, the enzymatic production of acrylamide, a typical commodity chemical, was started on an industrial scale, making microbial transformations in the industrial production of commodity chemicals a rapidly developing field.

Microbial transformations of nitriles

Nitriles are widely used in organic synthesis as precursors for compounds such as amides and organic acid. however, chemical conversion of nitriles presents several

problems: reactions require either strongly acidic or basic media; energy consumption is high; and unwanted by-products (toxic substances or large amount of salts) are formed. 'Biological' procedures currently being developed, in which microorganisms are used as the catalyst, are attractive because pH and temperature conditions are less severe than those of chemical processes and because very pure products are formed without by-products. Nitrile-hydrolyzing enzymes, nitrile hydratase and nitrilase, have great potential as catalysts for converting nitriles to higher-value amides or acids on an industrial scale.

(1) Acrylamide

Acrylamide is one of the most important chemical commodities, being in great demand (200 000 tons per year worldwide) as a starting material for the production of various polymers for use as flocculates and polymers for petroleum recovery. In conventional synthesis, an acrylonitrile solution is passed over a Raney copper catalyst at about 100 °C. However, the complex nature of the preparative procedure for the catalyst, difficulties in regenerating the used catalyst and problems associated with separating and purifying the acrylamide formed are drawbacks. Furthermore, because acrylamides are readily polymerized, their production under moderate conditions is desirable.

An acrylamide production process using the biocatalyst nitrile hydratase was established by our group at Kyoto University and the Nitto Chemical Industry, Ltd. research group in Japan. Now approximately 10 000 tons per year of acrylamide is produced using this process.

During the course of the studies on the degradation of nitrile compounds by microorganisms, the new enzyme termed 'nitrile hydratase' was found which catalyzes the hydration reaction of nitrile to the amide (equation 1)(Asano *et al.*, 1980).

$$CH_2{=}CHCN + H_2O \longrightarrow CH_2{=}CHCONH_2 \quad (1)$$

An extensive screening was carried out to isolate an acrylamide-accumulating strain. Various nitrile-assimilating bacteria were isolated and cultivated in different nitrile-containing media. Using these resting cells, the bioconversion activity of acrylonitrile into acrylamide was assayed. Thus, *Pseudomonas chlororaphis* B23(Asano *et al.*, 1982) and *Rhodococcus* sp. N-774(Watanabe, *et al.* 1979, 1986 and 1987) were selected as efficient catalysts for the production of acrylamide. When resting cells were incubated with acrylonitrile, provided the acrylonitrile was added gradually enough to the reaction mixture to avoid inhibition of the nitrile hydratase activity, up to 400 g of acrylamide per liter accumulated at 10 °C. More than 99% of the acrylonitrile was converted into acrylamide without the formation of acrylic acid as a by-product(Asano *et al.*, 1982). In developing this enzymatic reaction, various culture conditions were tested (Yamada, *et al.*, 1986), and the enzyme activity of strains was improved by mutagenesis(Ryuno *et al.*, 1988). Together, the culture medium and mutation studies significantly improved nitrile hydratase activity (Nagasawa *et al.*, 1989a). With Nitto Chemical Industry, we developed the process for industrial use. A new bioreactor using cells entrapped in a cationic acrylamide-based polymer gel was designed(Watanabe *et al.*, 1987), and a compact and efficient commercial plant was built (Nakai *et al*,, 1988).

In contrast to the conventional chemical process for acrylamide (Figure 1), the recovery of unreacted acrylonitrile is not necessary in the biochemical process because the conversion of the latter is more than 99.99%. Also, the removal of copper ions from the product is no longer necessary (Nakai *et al*,, 1988, Nagasawa et al., 1989b). Overall, the enzymatic process is simpler and more economical. It is carried out below 10°C under mild reaction conditions and requires no special energy source (Table 1). The immobilized cells are used repeatedly and a very pure product is obtained.

The industrial production of acrylamide was started using *Rhodococcus* sp. N-774 in 1985; later in 1988, a more efficient catalyst, *P. chlororaphis* B23 (Nagasawa et al., 1990, Yamada et al., 1990) was introduced. This change of biocatalyst led, to about 50% increase in yearly productivity of acrylamide (Table 2).

Compared to conventional organic synthesis, biocatalysts can have a potential for improving productivity and yields. To further enhance the productivity of acrylamide,

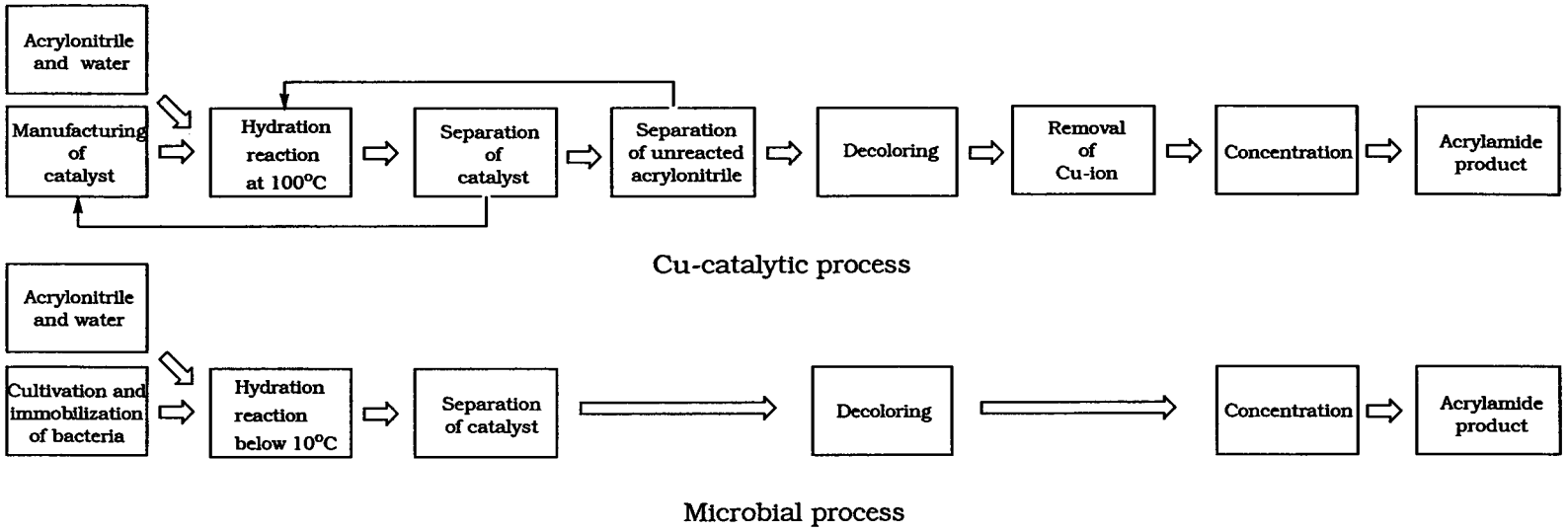

Figure 1: Comparison of microbial and conventional processes for acrylamide.

Table 1: Operating conditions for acrylamide production

Reaction conditions		Productivity	
pH 7.5-8.5		Conversion of acrylonitrile to acrylamide	99.97%
Temperature	0-5 °C	Yield of acrylamide	99.99%
Acrylonitrile mass concentration in the reactor	15-20 g/L	Acrylamide mass concentration	27-30 g/L

Table 2: Comparison of three kinds of biocatalyst

	Rhodococcus sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
Tolerance to acrylamide (g/L)	27	40	50
Acrylic acid formation	very little	barely detected	barely detected
Cultivation time (h)	48	45	72
Activity of culture broth (units/ml)	900	1 400	2 100
Specific activity (units/mg cells)	60	85	76
Cell yield (g/L)	15	17	28
Acrylamide productivity (g/g cells)	500	850	>7 000
Total amount of production (ton/year)	4 000	6 000	30 000
Final concentration of acrylamide (g/L)	20	27	40
First year of production	1985	1988	1991

we further improved the biocatalyst. Very recently *Rhodococcus rhodochrous* J1 was found to have a more powerful ability to produce acrylamide the *P. chlororaphis* B23. *P. chlororaphis* B23 and *Brevibacterium* R312 nitrile hydratases contain ferric ions as a cofactor (Nagasawa *et al.*, 1986 and 1987, Sugiura *et al.*, 1987); on the other hand, *R. rhodochrous* J1 nitrile hydratase contains cobalt ions as a cofactor (Nagasawa *et al.*, 1988a, Nagasawa *et al.*, 1991a)(Table 3). When *R. rhodochrous* J1, was cultivated in a nutrient medium supplemented with cobalt ions and urea as an inducer, an enormous amount of nitrile hydratase, corresponding to more than 40% of all soluble protein, was produced in the cells of *R. rhodochrous* J1 (Nagasawa et a., 1991b).

Table 3: Comparison of three kinds of nitrile hydratases

	Rhodococcus sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
Molecular mass	70 000	100 000	505 000
Subunit molecular mass	α 27 000	α 25 000	α 26 000
	β 27 500	β 25 000	β 29 000
Metal	Fe(III)	Fe(III)	Co
Optimum temperature(^{0}C)	35	20	35-40
Heat stability(^{0}C)	30	20	50
Optimum pH	7.7	7.5	6.5
pH stability	7.0-8.5	6.0-7.5	6.0-8.5
Substrate specificity	aliphatic nitriles	aliphatic nitriles	aliphatic & aromatic nitriles
Activation by light irradiation	+	-	-
Formation type	constitutive	inducible	inducible

R. rhodochrous J1 nitrile hydratase is much more heat stable and more tolerant to high concentrations of acrylonitrile when compared to the *P. chlororaphis* B23 and *Brevibacterium* R312 enzymes. Also, the *R. rhodochrous* J1 enzyme has a characteristic high tolerance to high concentrations of acrylamide (Figure 2). *R. rhodochrous* J1 cells exhibited catalytic activity even in the presence of 50% acrylamide; on the other hand, *P. chlororaphis* B23 and *Brevibacterium* R312 nitrile hydratases did not exhibit their full activity in the presence of 20% acrylamide.
Using *R. rhodochrous* J1 resting cells, the accumulation of acrylamide was determined by feeding acrylonitrile to maintain a constant concentration of 60 g/L. After 10 h incubation at 10 °C, 656 g/L of acrylamide accumulated. Similarly, at 15 °C, 567 g/L and at 20 °C, 560 g/L of acrylamide accumulated (Nagasawa et al., 1993). The acrylamide production by the three kinds of biocatalysts are summarized in Table 2.

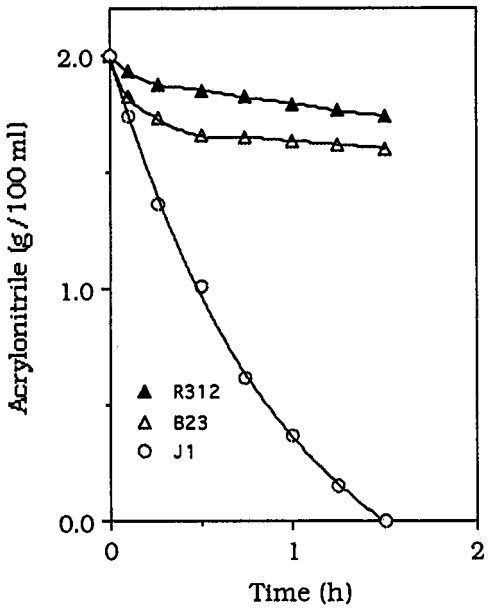

Figure 2:

Effect of acrylamide on the activity of three kinds of nitrile hydratase. The reaction was carried out in a mixture containing 2 g/100 ml acrylonitrile and 30 g/100 ml acrylamide.

Because of the high stability and high tolerance to acrylamide, at least 40% acrylamide accumulation can be attained in large scale production. In spite of a change of biocatalyst, almost no change in the plant is required. Thus, the importance of the selection of biocatalyst can be appreciated. Since 1991, *R. rhodochrous* J1 has been used for industrial production of acrylamide and at present about 10,000 tons/year of acrylamide is produced by Nitto Chemical Industry Ltd.
R. rhodochrous J1 nitrile hydratase exhibits broad substrate specificity. For example, using *R. rhodochrous* J1 cells, the highest yield achieved was almost 1.5 kg nicotinamide per liter of reaction mixture from 3-cyanopyridine without the formation of nicotinic acid (Nagasawa et al., 1988). Due to the high yields of this process, the ease of cultivation of *R. rhodochrous* J1 cells and the stability of the cells (which are stable at -20 °C for more than 2 years), the use of this enzymatic hydration process is promising for industrial production of various aliphatic, aromatic and heterocyclic amides (Mauger et al., 1988 and 1989, Nagasawa et al., 1990a).
Three kinds of nitrile hydratases of *P. chlororaphis* B23 (Nagasawa et al., 1987), *Rhodococcus* N-774 (Endo and Watanabe, 1989) and *R. rhodochrous* J1 (Nagasawa et al., 1991a) were purified and characterized (Table 3). These enzymes are composed of two subunits of different sizes (a and b subunits). *R. rhodochrous* J1 nitrile hydratase has much higher molecular mass and is composed of 10 a and 10 b subunits. The genes for these subunits were cloned and the open reading frame for the b subunits was located just upstream of that for the a subunits in the *R. rhodochrous* J1 gene (Kobayashi et al., 1991); however, the arrangement of the coding sequences for the two subunits was in the reverse order found in the nitrile hydratase genes of *Rhodococcus* N-774 (Ikehara *et al.*, 1989) and *P. chlororaphis* B23 (Nishiyama *et al.*, 1991).

The primary structure of each subunit of these nitrile hydratases were determined. The amino acid sequences of the subunits showed generally significant similarities. Each of the nitrile hydratase genes was expressed in *Escherichia coli* cells under the control of the lac promoter, when cultured in a medium supplemented with CoCl2 or FeCl3.

(2) Acrylic acid and metacrylic acid

Other nitrile converting enzymes such as nitrilase and amidase are also expected to have great potential as catalysts in organic chemical processing. These enzymes may be utilized for the preparation of a wide range of compounds in a flexible manner: for example, for the synthesis of optically pure amino acids and hydroxy acids form the corresponding amino nitriles or cyanohydrins, respectively.

Acrylic acid and methacrylic acid are important starting materials for the synthesis of various kinds of polymers. Acrylic acid and methacrylic acid are manufactured through gas-phase oxidation of propene and 2-methylpropene, respectively, by the use of chemical catalysts at high temperatures. Severe problems, such as damage to catalysts and polymerization of the products, cannot by by-passed. Therefore, the application of biocatalysts is attractive for the production of acrylic acid and methacrylic acid.

R. rhodochrous J1 exhibits metabolic versatility: when 3-methylbutyronitrile or hexano-d-lactam is used as an inducer without supplementation of cobalt ions, it can produce nitrilase abundantly (Nagasawa et al., 1988b and 1990b). We attempted the enzymatic conversion of acrylonitrile and methacrylonitrile into acrylic acid methacrylic acid, respectively (Equation 2 and 3), using hexano-δ-lactam-induced *R. rhodochrous* J1 cells.

$$CH_2{=}CHCN + 2H_2O \longrightarrow CH_2{=}CHCOOH \quad (2)$$

$$CH_2{=}C(CH_3)CN + 2H_2O \longrightarrow CH_2{=}C(CH_3)COOH \quad (3)$$

Under a periodic substrate feeding strategy, the highest accumulations, 390 g/L acrylic acid and 260 g/L methacrylic acid, were attained with almost 100% molar conversion yield (Nagasawa et al., 1990c).

Table 4: Various nitrilases produced by microorganisms

	Rhodococcus rhodochrous K22	*Rhodococcus rhodochrous* J1	*Alcaligenes faecalis JM3*	*Klebsiella ozaenae*
Molecular mass (kDa)	650	78	260	72
Subunit molecular mass (kDa)	41	41.5	44	38.1
Number of subunits	16	2	6	2
Optimum temperature(^{0}C)	50	45	45	35
Heat stability(^{0}C)	40	45	30	-
Optimum pH	5.5	7.7	7.5	9.2
Substrate	aliphatic nitriles	aliphatic &aromatic nitriles	arylacetonitriles	bromoxynil

R. rhodochrous J1 also converts 100% of 3-cyanopyridine and *p*-aminobenzonitrile added to nicotinic acid (172 g/L) and *p*-aminobenzoic acid (110 g/L), respectively (Mathew *et al.*, 1988, Kobayashi *et al.*, 1989). Several aromatic and heterocyclic nitrile compounds are substrates for this nitrilase. The enzyme exhibits regiospecificity for dicyaobenzenes enabling the synthesis of 3- and 4-cyanobenzoic acids from isophthalonitrile and terephthalonitrile, respectively, with a conversion rate of more than 99% (Kobayashi et al., 1988).

Recently we found that *R. rhodochrous* K22 and *Alcaligenes faecalis* JM3 produce novel nitrilases, which act on aliphatic nitriles (Kobayashi et al., 1990) and arylacetonitriles (Mauger et al., 1990, Nagasawa et al., 1990d), respectively (Table 4). Stalker et al (1987) reported that *Klebsiella ozaenae* nitrilase exhibits high specificity for bromoxynil as a substrate.

Using *Brevibacterium* R312 amidase, a continuous immobilized cell reactor was designed to produce acrylic acid via hydrolysis of acrylamide (Arnaud et al., 1979). The enzymatic hydrolysis of a dinitrile, for example, may give rise to five different products, i.e., monoamide mononitrile, monoacid mononitrile, diamide, monoamide monoacid, and diacids, whereas acid- or base-catalyzed hydrolysis gives only diamide and diacid. Many other useful compounds may be derived from a nitrile in connection with other chemical or enzymatic reactions (Godfredsen *et al.*, 1985).

(II) Other chemicals

(1) Propene oxide

Propene oxide is the raw material for the synthesis of propane-1,2-diol or homologous glycols. The direct air oxidation of propene to propene oxide proved impractical. Therefore, the conventional chemical synthesis of propene oxide from propene is carried out through halohydrin or by the use of peroxide. The advantages of microbial processes, the oxidation under the mild conditions (at ordinary temperature and pressure) and almost no formation of sub-products, have been pointed out.
The Cetus process was the first noticeable enzymatic process for the production of propene oxide (Figure 3) (Neidleman et al., 1981). In this process, propene halohydrin is synthesized from propene using *Caldariomyces fumago* chloroperoxidase in the presence of hydrogen peroxide (Geigert et al., 1983a). Halohydrin is further converted to propene oxide by *Flavobacterium* halohydrin epoxidase (Geigert et al., 1983b). Hydrogen peroxide can be supplied through the oxidation reaction of glucose by glucose-2-oxidase. The arabino-2-hexosulose formed is chemically reduced to D-fructose.

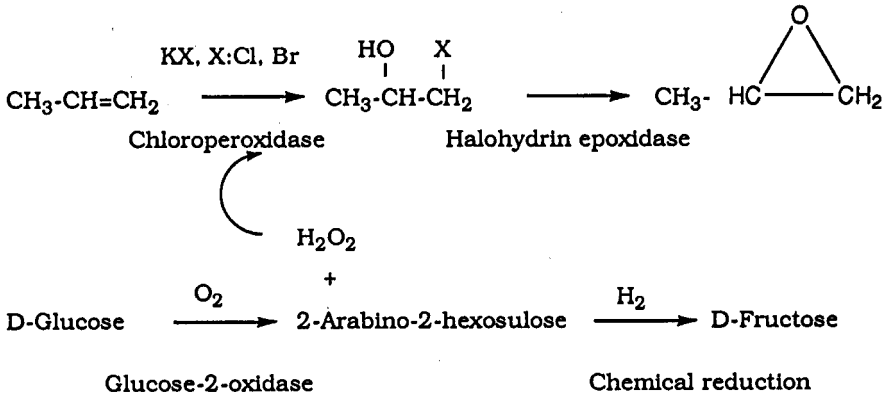

Figure 3: Outline of the Cetus process.

This process was not performed on an industrial scale because of economic and technical problems. Nevertheless, the concept of the Cetus process was novel and epoch-making at that time. In a sense, this process suggested a direction for biotechnology development.
Methane monooxygenase of methane-assimilating bacteria acts on C_2-C_4 alk-1-enes to produce the corresponding epoxide using NADH as a cofactor (Hou *et al.*, 1984)(Figure 4). A bioreactor containing methane-assimilating bacteria was constructed for the production of propene oxide. In this reactor NADH was regenerated by the oxidation reaction of methanol (Suzuki *et al.*, 1991). The weak point of this process is the instability of the biocatalyst. Simplification of the reactor, regeneration of the biocatalyst and improvement of downstream processing need to be investigated.

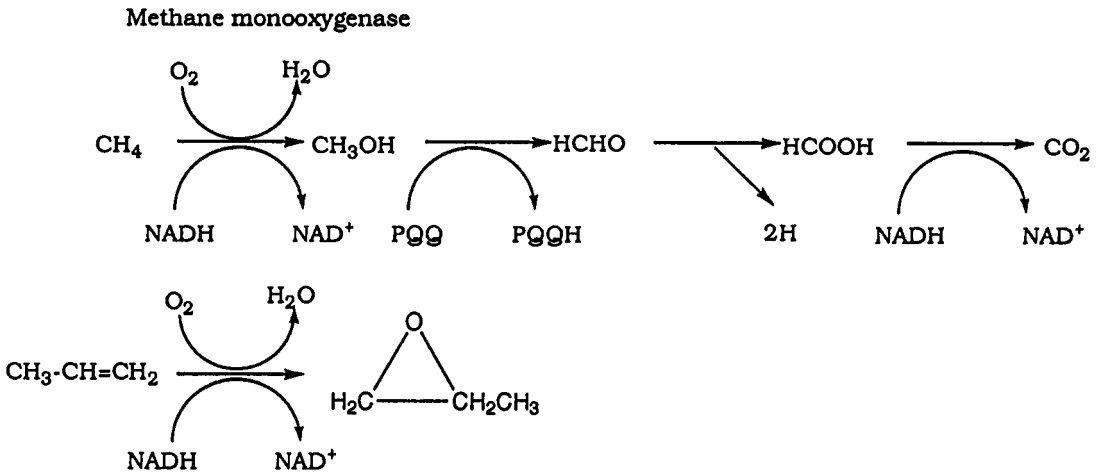

Figure 4: Oxidation of methane and propene by methane monooxygenase.

(2) Formaldehyde and formic acid

Formaldehyde is synthesized from methanol through chemical oxidation and 7-8 million tons/year of formaldehyde are produced in the world. A methanol oxidation system with alcohol oxidase and catalase from methylotrophic yeasts was utilized for the production of formaldehyde from methanol (Baratti, et al., 1978, Sakai et al., 1986). When formaldehyde production was investigated with cells of a mutant, AOU-1, methanol yeast, *Candida boidinii* S2 grown in methanol-limited chemostat culture, the highest productivity was shown with cells at a dilution rate of 0.075 h^{-1}. Under optimal reaction conditions, 1.5 M formaldehyde was produced in 1 hour from the cells. In a similar manner, from ethanol, allyl alcohol and propane-1-ol, the corresponding aldehydes were produced efficiently.

Formaldehyde dismutase from *Pseudomonas putida* catalyzes the dismutation of various aldehydes (including formaldehyde), leading to the formation of equimolar amounts of the corresponding alcohols and acids. An enzymatic process for the production of formate from methanol involving a combination of methanol oxidase and formaldehyde dismutase was developed (Kato *et al.*, 1988)(Figure 5). In this process, methanol is first oxidized to formaldehyde, which is converted to formate and methanol by the formaldehyde dismutase. On repetition of the sequential enzyme reactions, all methanol added is stoichiometrically converted to formate. The 600 mM methanol added periodically was converted to formate in a 75% yield in 12 h.

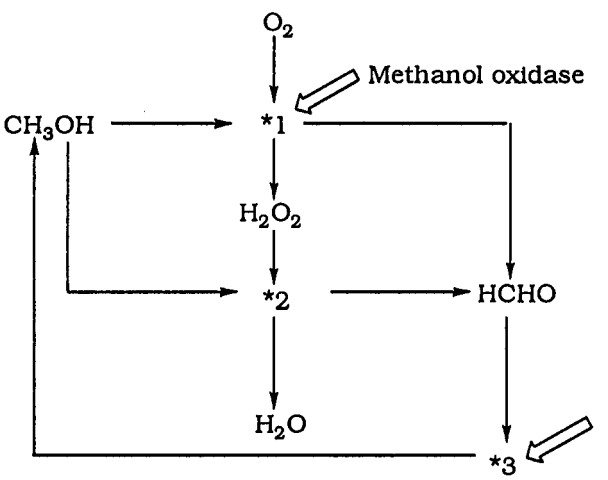

Figure 5: Conversion of methanol into formate by formaldehyde dismutase.

(3) Hydroquinone

Hydroquinone is manufactured by an organic chemical process and marked mainly as a photographic developer. Recently a process for the total microbial production of hydroquinone from phenol was investigated (Yoshida et al., 1990). A newly isolated *n*-butane-assimilating bacterium, *Mycobacterium* sp.B-394, catalyzing the position-specific hydroxylation of phenol (Figure 6), was used as the biocatalyst. Highly active cells were produced by nitrogen-restricted continuous cultivation using methyl ethyl ketone as the sole carbon source. A membrane reactor with recycling of suspended cells was employed for the continuous reaction. The reaction could be continued for 120 h with continuous supplementation of fresh cells. Throughout the reaction, a production of 3.0 g L^{-1} h^{-1} and a hydroquinone concentration of 2.2 g L^{-1} were maintained, and the selectivity and the yield of hydroquinone from phenol remained over 99%. In the downstream process, concentration by reverse osmosis and crystallization at low temperature were employed. The hydroquinone produced was readily recovered from the reaction mixture by these two steps. However, at present, membrane separation are expensive for cell separation and hydroquinone concentrations. To enable the industrialization of this microbial process, further investigation is required.

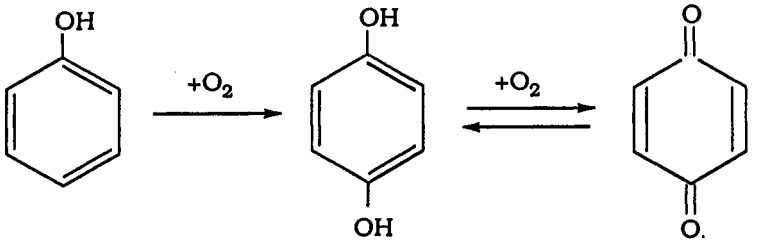

Figure 6: Hydroquinone production by *Mycobacterium* sp.

(4) (E,E)-Hexa-2,4-dienedioic acid

(E,E)-Hexa-2,4-dienedioic acid is suggested to be potentially useful as a raw material for new functional resins, pharmaceuticals and agrochemicals. This compound can be easily converted to adipic acid, a commodity chemical for nylon production. Recently the microbial production of hexadienedioic acid was studied systematically (Yoshikawa *et al.*, 1990). Initially, microorganisms with a high capacity for producing this acid from benzoic acid were selected (Figure 7). A mutant, identified as *Arthrobacter* sp., lacking an appropriate lactonizing enzyme, was obtained by ultraviolet irradiation. Next, a total bioreactor system for producing hexadienedioic acid using growing cells of this mutant was developed. With this system, consisting of a membrane separating type reactor and downstream processing, the pure product was almost quantitatively obtained from benzoic acid an continuous reaction. The highest production rare was 2.1-2.5 g L^{-1} h^{-1} and the overall yield from benzoic acid through the entire process was more than 90%.

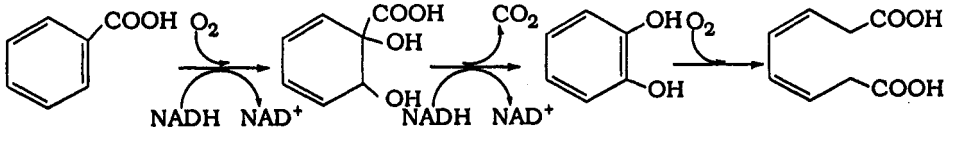

Figure 7: Conversion of benzoic acid into *cis,cis*-mucoic acid.

(5) Benzene-1,2,3-triol

Benzene-1,2,3-triol is polyphenol widely used in industry. It is used as developer in photography, for staining fur, leather and hair, for manufacturing various dyes, for determing oxygen in gas analysis and so on. It is industrially produced by autoclaving 3,4,5-trihydroxybenzoic acid, which is obtained from tannins, under strong acidic conditions. A bacterial strain *Citrobacter* sp. showed an inducible trihydroxybenzoic acid decarboxylase activity producing benzene-1,2,3-triol from trihydroxybenzoic acid (Figure 8)(Yoshida *et al.*, 1982). Cultural conditions for this reaction were investigated. To avoid oxidation of the substrate and the product by oxygen, anaerobic conditions were chosen. The maximum amount of benzene-1,2-3-triol produced under these conditions was 54.1 g/L with a conversion yield of 97.4% after 8 hours reaction time (Yoshida *et al.*, 1985). This biotransformation carried out in liquid two-phase systems is expected to offer a higher productivity due to a higher solubility of benzene-1,2,3-triol in organic solvent.

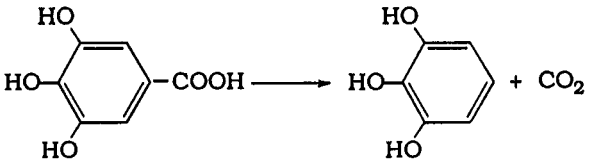

Figure 8: Production of pyrogallol from gallic acid by microbial decarboxylation.

(6) Indigo

Indigo is a blue pigment widely used to dye textiles. Therefore, indigo production occurred naturally only in plants. The chemical process developed by Bayer involves quite difficult and relatively severe procedures. Ensley *et al.*, (1983) discovered that expression of the *Pseudomonas* naphthalene dioxygenase system in *Escherichia coli* can result in the synthesis of indigo. The naphthalene dioxygenase system catalyzes the oxidation of indole to form indigo. Indole is produced from tryptophan in *E. coli* by the action of tryptophanase (Figure 9). Such combinations of microbial reactions can lead to the development of useful, new applications in the chemical industry.

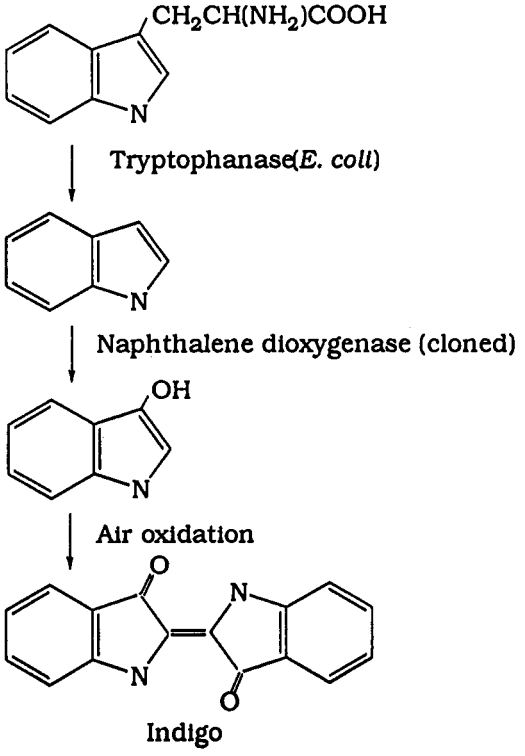

Figure 9: Production of indigo by *E. coli* carrying a cloned napthalene dioxygenase gene.

CONCLUDING REMARKS

Many chemical processes require severe reaction conditions which consume large amounts of energy and can damage the environment. Even if alternative biotechnological production processes exist, they are often hampered by economics, in spite of being potentially advantageous with respect to environmental protection. Nevertheless, increasingly environmental constraints with favour processes which can be run under milder conditions. An example realized in Japan typifies the substitution of a well-established large-scale chemical process by a biotransformation: the hydration of acrylonitrile producing acrylamide. This encouraging example reaffirms the potential of biocatalysis not only for fine chemicals synthesis but also for commodity chemicals.

Microorganisms show a great ability to adapt to a wide variety of environments. Consequently, they have tremendous potential for inducing new or novel enzyme systems capable of converting new substrates. It is possible to obtain and cultivate microorganisms that can survive or grow in thermophilic, acidophilic, and alkalophilic environments. These microorganisms are capable of producing unique enzymes stable towards heat, alkali or acid. Moreover, the latent abilities of microorganism are not fully elucidated, because our present techniques allow us to isolate only 10% of all microorganisms. With advances in the techniques for cultivation and enzyme purification, coincident with recent developments in gene technology and protein engineering, it is possible to enhance the special functions of particular microorganisms. In view of these advances, there must be far more ways than currently imaginable of using microorganisms for the production of commodity chemicals.

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