3

Methylotrophs for Biotechnology; Methanol as a Raw Material for Fermentative Production

YOSHIKI TANI

Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Introduction

The international symposium on 'Microbial Growth on C_1 Compounds', held on September 1983, at Minneapolis, Minnesota (Crawford and Hanson, 1984), was the fourth symposium on C_1 micro-organisms, following those in 1974 (Tokyo), 1977 (Puschino) and 1981 (Sheffield). In biotechnology which uses micro-organisms as tools, selection of the raw material for the culture substrate is of paramount importance, as is the pinpointing of the target product and the screening and breeding of micro-organisms. C_1 compounds have attracted much attention as the culture substrate for these micro-organisms, ever since the 1960s when methanol was anticipated to be the raw material for single-cell protein (SCP) production in place of *n*-paraffins. In 1982, SCP from methanol was given the brand name PRUTEEN and was marketed by Imperial Chemical Industries (ICI), England (King, 1982; Vasey and Powell, 1984).

The fundamental field of C_1 microbiology has come a long way since the reisolation of *Pseudomonas methanica* (Söhngen) (Dworkin and Foster, 1957). This progress has been accelerated by increasing interest in the practical use of C_1 compounds. This is a typical pattern in the development of applied microbiology, in which applied and fundamental studies are interrelated. As shown in *Figure 1*, C_1 compounds at all oxidation levels including carbon monoxide, carbon dioxide, methane, methanol and methylamines are now known to be utilized or synthesized by micro-organisms.

Among the C_1 micro-organisms, methylotrophs offer promise for the production of fine and commodity chemicals, as well as other useful materials

Abbreviations: G_f, G', free energy change; ICI, Imperial Chemical Industries; *Ke*, energy efficiency; Ke_p, practical energy efficiency; Ke_t, theoretical energy efficiency; P_i, inorganic phosphate; PQQ, pyrroloquinoline quinone; SCP, single-cell protein.

Biotechnology and Genetic Engineering Reviews — Vol. 3, September 1985
0264-8725/85/03/111-25$10.00 + $0.00

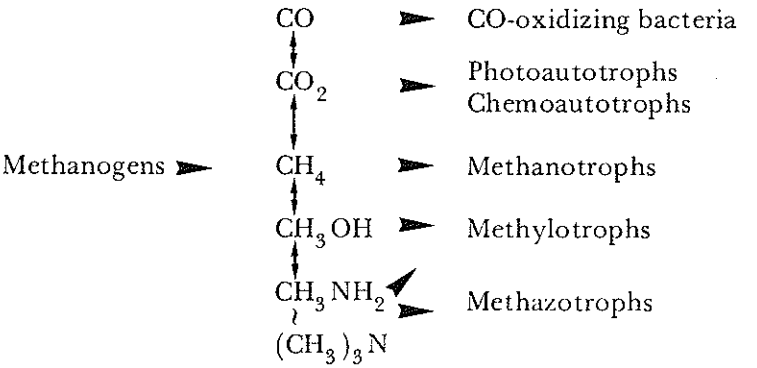

Figure 1. C_1 compounds and micro-organisms.

in the near future because of the advantageous properties of the carbon source, methanol, as the raw material. A variety of investigations have been carried out to establish the new process for fermentative production, in which both the unique and conventional metabolisms of methylotrophs are utilized.

The use of methylotrophs for industrial production can be summarized as follows:

1. Use of metabolites of methylotrophs. Examples of the intracellular and extracellular products derived from the anabolic metabolism of methylotrophs are proteins, amino acids, vitamins and coenzymes. Results so far demonstrate the superiority of methanol as a raw material in some instances, but problems in yield and growth are also noted in comparison with conventional carbon sources.
2. Use of the catalytic function of methylotrophs. Biochemical studies of methylotrophs demonstrate their use in biotransformation systems.
3. Use of the character of methylotrophic growth. Methylotrophs, to which functions from other organisms may be transferred, can exhibit the foreign activity but still retain useful features of growth on methanol as the carbon and energy source.

This chapter reviews research on the fermentative production of useful materials by methylotrophs. Recent reviews (Tani, Kato and Yamada, 1978; Colby, Dalton and Whittenbury, 1979; Kato, Tani and Yamada, 1983) and monographs (Anthony, 1982; Hou, 1984) may be referred to for the biochemical aspects of methylotrophs.

Methanol as the raw material

Of the C_1 compounds indicated in *Figure 1*, the most promising as a raw material for the fermentation industry is considered (at present) to be methanol. Carbon dioxide, which can be assimilated by photoautotrophs and chemoautotrophs, is an inexhaustible resource but problems arise from its

low concentration, and the need for energy if it is to be utilized. The use of methane and hydrogen is hampered by their flammability and gaseous nature, while the cost of hydrogen presents further problems.

Methanol can easily be synthesized from methane, which is a major component of natural gas from oil, coal or biogas. The world resource of natural gas is estimated at over 70 trillion m^3, i.e. several hundred times that of petroleum oil. The present annual world production of methanol is 14 million tons: most of this is used for the synthesis of chemicals such as formaldehyde. Recently, developments in C_1 synthetic chemistry offer the possibility of replacing ethylene from petroleum by carbon monoxide or methanol as the starting material. One example is the production of acetic acid from methanol (Monsanto process) and another is the partial substitution of gasoline by methanol. The use of methanol by the fermentation industry is attractive in view of its low cost and large-scale production. *Figure 2* illustrates the interrelations of methanol utilization.

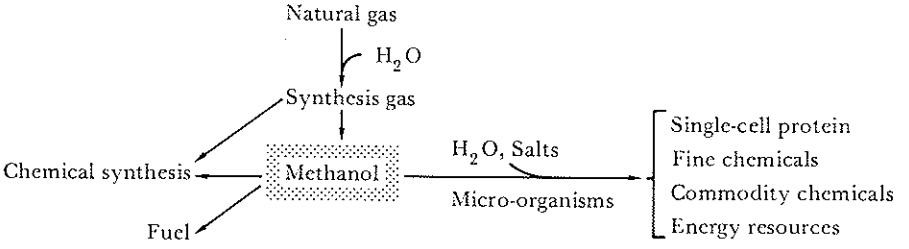

Figure 2. Utilization of methanol.

Molasses is now widely used as a carbon source in the fermentation industry. As production depends on agriculture, the supply is not stable. The presence of non-fermentable substances in molasses necessitates extra cost in waste treatment. On the other hand, methanol is supplied from an abundant resource, natural gas, while treatment of methanol broth waste is easier because of the high purity of the feedstock. *n*-Paraffins, which once were considered to be the preferred raw material for SCP production, have become less attractive since the price increases which followed the 'oil crisis' of the 1970s.

Unlike methane and *n*-paraffins, methanol is miscible with water. This ensures its distribution in the medium and, because methanol is partly oxidized, it creates a smaller oxygen demand and generates less heat during the fermentation process than *n*-paraffins and methane. These factors greatly reduce agitation and cooling costs. However, the energy content per unit volume is lower than that of methane but, being a liquid, it can be transported without the need for pressure-resistant tanks.

Methanol in high concentrations inhibits the growth of micro-organisms. The low optimum concentration for growth and biomass production has led to

fed-batch culture methods in which intermittent feeding of methanol is used to increase the cell concentration in a fermenter (Tani and Yamada, 1980). Another disadvantage of methanol as a feedstock is the extra high-energy consumption necessitated by the anabolic reactions required to synthesize essential metabolites from C_1 units.

Production of metabolites by methylotrophs

PROTEIN

The first application of methanol for fermentation has been to produce SCP. Basic information on the use of methylotrophs as a source of SCP, including the estimation of growth characteristics, efficiency of cell yield, nutritional value and safety as foodstuffs has been acquired over the past 10–15 years (Cooney and Levine, 1975). Some plants for SCP production from methanol are now in operation (ICI) or are reaching the commercial stage (Vasey and Powell, 1984).

Pathways to dissimilate and assimilate methanol have been studied (Anthony, 1982; Hou, 1984). The knowledge may be sufficient to enable us to produce a theoretical value of cell yield. The oxidation of methanol to carbon dioxide functions as an ATP-producing system (*Figure 3*). The type of electron acceptor directly determines the amount of ATP formed by the sequential oxidation of methanol. Pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (EC 1.1.99.8) (Salisbury *et al.*, 1979) and FAD-containing alcohol oxidase (EC 1.1.3.13) (Tani *et al.*, 1972) are characteristic enzymes which are distributed in almost all methylotrophic bacteria and yeasts, respectively. The two enzymes have a wide substrate specificity to alcohols, whereas oxidations of formaldehyde and formate are catalysed by specific NAD-dependent dehydrogenases (EC 1.2.1.1 and EC 1.2.1.43, respectively).

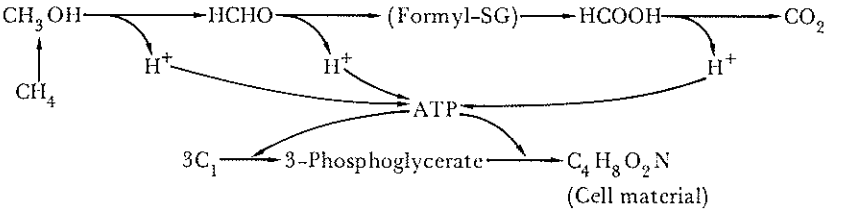

Figure 3. Dissimilation pathway of methanol.

At least three pathways are known for the assimilation of methanol (*Figure 4*). All obligate methylotrophic bacteria use the ribulose monophosphate pathway, in which formaldehyde is fixed to ribulose 5-phosphate to form a unique compound, D-arabino-3-hexulose 6-phosphate. Facultative methylotrophic bacteria use the serine pathway, which is initiated by the formation of

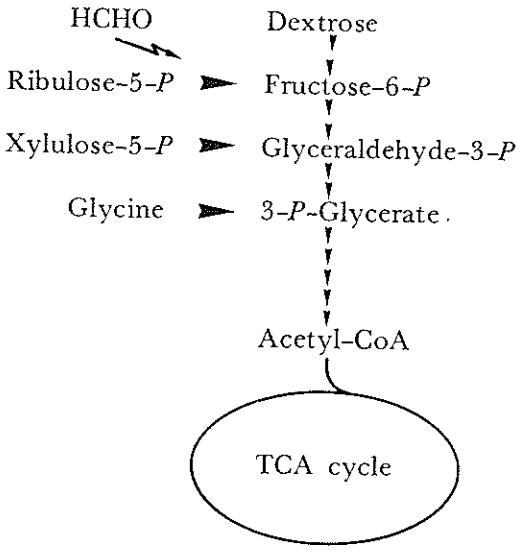

Figure 4. Assimilation pathway of methanol.

serine from formaldehyde and glycine. The third pathway is the xylulose monophosphate pathway in yeasts. Xylulose 5-phosphate as the acceptor of formaldehyde is cleaved to form glyceraldehyde 3-phosphate and dihydroxyacetone by the catalysis of dihydroxyacetone synthase, a kind of transketolase (EC 2.2.1.1).

The ATP balance of the assimilation pathway of methanol can be roughly calculated in comparisons between yeasts and bacteria in spite of variations in the dissimilation and assimilation pathways and in the efficiency of the oxidative phosphorylation systems. The serine pathway consumes three molecules of ATP and one molecule of NADH, and reduces one molecule of flavoprotein in the formation of one molecule of 3-phosphoglycerate. In the formation of this compound, the ribulose monophosphate pathway provides one molecule of NADH. The xylulose monophosphate pathway in methylotrophic yeasts consumes three molecules of ATP to form one molecule of glyceraldehyde 3-phosphate in phosphorylation of dihydroxyacetone, regenerating three molecules of xylulose 5-phosphate. The consumed ATP can be recovered by the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate yielding one molecule each of ATP and NADH.

Van Dijken and Harder (1975) reported the yield of micro-organisms grown on methanol, based on a value of Y_{ATP} of 10·5. The calculation showed a lower cell yield of methylotrophic yeasts (0·54 g/g methanol) than that of the bacteria (0·63 g/g methanol). Further, they obtained a value for the low yield of *Hansenula polymorpha* grown in a methanol-limited chemostat, of 0·38 g/g methanol, which was explained by the absence of ATP production during oxidation of methanol by alcohol oxidase (van Dijken, Otto and Harder, 1976).

One specific drawback to the use of methanol as the sole source of carbon and energy is its toxicity to the growth of micro-organisms. Some fed-batch cultures have been carried out to increase the cell concentration of methylotrophic yeasts and bacteria by intermittent feeding of methanol. In contrast to the usual continuous culture, the fed-batch method keeps the methanol concentration low so that the maximum specific growth rate can be maintained during the cultivation.

Several methods of controlling methanol fermentations have been reported. In a fed-batch culture of *Candida boidinii* the methanol concentration was controlled by measuring the amount of methanol in the exhaust gas (Reuss *et al.*, 1975). Yamane, Kishimoto and Yoshida (1976) have designed a fed-batch culture which maintains the exponential growth of methylotrophic bacteria with a feed-rate programmer. A culture controlled by pH, in which a methanol–ammonia mixture was fed in response to the direct signal of pH change, was employed by Nishio *et al.* (1977a) for the cultivation of the bacteria. The cell concentration of *Protaminobacter ruber* was found to reach about 85 g/litre with a fed-batch culture when dissolved oxygen tension was used as the control indicator (Yano, Kobayashi and Shimizu, 1978).

Fermenter design has been improved and allows the use of methanol for the industrial production of SCP. The cultivation of a methylotrophic yeast, *Pichia aganobii*, was carried out in an air-lift fermenter as a pilot-plant fermenter to obtain high oxygen transfer and miscibility (Kuraishi *et al.*, 1977). A high cell-density culture at a normal dilution rate could be obtained.

The cloning of the glutamate dehydrogenase gene of *Escherichia coli* into broad host-range plasmids to complement glutamate synthase mutants of *Methylophilus methylotrophus* (the ICI strain for SCP) is the first improvement of the cell yield of a methylotroph by a genetic engineering method (Windass *et al.*, 1980). Assimilation of ammonia through glutamate dehydrogenase is more energy-efficient than through glutamate synthase. Thus the recombinant organism converts methanol more efficiently into cellular carbon.

AMINO ACIDS

The fermentative production of amino acids by methylotrophs appeared in a patent in 1970. The accumulation of L-glutamate in the culture medium of *Methanomonas methylovora* (Oki *et al.*, 1973) was the first report in this field. Subsequently, amino-acid production using methylotrophs has been developed from knowledge of methylotroph metabolism.

Work so far published on the production of amino acids by methylotrophs can be classified into two groups: in one, the biosynthetic pathways for L-glutamate, L-leucine, L-valine, L-tryptophan, L-phenylalanine and L-tyrosine production are identical to those of conventional organisms. In the other group, the biosynthesis of L-serine, L-methionine and *O*-methyl-L-homoserine depends on incorporation of a C_1 unit in the biosynthetic pathway; the C_1 unit is methanol or may be derived from it by oxidation.

L-Glutamate

M. methylovora, a thiamin-requiring strain, excreted L-glutamate (6·8 g/litre) into the culture medium consisting initially of 2% methanol and inorganic salts (Oki *et al.*, 1973). The yield was about 11% (w/w) of methanol consumed. The glutamate production depends on the concentration of thiamin, and ferrous and magnesium ions in the medium. Other metabolic products, pyruvate and α-ketoglutarate, were also detected in the culture medium at concentrations of 0·5–1·0 g/litre.

L-Serine

Production of L-serine by methylotrophs is one use of their unique metabolic pathway, the serine pathway (*Figure 5*). Serine hydroxymethyltransferase (EC 2.1.2.1), which fixes formaldehyde to glycine to form L-serine, is the key enzyme in this pathway.

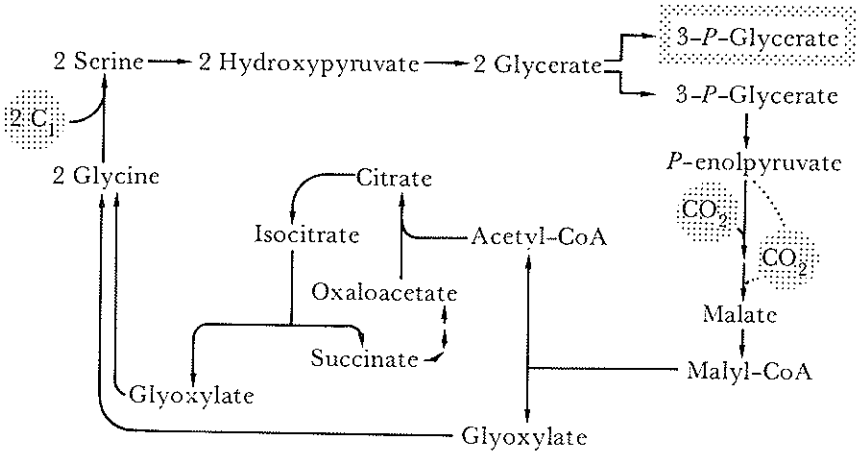

Figure 5. Serine pathway.

Pseudomonas 3ab is a facultative methylotroph with ability to produce L-serine on the addition of glycine and methanol at the end of the exponential growth phase (Keune, Sahm and Wagner, 1976). The maximum yield of L-serine (4·7 g/litre) was obtained when 20 g/litre glycine and 8 g/litre methanol were added and the pH of the culture medium was changed to 8·5. *Pseudomonas* MS 31 also produced L-serine when glycine was added at the late exponential growth phase (Morinaga, Yamanaka and Takinami, 1981a). The strain can rapidly degrade L-serine. This activity was first prevented by the addition of chelating agents and metal ions. From this organism was

derived a temperature-sensitive mutant, defective in degradation activity and growth on methanol above 37°C (Morinaga, Yamanaka and Takinami, 1981b). This mutant strain showed a relatively high conversion rate of glycine (12 g/litre) to L-serine (6·8 g/litre) when the fermentation temperature was changed from a permissive (30°C) to a non-permissive state (38–42°C), together with the addition of glycine and methanol after adequate growth. From this mutant was then derived a strain resistant to *O*-methyl-DL-serine, an analogue of L-serine (Morinaga, Yamanaka and Takinami, 1983). By this mutation the serine hydroxymethyltransferase was increased by consequent desensitization to glycine. The mutant strain accumulated 12 g/litre of L-serine after 5 days' cultivation at 34°C when cultured with 4% (v/v) methanol and 15 g/litre glycine for 40–48 h. The molar conversion rate of glycine to L-serine was 57%. Somewhat higher amounts of L-serine may be produced by non-methylotrophic micro-organisms. However, the theoretical conversion rate of glycine to L-serine cannot exceed 50% in these processes, because the C_1 unit is provided by the cleavage of another glycine molecule.

A Gram-positive methylotroph, *Arthrobacter globiformis*, was isolated as a glycine-tolerant L-serine producer (Tani *et al.*, 1978). The strain excreted a considerable amount of the amino acid when glycine and dextrose were added to a methanol medium during cultivation. Development of mutants increased productivity. A maximum value (5·2 g/litre) is obtained with an L-methionine auxotroph.

The use of an increased concentration of cells in the resting phase has been shown to increase the amount of L-serine in the reaction mixture (Izumi *et al.*, 1982). Cells of an obligate methylotroph, *Hyphomicrobium methylovorum*, converted glycine (100 g/litre) to L-serine (24 g/litre).

L-Methionine

Although L-methionine is synthesized by alternate pathways in micro-organisms, L-homoserine, sulphide, 5-methyltetrahydrofolate and L-serine are essential intermediates in the biosynthesis. Thus, several steps of C_1 unit incorporation are included in the biosynthesis of L-methionine. There is, therefore, a possibility that methionine, which is now supplied by chemical synthesis, could be manufactured by a methylotrophic fermentation.

An obligate methylotroph, strain OM 33, which was selected from more than 400 methanol-utilizing isolates, excreted 70 mg/litre of L-methionine when L-homoserine (5 g/litre) and sodium sulphite (2 g/litre) were added to the culture medium (Yamada, Morinaga and Tani, 1982). A mutant from OM 33 resistant to ethionine (an analogue of L-methionine) accumulated 420 mg/litre of L-methionine without any L-homoserine supplement. Morinaga, Tani and Yamada (1982a) describe the enzymes involved in L-methionine biosynthesis by strain OM 33 and the desensitization of homoserine succinyltransferase (EC 2.3.1.46) in this organism from feedback inhibition by *S*-adenosyl-L-methionine.

5-Methyltetrahydrofolate and L-serine, biosynthetic intermediates of L-methionine, are directly related to the serine pathway. Methylotrophs

utilizing the serine pathway should be able to synthesize L-methionine. In fact, a facultative methylotroph, *Pseudomonas* FM518, showed relatively high productivity of L-methionine (Morinaga, Tani and Yamada, 1982b). An ethionine-resistant mutant of this strain produced 0·8 g/litre L-methionine in a methanol medium. The strain displays both cystathionine β-lyase (EC 4.4.1.8) and *O*-acetylhomoserine (thiol)-lyase (EC 4.2.99.10) activity, indicating the presence of two pathways for the synthesis of L-homocysteine (Morinaga, Tani and Yamada, 1983). The production of L-methionine from DL-homocysteine and methanol by transmethylation was studied in this strain (Morinaga, Tani and Yamada, 1984): L-methionine (388 mg/litre) was formed from DL-homocysteine (4 g/litre) and methanol (26 g/litre).

O-*Methyl-L-homoserine*

Several kinds of *O*-alkyl-L-homoserine are formed in the culture medium of alcohol-utilizing bacteria, when these are grown on corresponding alkyl alcohols. Mutants derived from facultative methylotrophs, *Microcyclus eburneus* and *Protaminobacter ruber*, have been found to accumulate 0·5–3·8 g/litre of *O*-methyl-L-homoserine in a methanol medium without supplements of L-homoserine (Tanaka, Araki and Nakayama, 1980). The wild-type strains form the amino acid when L-homoserine is added to the medium: the accumulation of *O*-methyl-L-homoserine by the mutants is therefore ascribed to an increased L-homoserine pool in the cells.

Others

L-Tryptophan, L-phenylalanine and L-tyrosine are now much in demand. The biosynthesis of aromatic amino acids starts at the synthesis of 3-deoxy-D-arabino-heptulosonate 7-phosphate from erythrose 4-phosphate and phosphoenolpyruvate. In this sense, one can assume that the methylotroph which possesses the ribulose monophosphate or xylulose monophosphate pathway will be able to support production of these amino acids.

An obligate methylotroph, *Methylomonas methanolophila*, was isolated as a producer of aromatic amino acids (Suzuki *et al.*, 1977a). A mutant given resistance to aromatic amino-acid analogues, β-2-thienylalanine, 5-methyltryptophan and 3-aminotyrosine, produced L-phenylalanine (4 g/litre), L-tyrosine (1·1 g/litre) and L-tryptophan (0·2 g/litre) in a methanol–inorganic salts medium supplemented with biotin and thiamin (Suzuki *et al.*, 1977b).

The production of L-tryptophan has been studied with a methylotrophic yeast, *Hansenula polymorpha*. Mutants of the yeast excreted a low but significant level of L-tryptophan (Sanchez and Demain, 1978; Denenu and Demain, 1981). The optimization of production of indole-containing metabolites (L-tryptophan, tryptophol, indoleacetic acid and indoleacetaldehyde) by a mutant was studied with batch and continuous cultures, and a washed-cell system (Longin, Cooney and Demain, 1982). Although L-serine is involved in the biosynthesis of L-tryptophan, the use of the serine pathway for the production of L-tryptophan has not been reported.

Methylomonas aminofaciens was isolated as a producer of branched-chain amino acids in the screening of methylotrophs producing amino acids (Ogata *et al.*, 1977). A mutant of this strain, resistant to valine hydroxamate, accumulated L-valine (2·2 g/litre) and L-leucine (0·8 g/litre) (Izumi *et al.*, 1977).

VITAMINS AND COENZYMES

Vitamin B_{12}

Vitamin B_{12} has a complicated corrinoid structure, and has been produced by microbial processes. The development of a more efficient production method is still being sought. Methylotrophs were thought to be potentially useful as corrinoid producers because the coenzymatic function of vitamin B_{12} involves the transfer of a methyl group and some essential reactions in methylotrophs are vitamin B_{12}-dependent (Sato, Ueda and Shimizu, 1976; Ueda, Sato and Shimizu, 1981).

The production of vitamin B_{12} was attempted with methylotrophs such as unidentified bacteria (Tanaka *et al.*, 1974; Toraya *et al.*, 1975, 1976; Dumenil *et al.*, 1979), *Klebsiella* sp. (Nishio, Yano and Kamikubo, 1975a,b), *Protaminobacter ruber* (Sato, Ueda and Shimizu, 1977), and *Pseudomonas* AM-1 and *Microcyclus eburneus* (Nishio *et al.*, 1977a) using a methanol medium, and an unidentified bacterium (Ohsugi *et al.*, 1983) with a medium based on formate. A practical fed-batch culture, in which a methanol–ammonia mixture was fed in response to a direct signal of pH change of the culture, was used to investigate vitamin B_{12} production (Nishio *et al.*, 1977b). A methanol-limited chemostat culture also showed an increase in intracellular content of vitamin B_{12} with increase of dilution rate (Tsuchiya, Nishio and Nagai, 1980), but the productivity of these bacteria was found to be low, not more than 3·2 mg/litre after cultivation of *Pseudomonas* AM-1 for 85 h (Nishio *et al.*, 1977b).

Coenzyme Q

Coenzyme Q (or ubiquinone), is a fat-soluble coenzyme and an essential component in the respiratory chain. Homologues with side chains of different lengths are widely distributed in nature. Among them, coenzyme Q_{10} is used in some treatments of heart disease; methylotrophic bacteria have been studied for the development of fermentative production. Consequently, the Mitsubishi Gas Chemical Co., Japan, started commercial production of coenzyme Q_{10} on the basis of continuous culture in the spring of 1980 (Urakami, Terao and Nagai, 1981).

The coenzyme Q system is important for the chemotaxonomic differentiation and classification of Gram-negative methylotrophic bacteria (Urakami and Komagata, 1979). The classification of these bacteria into four major groups based on the coenzyme Q system and on cellular fatty-acid composition showed a good correlation with the electrophoretic comparison of some enzymes (Urakami and Komagata, 1981). Coenzyme Q_{10} is the quinone system present in strains of *Pseudomonas*, *Protaminobacter* and *Microcyclus*.

The production of coenzyme Q_{10} in a methanol medium by a facultative methylotroph, *Pseudomonas* sp., was reported by Natori *et al.* (1978). The improvement of productivity by mutation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and the optimization of culture conditions gave a high coenzyme Q_{10} content in cells, 10–15 mg/g dry cells and 280 mg/litre of culture broth, in a medium containing dextrose as the carbon source but not methanol (Natori and Nagasaki, 1981). Some mutants also produced significant amounts of the homologues coenzyme Q_{11} (Natori and Nagasaki, 1979), and coenzyme Q_{12} and coenzyme Q_{13} (Natori and Nagasaki, 1980).

FAD

A unique feature of the methanol oxidation system of yeast appears in the first step, methanol to formaldehyde (Tani, 1984). All yeasts have an alcohol oxidase. The enzyme is specifically induced during the growth of yeast on methanol. The level of the enzyme increases from 7% to approximately 20% of the soluble protein in cells when the dilution rate in a methanol-limited continuous culture is increased from 0·16 to 0·3/h (van Dijken, Otto and Harder, 1976). The high content of alcohol oxidase, which has eight molecules of FAD in each molecule, is correlated with the content of flavin in the yeast cells.

When *Kloeckera* sp. No. 2201 was grown on methanol, the amount of FAD and the activity of FMN adenylyltransferase (EC 2.7.7.2) were three and five times higher, respectively, than those in the cells grown on dextrose, ethanol, or glycerol (Shimizu *et al.*, 1977a,b). A positive correlation between the formation of alcohol oxidase and FMN adenylyltransferase was also shown with a mutant of *Candida boidinii* (Eggeling, Sahm and Wagner, 1977). The induction of FAD biosynthesis by methanol led to the investigation of methylotrophic yeasts for FAD production. Riboflavin or FMN added to a yeast culture on a methanol medium was converted to FAD, 45·4 mg/litre (Shimizu *et al.*, 1977c).

Cytochrome c

Cytochrome *c* is important for the utilization of oxygen and is used medicinally in some countries for diseases caused by oxygen deficiency. The coenzyme is an intrinsic electron acceptor of the oxidation sequence of methanol: thus, the growth substrate is directly connected with cytochrome *c* in methylotrophs. A cyanide-resistant mutant of an obligate methylotrophic bacterium produced a large amount (60 mg/litre) of cytochrome *c* intracellularly (Tani, Yoon and Yamada, 1985).

ENZYMES

In the dissimilation and assimilation pathways of methanol, there are several unusual enzymes, such as PQQ-dependent alcohol dehydrogenase, alcohol oxidase, 3-hexulosephosphate synthase and dihydroxyacetone synthase (Hou, 1984).

Alcohol oxidase and formate dehydrogenase (EC 1.2.1.2) from a methylotrophic yeast, *Candida boidinii*, are now commercially available for analytical use. Alcohol oxidase is almost equally active on ethanol and methanol, and does act on several primary alcohols but to a lesser extent (Tani, Miya and Ogata, 1972). Formate dehydrogenase (NAD dependent) catalyses specifically the oxidation of formate to carbon dioxide (Kato *et al.*, 1974).

POLYSACCHARIDES

The extracellular accumulation of polysaccharide in a methanol medium was briefly reported as anthrone reaction-positive substances at a concentration of 4 g dextrose/litre (Oki *et al.*, 1973). Since then, other methylotrophic bacteria such as *Methylomonas methanolica* (2 g/litre) (Häggström, 1977), *Pseudomonas* S46B1 (3.2 g/litre) (Kodama *et al.*, 1977), *Methylocystis parvus* (9 g/litre) (Hou, Laskin and Patel, 1978), *Pseudomonas* sp. (Misaki, Tsumuraya and Kakuta, 1979) and *Hyphomicrobium* sp. (1·5 g/litre) (Kanamaru *et al.*, 1982a), and a thermophilic methane-oxidizing bacterium (1·8 g/litre) (Chida *et al.*, 1983), have been found to produce extracellular carbohydrate.

A polysaccharide produced by *Pseudomonas* sp. is acidic, consisting of D-galactose (55·4%), D-mannose (13·3%), D-glucose (10·7%), D-glucuronic acid (11·0%) and D-allose (9·8%) as the sugar component (Misaki, Tsumuraya and Kakuta, 1979). D-Allose is rarely found in natural polysaccharides. The polysaccharide from *Hyphomicrobium* sp. contains a new monomethyl sugar, methyl-D-mannose, as well as D-glucose, D-mannose and pyruvate residues in the relative proportions of 2:1:1:1 (Kanamaru *et al.*, 1982b). The methylation of D-mannose may be characteristic of the obligate methylotroph. However, polysaccharides produced by methylotrophs have not yet found any practical utility.

PIGMENTS

Pink-pigmented bacteria have frequently been isolated among the facultative methylotrophs. The pink pigment of *Protaminobacter ruber* was shown to be a carotenoid containing a carbonyl group, sugar moiety and unidentified UV-absorbing substance (Sato *et al.*, 1982). The bacterium produced the carotenoid (2 mg/litre) on a 1,2-propane-diol medium (Shimizu *et al.*, 1982). *Protaminobacter ruber* and *Pseudomonas* AM1 both produced bacteriochlorophyll *a*, despite being non-phototrophic bacteria (Sato, 1978). Biosynthesis by these bacteria on the 1,2-propanediol medium differs from that of phototrophic bacteria, in that pigment synthesis as well as growth of these methylotrophs absolutely requires oxygen while continuous light exposure completely prevents pigment formation (Sato and Shimizu, 1979).

ORGANIC ACIDS

Candida lipolytica and many other strains of the genus *Candida* are known to accumulate citric acid extracellularly in a medium containing *n*-paraffins or

other carbon compounds. Mutant strains of these yeasts which are sensitive to fluoroacetate, a potent inhibitor of aconitase, have a lower aconitase activity and higher citric acid productivity.

A methylotrophic yeast, *Candida boidinii*, is cited in a review (Sahm, 1977) as secreting citric acid. The yeast produced about 1 g/litre when fluoroacetate was added to a methanol medium. On the other hand, mutants of *Candida* sp., resistant to fluoroacetate, were found to produce a considerable amount of citric acid (5 g/litre after 4 days) in a methanol medium (Y. Tani, S. Cho, Y. Sakai and H. Yamada, unpublished data).

The accumulation of poly-β-hydroxybutyrate, an aliphatic polyester, in cells of a hydrogen bacterium, *Alcaligenes eutrophus*, is noted in a review by King (1982). The degree of polymerization lies between 5000 and 10 000 and the quantity obtained is up to 80% of cell mass in a dextrose medium. A methylotroph, *Protaminobacter ruber*, has been shown (Sato and Shimizu, 1979) to produce 137 g poly-β-hydroxybutyrate per litre in a nitrogen-limited fed-batch culture in which the cell mass was 207 g dry cells/litre and the polymer content was 66% (S. Shimizu, T. Suzuki, T. Yamane and S. Shimiqu, unpublished data). The biodegradable polymer is a fascinating candidate for large-scale biotechnology.

LIPIDS

Suzuki, Jigami and Nakasato (1979) and Jigami, Suzuki and Nakasato (1979) have reported changes in lipid composition of methanol-grown *Candida guilliermondii* under different growth conditions and a decrease in lipid content when the micro-organism is grown on methanol. Methanol-grown cells contained 12% total lipids (67% neutral lipids) whereas cells grown on dextrose or ethanol contain 21–22% total lipids, 80% of which are neutral lipids. In methanol-grown cells, 18:1 acids are decreased and 18:2 acids are increased in comparison with dextrose- or ethanol-grown cells.

Utilization of catalytic functions of methylotrophs

As described above, a variety of useful metabolites have been obtained by intracellular or extracellular accumulation during the growth of methylotrophs. The special ability of methylotrophs, especially their oxidative functions, offers the possibility of novel biotechnological applications to commodity as well as fine chemical production.

FINE CHEMICALS

ATP

Fermentative production of ATP has been established with substrate level phosphorylation of yeast and with salvage synthesis in *Brevibacterium ammoniagenes*. However, we do not know of any report on ATP production from AMP or adenosine using the oxidative phosphorylation system. Methylo-

trophic yeasts reduce NAD^+ in the oxidation pathway to supply energy for the assimilation of C_1 compounds through glyceraldehyde 3-phosphate by the xylulose monophosphate pathway (Kato, Tani and Yamada, 1983; Tani, 1984). NADH thus formed is led to the respiratory chain and ADP is phosphorylated in the oxidative phosphorylation system by the proton-driving force.

A reaction system producing ATP from AMP and reduced C_1 compounds was constructed with cells of *Candida boidinii* (*Kloeckera* sp.) No. 2201 (Tani, Mitani and Yamada, 1982). Yeast cells were treated with Zymolyase®, a cell wall-lytic bacterial enzyme. The treated cells release protoplasts on transfer to a hypotonic reaction mixture from a hypertonic suspension, and then burst to yield intact mitochondria and cytoplasmic enzymes. A reaction mixture containing AMP, methanol (or formate), NAD^+, inorganic phosphorus and the treated cells was used for ATP production. Production of ATP could be inhibited by replacement of air in the reaction tube by nitrogen gas, and by addition of an uncoupler, an electron-transport inhibitor or an ATPase inhibitor. Involvement of substrate-level phosphorylation in the presence of xylulose 5-phosphate, an initiator of the xylulose monophosphate pathway, was negligible.

Thus, Zymolyase-treated cells of *Candida boidinii* No. 2201 produce ATP from AMP by a sequence of reactions including phosphorylation of AMP to ADP by adenylate kinase (EC 2.7.4.3), oxidation of methanol by formaldehyde and formate dehydrogenases to reduce NAD^+ and oxidative phosphorylation of ADP to ATP in the respiratory chain as shown in *Figure 6*.

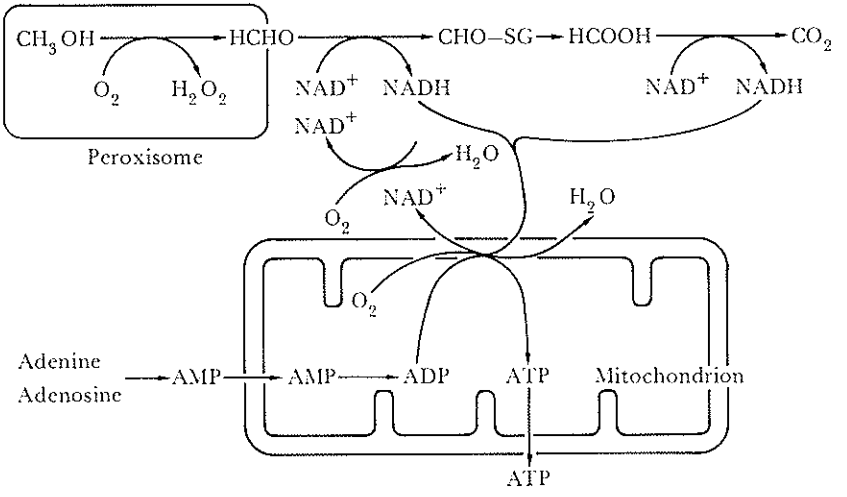

Figure 6. ATP-producing system by methylotrophic yeast.

The phosphorylation of AMP proceeds by the following scheme:

$$\text{AMP} + \text{ATP} \xrightarrow{(1)} 2\text{ADP} \xrightarrow{(2)} 2\text{ATP}$$

ATP for reaction 1, adenylate kinase, is initially supplied from the endogenous pool in the protoplast and then by reaction 2, oxidative phosphorylation. Thus, oxidative phosphorylation is required twice for the formation of one molecule of ATP in this system.

Reaction conditions for ATP production were optimized in respect of substrate and coenzyme concentrations, pH and temperature, osmotic pressure, and oxygen supply. Under optimal conditions, 13 g/litre and 4 g/litre of ATP were produced with methanol and formate as C_1 substrate, respectively (Tani, Mitani and Yamada, 1984a).

Subsequently, cell treatment was simplified to obtain yeast cells with stable ATP production ability, by incubation with added sorbitol (Tani, Mitani and Yamada, 1984b). The sorbitol-treated cells did not lose this activity even in the presence of 3 M methanol or after incubation for 36 h. Under optimal reaction conditions, the amount of ATP in the reaction mixture reached 30 g/litre (Tani *et al.*, 1984).

ATP production can be characterized as a biotransformation system or in terms of energy conversion. The conversion of the free energy in methanol to other compounds could be employed to compare methanol with other feedstocks in bioconversion systems.

Energy efficiency can be calculated as follows: *Table 1* shows the free energy change of each reaction involved in ATP production by the oxidation of methanol and phosphorylation of AMP. Assuming the value of the P:O ratio in the methylotrophic growth of yeast to be 2·0 (van Dijken, Harder and Quayle, 1981), the sequence can be summarized as in equation 1, as one molecule of ATP is consumed to phosphorylate AMP to ADP:

$$\text{AMP} + 2\text{P}_i + \tfrac{3}{4}\text{O}_2 + \tfrac{1}{2}\text{methanol} \rightarrow \text{ATP} + \tfrac{1}{2}\text{CO}_2 + 4\text{H}_2\text{O} \qquad (1)$$

The theoretical energy efficiency (Ke_t) in equation 1 is:

$$Ke_{(t)}(\text{methanol} \rightarrow 2\text{ATP}) = (61 \times \tfrac{2}{703}) \times 100 = 17{\cdot}4\%$$

Table 1. Free energy change of reactions involved in ATP production

Reaction	$-\Delta G$ (kJ/mol)
$CH_3OH + 3/2O_2 \rightarrow CO_2 + 2H_2O$	703
$NADH + H^+ + 1/2O_2 \rightarrow NAD^+ + H_2O$	202
$AMP + 2P_i \rightarrow ATP + 2H_2O$	−61
$2ADP + 2P_i \rightarrow 2ATP + 2H_2O$	-30×2
$AMP + ATP \rightarrow 2ADP$	0

The practical energy efficiency, $Ke_{(p)}$, is calculated using a result of ATP production where 19·46 mM ATP and 18·50 mM ADP are formed in the reaction mixture on the consumption of 906 mM methanol. Free energy changes in the reaction are:

$$G_f(\text{methanol}\rightarrow CO_2) = (906/1000) \times 703 = 640\,\text{kJ/litre}$$

$$G'(\text{AMP}\rightarrow\text{ADP}) = (18{\cdot}5/1000) \times 30 = 0{\cdot}58\,\text{kJ/litre}$$

$$G'(\text{AMP}\rightarrow\text{ATP}) = (19{\cdot}46/1000) \times 61 = 1{\cdot}1\,\text{kJ/litre}$$

Therefore, the energy efficiency of the phosphorylation of AMP to ATP versus the consumption of methanol is:

$$Ke_{(p)} = \{(0{\cdot}58 + 1{\cdot}1)/640\} \times 100 = 0{\cdot}3\%$$

The ratio of the practical efficiency versus the theoretical efficiency is:

$$Ke(\text{methanol}\rightarrow\text{ATP}) = (Ke_{(p)}/Ke_{(t)}$$
$$= \{(0{\cdot}58 + 1{\cdot}1)/640/(61 \times 2)/703\} \times 100 = 1{\cdot}3\%$$

Adenosine or adenine, which are more economical substrates than AMP, can be used as the substrate instead of AMP (Tani and Yonehara, 1985). The amount of ATP produced (100 g/litre) and the conversion rate (77% from adenosine) are thus greatly increased and the ratio of the practical energy efficiency against the theoretical efficiency is improved to over 30% (T. Yonehara and Y. Tani, unpublished data).

NADH and NADPH

In the oxidation sequence of methanol in methylotrophs, two dehydrogenases are involved in the reduction of NAD^+. Formate dehydrogenase is effective for the NADH-requiring system in the bioconversion process, because the substrate is fairly inexpensive and the product, carbon dioxide, does not inhibit the conversion reaction(s). Freeze-thawed, air-dried or acetone-dried cells of a facultative methylotroph, *Arthrobacter* sp., convert NAD^+ to NADH in the presence of formate, with a good yield (Izumi *et al.*, 1983). The conversion ratio and the amount of NADH reached 90% and 30 g/litre, respectively.

The oxidation of formate or hydrogen by methanogenic bacteria is mediated through F_{420} and coupled by F_{420} $NADP^+$ oxidoreductase with concomitant reduction of $NADP^+$. Resting cells of a methanogen produce NADPH from $NADP^+$ in the presence of 0·2% Triton X-100 (Eguchi, Nishio and Nagai, 1983). The conversion ratio and the amount of NADPH were 60% and 6 g/litre, respectively.

EPOXIDES

Epoxides of olefins are important for the chemical industry. The application of biotechnology in the production of commodity chemicals such as ethylene oxide and propylene oxide by microbiological processes is attracting much attention. A direct conversion process producing 1,2-epoxyalkanes from 1-alkenes by the cultivation of *Nocardia corallina* (Furuhashi *et al.*, 1981) has been developed, as well as conversion of α,β-halohydrins to the corresponding epoxides by cells of *Flavobacterium* sp. (Geigert *et al.*, 1983).

Methane mono-oxygenase of an obligate methylotroph, *Methylococcus capsulatus*, is the enzyme responsible for the initial oxygenation of methane to methanol in the presence of NADH and oxygen (Patel, 1984), and is an oxygenase of broad specificity (Colby, Stirling and Dalton, 1977), in that it can oxidize various substituted methane derivatives, *n*-alkanes, terminal alkenes, dimethyl ether, cyclic alkanes and aromatic compounds. The application of this enzyme to the epoxidation system has been studied by Hou, Patel and Laskin (1980) and by Dalton (1980). Resting cells and cell-free extracts of *Methylococcus capsulatus*, *Methylosinus trichosporium* and *Methylobacterium organophilum* oxidize C_2–C_4 1-alkenes to their corresponding 1,2-epoxides (Higgins *et al.*, 1979; Hou *et al.*, 1979a; Patel *et al.*, 1979a). Of the gaseous alkenes, propylene is oxidized at the highest rate (2·8 μmol/h/mg dry cells). Methanol-grown cells do not have this epoxidation activity. An advantage of this oxidation system is that the products, 1,2-epoxides, are not further metabolized because they are not assimilated and hence accumulate extracellularly.

METHYL KETONES

Methyl ketones such as acetone, 2-butanone, 2-pentanone and 2-hexanone are important raw materials for the chemical industry. Oxidation of secondary alcohols to their corresponding methyl ketones occurs in cell suspensions of obligate and facultative methylotrophic bacteria and methylotrophic yeasts (Hou *et al.*, 1979b; Patel *et al.*, 1979b; Hou, Patel and Laskin, 1980). 2-Propanol, 2-butanol, 2-pentanol and 2-hexanol are converted to acetone, 2-butanone, 2-pentanone and 2-hexanone, respectively, and accumulated extracellularly. Among the secondary alcohols, 2-butanol is oxidized at the highest rate (6·8 μmol/h/mg protein) by cell suspension of methanol-grown *Candida utilis*.

An NAD-dependent secondary alcohol dehydrogenase specifically catalyses the oxidation. The activity is found in cell-free extracts of methane-, methanol- and methylamine-grown cells but not in those of succinate-grown cells. Oxidation of 2-heptanol to 2-decanol, formaldehyde, butanal to decanal, benzaldehyde, methanol to *n*-decanol, isobutanol, phenol, butane, 1,2-diol and succinate does not occur. The newly discovered enzyme has been purified and characterized (Hou *et al.*, 1979c, 1981; Patel *et al.*, 1979c).

ALDEHYDES

Methanol produced from natural gas is mostly used in the production of formaldehyde, which is employed as a raw material for resins, antiseptics, pesticides, etc. The conversion of methanol to formaldehyde is carried out by a chemical process at 500–600°C. The introduction of a microbial process for the conversion should enable energy to be saved and side-product formation to be minimized. Alcohol oxidase, which catalyses the oxidation of methanol to formaldehyde in methylotrophic yeasts, uses oxygen as the electron acceptor and, therefore, does not require regeneration or an exogenous supply of cofactor.

Cell-free extracts, partially purified alcohol oxidase and cells of *Hansenula polymorpha* are used for the enzymatic oxidation of methanol to formaldehyde (Baratti *et al.*, 1978; Couderc and Baratti, 1980). The enzyme (immobilized on DEAE-cellulose) and the cells (entrapped in polyacrylamide gel) have been used in batch and continuous reactors. The conversion yield is 70% and 60% for methanol concentrations of 50 and 100 mM, respectively.

A mutant of *Candida* sp. with increased alcohol oxidase activity has been investigated for the production of high concentrations of formaldehyde (Tani, Sakai and Yamada, 1985), and a formaldehyde concentration of 0·8 M has been obtained.

The use of alcohol oxidase has also been suggested for the conversion of ethanol to acetaldehyde (Kierštan, 1982). Yeast ethanol fermentation has increased in importance with increased fuel demand. However, the inhibition of yeast growth by ethanol limits its use as an economical process. Acetaldehyde has a relatively low boiling point and therefore readily evaporates from systems operating above 21°C. The oxidized ethanol can then readily be condensed and can be utilized or chemically converted to other products.

Utilization of growth characteristics of methylotrophs

The final item of microbial utilization of methanol is achieved by the incorporation of foreign genes into methylotrophs to use the character of methylotrophic growth. Methylotrophs, especially obligate ones, have several cultural advantages: they can grow on cheap substrates—methanol, ammonia and mineral salts—are not infectious to humans and do not become contaminated.

The expression of two eukaryotic cDNAs encoding chicken ovalbumin and mouse dihydrofolate reductase in an obligate methylotroph, *Methylomonas methylotrophus*, has been studied using the broad host-range cloning vector, pGSS15 (Hennam *et al.*, 1982). Although this is the only example so far reported under this heading, further application of the technique may give rise to exciting developments such as the production by methylotrophs of useful compounds, including mammalian peptides.

Conclusion

A variety of studies using methylotrophs for the production of useful materials have been reviewed in this chapter. At present, commercial

processes involving methylotrophs are limited to the production of SCP and coenzyme Q. An increasing demand for methanol is now expected, not only in the field of biotechnology but also in that of the chemical industry and petroleum-(gasoline)-substitute fuels. In the interrelationship between these fields, a trigger for large-scale consumption in any field can lower the price of methanol through economies of scale and then increase the consumption in competition with other raw materials. The use of methanol for denitrification in waste-water treatment plants should be also noted.

In addition, the usefulness of methylotrophs is enhanced by their application as analytical tools. A marine methylotrophic bacterium requiring vitamin B_{12} for growth can be used for bioassay of the vitamin (Yamamoto, Okamoto and Inui, 1979). The bacterium is ten times more sensitive to the vitamin than one of the conventional assay micro-organisms, *Lactobacillus leichmannii*, and is also useful for determination of vitamin B_{12} in sea water. Cells of *Methylomonas* sp. and methanol dehydrogenase have been used for the determination of methane in a microbial sensor system (Okada, Karube and Suzuki, 1981; Karube, Okada and Suzuki, 1982; Karube, 1984), and for a bioelectrochemical cell and alcohol detector (Plotkin, Higgins and Hill, 1981; Davis *et al.*, 1983), respectively. The development of bioelectrochemical fuel cells is expected to make use of the catalytic function of methylotrophs for energy production (Higgins *et al.*, 1984; *see also* Aston and Turner, 1984).

References

Anthony, C. (1982). *The Biochemistry of Methylotrophs*. Academic Press, London.

Aston, W. J. and Turner, A. P. F. (1984). Biosensors and biofuel cells. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 1, pp. 89–120. Intercept, Ponteland, Newcastle upon Tyne.

Baratti, J., Couderc, R., Cooney, C. L. and Wang, D. I. C. (1978). Preparation and properties of immobilized methanol oxidase. *Biotechnology and Bioengineering* **20**, 333–348.

Chida, K., Shen, G.-J., Kodama, T. and Minoda, Y. (1983). Acidic polysaccharide production from methane by a new methane-oxidizing bacterium H-2. *Agricultural and Biological Chemistry* **47**, 275–280.

Colby, J., Dalton, H. and Whittenbury, R. (1979). Biological and biochemical aspects of microbial growth on C_1 compounds. *Annual Review of Microbiology* **33**, 481–517.

Colby, J., Stirling, D. I. and Walton, 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.

Cooney, C. L. and Levine, D. W. (1975). SCP production from methanol by yeast. In *Single Cell Protein*, (S. R. Tannenbaum and D. I. C. Wang, Eds), volume 2, pp. 402–423. MIT Press, Cambridge.

Couderc, R. and Baratti, J. (1980). Immobilized yeast cells with methanol oxidase activity: Preparation and enzymatic properties. *Biotechnology and Bioengineering* **22**, 1155–1173.

Crawford, R. L. and Hanson, R. S. (Eds) (1984). *Microbial growth on C_1 compounds: Proceeding of the Fourth International Symposium*. American Society for Microbiology, Washington, DC.

Dalton, H. (1980). Oxidation of hydrocarbons by methane mono-oxygenases from a variety of microbes. *Advances in Applied Microbiology* **26**, 71–87.

DAVIS, G., HILL, H. A. O., ASTON, W. J., HIGGINS, I. J. AND TURNER, A. P. F. (1983). Bioelectrochemical fuel cell and sensor based on a quinoprotein methanol dehydrogenase. *Enzyme and Microbial Technology* **5**, 383–388.

DENENU, E. O. AND DEMAIN, A. L. (1981). Derivation of aromatic amino acid mutants from a methanol-utilizing yeast, *Hansenula polymorpha*. *Applied and Environmental Microbiology* **41**, 1088–1096.

DUMENIL, G., CRÉMIEUX, A., COUDERC, R., CHEVALIER, J., GUIRAUD, H. AND BALLERINI, D. (1979). Production of vitamin B_{12} by Gram-variable methanol-utilizing bacteria. *Biotechnology Letters* **1**, 371–376.

DWORKIN, M. AND FOSTER, J. W. (1957). Studies on *Pseudomonas methanica* (Söhngen) nov. comb. *Journal of Bacteriology* **72**, 646–659.

EGGELING, L., SAHM, H. AND WAGNER, F. (1977). Induction of FMN adenylyltransferase in the methanol utilizing yeast *Candida biodinii*. *FEMS Microbiology Letters* **1**, 205–210.

EGUCHI, S. Y., NISHIO, N. AND NAGAI, S. (1983). NADPH production from $NADP^+$ by a formate-utilizing methanogenic bacterium. *Agricultural and Biological Chemistry* **47**, 2941–2943.

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. *European Journal of Applied Microbiology and Biotechnology* **12**, 39–45.

GEIGERT, J., NEIDLEMAN, S. L., LIU, T.-N. E., DEWITT, S. K., PANSCHAR, B. M., DALIETOS, D. J. AND SIEGEL, E. R. (1983). Production of epoxides from α,β-halohydrins by *Flavobacterium* sp. *Applied and Environmental Microbiology* **45**, 1148–1149.

HÄGGSTRÖM, L. (1977). Mutant of *Methylomonas methanolica* and its characterization with respect to biomass production from methanol. *Applied and Environmental Microbiology* **33**, 567–576.

HENNAM, J. F., CUNNINGHAM, A. E., SHARPE, G. S. AND ATHERTON, K. T. (1982). Expression of eukaryotic coding sequences in *Methylophilus methylotrophus*. *Nature* **297**, 80–82.

HIGGINS, I. J., HAMMOND, R. C., SARIASLANI, F. S., BEST, D., DAVIES, M. M., TRYHORN, S. E. AND TAYLOR, F. (1979). Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB 3b. *Biochemical and Biophysical Research Communications* **89**, 671–677.

HIGGINS, I. J., ASTON, W. J., BEST, D. J., TURNER, A. P. F., JEZEQUEL, S. G. AND HILL, H. A. O. (1984). Applied aspects of methylotrophy: Bioelectrochemical applications, purification of methanol dehydrogenase, and mechanism of methane monooxygenase. In *Microbial Growth on C_1 Compounds* (R. L. Crawford and R. S. Hanson, Eds), pp. 297–305. American Society for Microbiology, Washington DC.

HOU, C. T. (ED.) (1984). *Methylotrophs: Microbiology, Biochemistry and Genetics*. CRC Press, Florida.

HOU, C. T., LASKIN, A. I. AND PATEL, R. N. (1978). Growth and polysaccharide production by *Methylocystis parvus* OBBP on methanol. *Applied and Environmental Microbiology* **37**, 800–804.

HOU, C. T., PATEL, R. N. AND LASKIN, A. I. (1980). Epoxidation and ketone formation by C_1-utilizing microbes. *Advances in Applied Microbiology* **26**, 41–69.

HOU, C. T., PATEL, R., LASKIN, A. I. AND BARNABE, N. (1979a). Microbial oxidation of gaseous hydrocarbons: Epoxidation of C_2 to C_4 *n*-alkenes by methylotrophic bacteria. *Applied and Environmental Microbiology* **38**, 127–134.

HOU, C. T., PATEL, R., LASKIN, A. I., BARNABE, N. AND MARCZAK, I. (1979b). Microbial oxidation of gaseous hydrocarbons: Production of methyl ketones from their corresponding secondary alcohols by methane- and methanol-grown microbes. *Applied and Environmental Microbiology* **38**, 135–142.

HOU, C. T., PATEL, R. N., LASKIN, A. I., BARNABE, N. AND MARCZAK, I. (1979c). Identification and purification of a nicotinamide adenine dinucleotide-dependent

secondary alcohol dehydrogenase from C_1-utilizing microbes. *FEBS Letters* **101**, 179–183.

HOU, C. T., PATEL, R., BARNABE, N. AND MARCZAK, I. (1981). Stereospecificity and other properties of a novel secondary-alcohol-specific alcohol dehydrogenase. *European Journal of Biochemistry* **119**, 359–364.

IZUMI, Y., ASANO, Y., TANI, Y. AND OGATA, K. (1977). Mutants of an obligate methylotroph, formation of valine and leucine by analog-resistant *Methylomonas aminofaciens*. *Journal of Fermentation Technology* **55**, 452–458.

IZUMI, Y., TAKIZAWA, M., TANI, Y. AND YAMADA, H. (1982). L-Serine production by resting cells of a methanol-utilizing bacterium. *Journal of Fermentation Technology* **60**, 269–276.

IZUMI, Y., MISHRA, S. K., GHOSH, B. S., TANI, Y. AND YAMADA, H. (1983). NADH production from NAD^+ using a formate dehydrogenase system with cells of a methanol-utilizing bacterium. *Journal of Fermentation Technology* **61**, 135–142.

JIGAMI, Y., SUZUKI, O. AND NAKASATO, S. (1979). Comparison of lipid composition of *Candida guilliermondii* grown on glucose, ethanol and methanol as the sole carbon source. *Lipids* **14**, 937–942.

KANAMARU, K., HIEDA, T., IWAMURO, Y., MIKAMI, Y., OBI, Y. AND KISAKI, T. (1982a). Isolation and characterization of a *Hyphomicrobium* species and its polysaccharide formation from methanol. *Agricultural and Biological Chemistry* **46**, 2411–2417.

KANAMARU, K., IWAMURO, Y., MIKAMI, Y., OBI, Y. AND KISAKI, T. (1982b). 2-*O*-methyl-D-mannose in an extracellular polysaccharide from *Hyphomicrobium* sp. *Agricultural and Biological Chemistry* **46**, 2419–2424.

KARUBE, I. (1984). Possible developments in microbial and other sensors for fermentation control. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 2, pp. 313–339. Intercept, Ponteland, Newcastle upon Tyne.

KARUBE, I., OKADA, T. AND SUZUKI, S. (1982). A methane gas sensor based on methane-oxidizing bacteria. *Analytica chimica acta* **135**, 61–67.

KATO, N., KANO, M., TANI, Y. AND OGATA, K. (1974). Purification and characterization of formate dehydrogenase in a methanol-utilizing yeast, *Kloeckera* sp. No. 2201. *Agricultural and Biological Chemistry* **38**, 111–116.

KATO, N., TANI, Y. AND YAMADA, H. (1983). Microbial utilization of methanol: Production of useful metabolites. *Advances in Biotechnological Processes* **1**, 171–202.

KEUNE, H., SAHM, H. AND WAGNER, F. (1976). Production of L-serine by the methanol utilizing bacterium, *Pseudomonas* 3ab. *European Journal of Applied Microbiology* **2**, 175–184.

KIERŠTAN, M. (1982). The enzymatic conversion of ethanol to acetaldehyde as a model recovery system. *Biotechnology and Bioengineering* **24**, 2275–2277.

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

KODAMA, T., NAKAHARA, T., OHMORI, T., BINH, N. T., HOSHINO, N. T. AND MINODA, Y. (1977). Production of extracellular polysaccharides by a hydrogen bacterium and a methanol-utilizing bacterium. In *Microbial Growth on C_1-Compounds* (G. K. Suryabin, M. V. Ivanov, E. N. Kondratjeva, G. A. Zavarzin, Yu. A. Trotsenko and A. I. Nesterov, Eds), pp. 211–213. Scientific Centre for Biological Research, USSR Academy of Sciences, Puschino.

KURAISHI, M., OHKOUCHI, H., MATSUDA, N. AND TERAO, I. (1977). A study on the performance of air-lift fermentors in the production of methanol single cell protein. In *Microbial Growth on C_1-Compounds*, (G. K. Skryabin, M. V. Ivanov, E. N. Kondratjeva, G. A. Zavarzin, Yu, A. Trotsenko and A. I. Nesterov, Eds), pp. 180–181. Scientific Centre for Biological Research, USSR Academy of Sciences, Puschino.

LONGIN, R., COONEY, C. L. AND DEMAIN, A. L. (1982). Studies on the overproduction of indole-containing metabolites by a methanol-utilizing yeast, *Hansenula polymorpha*. *Applied Biochemistry and Biotechnology* **7**, 281–293.

MISAKI, A., TSUMURAYA, Y. AND KAKUTA, M. (1979). D-Allose-containing polysaccharide synthesized from methanol by *Pseudomonas* sp. *Carbohydrate Research* **75**, C8.

MORINAGA, Y., TANI, Y. AND YAMADA, H. (1982a). Regulatory properties of L-methionine biosynthesis in obligate methylotroph OM 33: Role of homoserine-*O*-transsuccinylase. *Agricultural and Biological Chemistry* **46**, 57–63.

MORINAGA, Y., TANI, Y. AND YAMADA, H. (1982b). L-Methionine production by ethionine-resistant mutants of a facultative methylotroph, *Pseudomonas* FM 518. *Agricultural and Biological Chemistry* **46**, 473–480.

MORINAGA, Y., TANI, Y. AND YAMADA, H. (1983). Biosynthesis of homocysteine in a facultative methylotroph, *Pseudomonas* FM 518. *Agricultural and Biological Chemistry* **47**, 2855–2860.

MORINAGA, Y., TANI, Y. AND YAMADA, H. (1984). Homocysteine transmethylation in methanol-utilizing bacteria and its application to L-methionine production. *Agricultural and Biological Chemistry* **48**, 143–148.

MORINAGA, Y., YAMANAKA, S. AND TAKINAMI, K. (1981a). L-Serine production by methanol-utilizing bacterium *Pseudomonas* MS31. *Agricultural and Biological Chemistry* **45**, 1419–1424.

MORINAGA, Y., YAMANAKA, S. AND TAKINAMI, K. (1981b). L-Serine production by temperature-sensitive mutants of methanol-utilizing bacterium *Pseudomonas* MS31. *Agricultural and Biological Chemistry* **45**, 1425–1430.

MORINAGA, Y., YAMANAKA, S. AND TAKINAMI, K. (1983). L-Serine production improved by analogue-resistant mutants of a methanol-utilizing bacterium. *Agricultural and Biological Chemistry* **47**, 2113–2114.

NATORI, Y. AND NAGASAKI, T. (1979). Formation of coenzyme Q_{11} by *Pseudomonas* M16, a mutant. *Agricultural and Biological Chemistry* **43**, 797–801.

NATORI, Y. AND NAGASAKI, T. (1980). Occurrence of coenzyme Q_{12} and coenzyme Q_{13} in facultative methanol-oxidizing bacteria. *Agricultural and Biological Chemistry* **44**, 2105–2110.

NATORI, Y. AND NAGASAKI, T. (1981). Enhancement of coenzyme Q_{10} accumulation by mutation and effects of medium components on the formation of coenzyme Q homologs by *Pseudomonas* N842 and mutants. *Agricultural and Biological Chemistry* **45**, 2175–2182.

NATORI, Y., NAGASAKI, T., KOBAYASHI, A. AND FUKAWA, H. (1978). Production of coenzyme Q_{10} by *Pseudomonas* N842. *Agricultural and Biological Chemistry* **42**, 1799–1800.

NISHIO, N., YANO, T. AND KAMIKUBO, T. (1975a). Isolation of methanol-utilizing bacteria and its vitamin B_{12} production. *Agricultural and Biological Chemistry* **39**, 21–27.

NISHIO, N., YANO, T. AND KAMIKUBO, T. (1975b). Further studies of vitamin B_{12} production by methanol utilizing bacterium, *Klebsiella* sp. No. 101. *Agricultural and Biological Chemistry* **39**, 207–213.

NISHIO, N., TANAKA, M., MATSUNO, R. AND KAMIKUBO, T. (1977a). Production of vitamin B_{12} by methanol-utilizing bacteria, *Pseudomonas* AM-1 and *Microcyclus eburneus*. *Journal of Fermentation Technology* **55**, 200–203.

NISHIO, N., TSUCHIYA, Y., HAYASHI, M. AND NAGAI, S. (1977b). A fed-batch culture of methanol-utilizing bacteria with pH stat. *Journal of Fermentation Technology* **55**, 151–155.

OGATA, K., IZUMI, Y., KAWAMORI, M., ASANO, Y. AND TANI, Y. (1977). Amino acid formation by methanol-utilizing bacteria. *Journal of Fermentation Technology* **55**, 444–451.

OHSUGI, M., YAMADA, M., YOSHIDA, Y., ISHIBASHI, F. AND INOUE, Y. (1983). Isolation of a formate-assimilating bacterium and its vitamin B_{12} formation. *Agricultural and Biological Chemistry* **47**, 1127–1128.

OKADA, T., KARUBE, I. AND SUZUKI, S. (1981). Microbial sensor system which uses *Methylomonas* sp. for the determination of methane. *European Journal of Applied Microbiology and Biotechnology* **12**, 102–106.

OKI, T., KITAI, A., KOUNO, K. AND OZAKI, A. (1973). Production of L-glutamic acid by methanol-utilizing bacteria. *Journal of General and Applied Microbiology* **19**, 79–83.

PATEL, R. N. (1984). Methane monooxygenase from *Methylobacterium* sp. strain CRL-26. In *Microbial Growth on C_1 Compounds* (R. L. Crawford and R. S. Hanson, Eds), pp. 83–90. American Society for Microbiology, Washington, DC.

PATEL, R. N., HOU, C. T., LASKIN, A. I., FELIX, A. AND DERELANKO, P. (1979a). Microbial oxidation of gaseous hydrocarbons. II. Hydroxylation of alkenes and epoxidation of alkenes by cell-free particulate fractions of methane-utilizing bacteria. *Journal of Bacteriology* **139**, 675–679.

PATEL, R. N., HOU, C. T., LASKIN, A. I., DERELANKO, P. AND FELIX, A. (1979b). Oxidation of secondary alcohols to methyl ketones by yeast. *Applied and Environmental Microbiology* **38**, 219–223.

PATEL, R. N., HOU, C. T., LASKIN, A. I., DERELANKO, P. AND FELIX, A. (1979c). Microbial production of methyl ketones. Purification and properties of a secondary alcohol dehydrogenase from yeast. *European Journal of Biochemistry* **101**, 401–406.

PLOTKIN, E. V., HIGGINS, I. J. AND HILL, H. A. O. (1981). Methanol dehydrogenase bioelectrochemical cell and alcohol detector. *Biotechnology Letters* **3**, 187–192.

REUSS, M., GNIESER, J., RENG, H. G. AND WAGNER, F. (1975). Extended culture of *Candida boidinii* on methanol. *European Journal of Applied Microbiology* **1**, 295–305.

SAHM, H. (1977). Metabolism of methanol by yeasts. *Advances in Biochemical Engineering* **6**, 77–103.

SALISBURY, S. A., FORREST, H. S., CRUSE, W. B. T. AND KENNARD, O. (1979). A novel coenzyme from bacterial primary alcohol dehydrogenases. *Nature* **280**, 844–845.

SANCHEZ, S. AND DEMAIN, A. L. (1978). Tryptophan excretion by a bradytroph of *Hansenula polymorpha* growing in methanol. *Applied and Environmental Microbiology* **35**, 459–461.

SATO, K. (1978). Bacteriochlorophyll formation by facultative methylotrophs *Protaminobacter ruber* and *Pseudomonas* AM1. *FEBS Letters* **85**, 207–210.

SATO, K. AND SHIMIZU, S. (1979). The conditions for bacteriochlorophyll formation and the ultrastructure of a methanol-utilizing bacterium, *Protaminobacter ruber*, classified as non-photosynthetic bacteria. *Agricultural and Biological Chemistry* **43**, 1669–1675.

SATO, K., UEDA, S. AND SHIMIZU, S. (1976). Methylmalonyl-CoA mutase in a methanol-utilizing bacterium, *Protaminobacter ruber*. *FEBS Letters* **71**, 248–250.

SATO, K., UEDA, S. AND SHIMIZU, S. (1977). Form of vitamin B_{12} and its role in a methanol-utilizing bacterium, *Protaminobacter ruber*. *Applied and Environmental Microbiology* **33**, 515–521.

SATO, K., MIZUTANI, T., HIRAOKA, M. AND SHIMIZU, S. (1982). Carotenoid containing sugar moiety from a facultative methylotroph. *Protaminobacter ruber*. *Journal of Fermentation Technology* **60**, 111–115.

SHIMIZU, S., ISHIDA, M., KANO, N., TANI, Y. AND OGATA, K. (1977a). Flavin changes of *Kloeckera* sp. No. 2201 during adaptation to methanol. *Agricultural and Biological Chemistry* **41**, 423–424.

SHIMIZU, S., ISHIDA, M., TANI, Y. AND OGATA, K. (1977b). Derepression of FAD pyrophosphorylase and flavin changes during growth of *Kloeckera* sp. No. 2201 on methanol. *Agricultural and Biological Chemistry* **41**, 2215–2220.

SHIMIZU, S., ISHIDA, M., TANI, Y. AND OGATA, K. (1977c). Production of flavin adenine dinucleotide by methanol-utilizing yeasts. *Journal of Fermentation Technology* **55**, 630–632.

SHIMIZU, S., SATO, K., HIRAOKA, M., YAMASHITA, F. AND KOBAYASHI, T. (1982). Carotenoid formation by a facultative methylotroph, *Protaminobacter ruber*. *Journal of Fermentation Technology* **60**, 163–166.

SUZUKI, M., KÜHN, I., BERGLUND, Å., UNDEN, A. AND HEDEN, C.-G. (1977a). Identification of a new methanol-utilizing bacterium and its characteristic re-

sponses to some chemicals. *Journal of Fermentation Technology* **55**, 459–465.

SUZUKI, M., BERGLUND, Å., UNDEN, A. AND HEDEN, C.-G. (1977b). Aromatic amino acids production by analogue-resistant mutants of *Methylomonas methanolophila* 6R. *Journal of Fermentation Technology* **55**, 466–475.

SUZUKI, O., JIGAMI, Y. AND NAKASATO, S. (1979). Changes in lipid composition of methanol-grown *Candida guilliermondii*. *Agricultural and Biological Chemistry* **43**, 1343–1345.

TANAKA, A., OHYA, Y., SHIMIZU, S. AND FUKUI, S. (1974). Production of vitamin B_{12} by methanol-assimilating bacteria. *Journal of Fermentation Technology* **52**, 921–924.

TANAKA, Y., ARAKI, K. AND NAKAYAMA, K. (1980). Accumulation of *O*-methyl-L-homoserine by methanol-utilizing bacteria. *Biotechnology Letters* **2**, 67–74.

TANI, Y. (1984). Microbiology and biochemistry of methylotrophic yeasts. In *Methylotrophs: Microbiology, Biochemistry and Genetics* (C. T. Hou, Ed.), pp. 55–85. CRC Press, Florida.

TANI, Y. AND YAMADA, H. (1980). Microbial utilization of C_1-compounds. *Biotechnology and Bioengineering* **22**, Suppl. 1, 163–175.

TANI, Y. AND YONEHARA, T. (1985). ATP production from adenosine or adenine by a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. *Agricultural and Biological Chemistry*, **49**, 637–642.

TANI, Y., KATO, N. AND YAMADA, H. (1978). Utilization of methanol by yeasts. *Advances in Applied Microbiology* **24**, 165–186.

TANI, Y., MITANI, Y. AND YAMADA, H. (1982). Utilization of C_1-compounds: Phosphorylation of adenylate by oxidative phosphorylation in *Candida boidinii* (*Kloeckera* sp.) No. 2201. *Agricultural and Biological Chemistry* **46**, 1097–1099.

TANI, Y., MITANI, Y. AND YAMADA, H. (1984a). ATP production by protoplasts of a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. *Agricultural and Biological Chemistry* **48**, 431–437.

TANI, Y., MITANI, Y. AND YAMADA, H. (1984b). Preparation of ATP-production cells of a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. *Journal of Fermentation Technology* **62**, 99–101.

TANI, Y., SAKAI, Y. AND YAMADA, H. (1985). Isolation and characterization of a mutant of a methanol yeast, *Candida boidinii* S2, with higher formaldehyde productivity. *Agricultural and Biological Chemistry*, in press.

TANI, Y., YOON, B.-D. AND YAMADA, H. (1985). Production of cytochrome *c* by an obligate methylotroph, *Methylomonas* sp. YK1. *Agricultural and Biological Chemistry*, in press.

TANI, Y., MIYA, T. AND OGATA, K. (1972). The microbial metabolism of methanol. Part II. Properties of crystalline alcohol oxidase from *Kloeckera* sp. No. 2201. *Agricultural and Biological Chemistry* **36**, 76–83.

TANI, Y., MIYA, T., NISHIKAWA, H. AND OGATA, K. (1972). The microbial metabolism of methanol. Part 1. Formation and crystallization of methanol-oxidizing enzyme in a methanol-utilizing yeast, *Kloeckera* sp. No. 2201. *Agricultural and Biological Chemistry* **36**, 68–75.

TANI, Y., KANAGAWA, T., HANPONGKITTIKUN, A., OGATA, K. AND YAMADA, H. (1978). Production of L-serine by a methanol-utilizing bacterium, *Arthrobacter globiformis* SK-200. *Agricultural and Biological Chemistry* **42**, 2275–2279.

TANI, Y., YONEHARA, T., MITANI, Y. AND YAMADA, H. (1984). ATP production by sorbitol-treated cells of a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. *Journal of Biotechnology* **1**, 119–127.

TORAYA, T., YONGSMITH, B., TANAKA, A. AND FUKUI, S. (1975). Vitamin B_{12} production by a methanol-utilizing bacterium. *Applied Microbiology* **30**, 477–479.

TORAYA, T., YONGSMITH, B., HONDA, S., TANAKA, A. AND FUKUI, S. (1976). Production of vitamin B_{12} by a methanol-utilizing bacterium. *Journal of Fermentation Technology* **54**, 102–108.

TSUCHIYA, Y., NISHIO, N. AND NAGAI, S. (1980). Vitamin B_{12} production from

methanol by continuous culture of *Pseudomonas* AM-1. *Journal of Fermentation Technology* **58**, 485–487.

UEDA, S., SATO, K. AND SHIMIZU, S. (1981). Glyoxylate formation from mesaconyl-CoA and its related reactions in a methanol-utilizing bacterium, *Protaminobacter ruber*. *Agricultural and Biological Chemistry* **45**, 823–830.

URAKAMI, T. AND KOMAGATA, K. (1979). Cellular fatty acid composition and coenzyme Q system in gram-negative methanol-utilizing bacteria. *Journal of General and Applied Microbiology* **25**, 343–360.

URAKAMI, T. AND KOMAGATA, K. (1981). Electrophoretic comparison of enzymes in the gram negative methanol-utilizing bacteria. *Journal of General and Applied Microbiology* **27**, 381–403.

URAKAMI, T., TERAO, I. AND NAGAI, I. (1981). Process for producing bacterial single cell protein from methanol. In *Microbial Growth on C_1-Compounds* (H. Dalton, Ed.), pp. 349–359. Heyden & Son Ltd, London.

VAN DIJKEN, J. P. AND HARDER, W. (1975). Growth yields of microorganisms on methanol and methane. A theoretical study. *Biotechnology and Bioengineering* **17**, 15–30.

VAN DIJKEN, H. P., HARDER, W. AND QUAYLE, J. R. (1981). Energy transduction and carbon assimilation in methylotrophic yeasts. In *Microbial Growth on C_1-Compounds* (H. Dalton, Ed.), pp. 191–201. Heyden & Sons Ltd, London.

VAN DIJKEN, J. P., OTTO, R. AND HARDER, W. (1976). Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. *Archives of Microbiology* **111**, 137–144.

VASEY, R. B. AND POWELL, K. A. (1984). Single-cell protein. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 2, pp. 285–311. Intercept, Ponteland, Newcastle upon Tyne.

WINDASS, J. D., WORSEY, M. J., PIOLI, E. M., PIOLI, D., BARTH, P. T., ATHERTON, K. T., DART, E. C., BYROM, D., POWELL, K. AND SENIOR, P. J. (1980). Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus*. *Nature* **287**, 396–401.

YAMADA, H., MORINAGA, Y. AND TANI, Y. (1982). L-Methionine overproduction by ethionine-resistant mutants of obligate methylotroph strain OM33. *Agricultural and Biological Chemistry* **46**, 47–55.

YAMAMOTO, M., OKAMOTO, R. AND INUI, T. (1979). Application of a marine methanol-utilizing bacterium for bioassay of vitamin B_{12} in sea water. *Journal of Fermentation Technology* **57**, 400–407.

YAMANE, T., KISHIMOTO, M. AND YOSHIDA, F. (1976). Semi-batch culture of methanol-assimilating bacteria with exponentially increased methanol feed. *Journal of Fermentation Technology* **54**, 229–240.

YANO, T., KOBAYASHI, T. AND SHIMIZU, S. (1978). Fed batch culture of methanol-utilizing bacterium with DO stat. *Journal of Fermentation Technology* **56**, 416–420.