Methanol: a fermentation substrate

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The present and future cost structure, the solubility, the purity and the ease of handling of methanol are factors which make methanol an attractive fermentation substrate. For these reasons, the possibility of producing single-cell protein or metabolites – for example amino acids, antibiotics or vitamins – from methanol is explored in several laboratories.

In recent years, many different species of bacteria and some species of yeasts capable of utilizing methanol were isolated. As shown in the Table, more than 100 strains of obligate and facultative methanol utilizing bacteria and about 20 species of methanol assimilating yeasts are known.

Methanol utilizing microorganisms

1. Bacteria

a) Obligate methylotrophic (CH_4, CH_3OH, CH_3-O-CH_3)

Methylobacter	*Methylocystis*
Methylococcus	*Methylosinus*
Methylomonas	

b) Facultative methylotrophic (CH_3OH, carbohydrates, organic acids)

Arthrobacter	Pseudomonas
Bacillus	*Rhodopseudomonas*
Hyphomiciobium	*Streptomyces*
Protoaminobacter	*Vibrio*

2. Yeasts and fungi

Facultative methylotrophic (CH_3OH, carbohydrates, organic acids)

Candida	*Pichia*
Hansenula	*Torulopsis*
Kloeckera	*Trichoderma*

Growth of microorganisms on reduced C_1-compounds requires that they are able to generate energy and reduction equivalents from these compounds. Furthermore, they must have acquired special metabolic pathways for the production of cell constituents from C_1-compounds via the synthesis of carbon-carbon bonds.

Dissimilation of methanol

Intensive studies over the past 10–15 years have shown that, in the majority of microorganisms, the oxidation of methanol is accomplished by 3 successive oxidative reactions via formaldehyde and formate to carbon dioxide (Figure 1).

In methanol-utilizing yeasts an alcohol oxidase was found to be responsible for the oxidation of methanol to formaldehyde[1]. This enzyme is strictly dependent on oxygen as an electron acceptor (X = O_2, see Figure 1). Furthermore, in methanol-grown yeast cells, catalase activity is increased about 5–20-fold in comparison with cells cultivated on glucose or ethanol[2]. In methane- and methanol-utilizing bacteria, an enzyme is present which requires ammonium ions for activity and catalyzes the oxidation of methanol with phenazine metosulphate as an artificial electron acceptor[3]. It has been suggested that the enzyme contains a pteridine derivative as a prosthetic group[4]; however, the precise nature of this prosthetic group is still unknown. There is some evidence to indicate that in vivo the enzyme can transfer the electrons obtained from the oxidation of methanol to an electron transport chain. It has been reported, for instance, that in *Pseudomonas* AM1 the reduced methanol dehydrogenase can be reoxidized by cytochrome C[5].

Two different enzymes have been shown to catalyze the oxidation of formaldehyde in methanol utilizing microorganisms. An NAD-linked glutathion-dependent formaldehyde dehydrogenase is present in methanol-grown *Micrococcus denitrificans*[6], yeasts[1], *Protaminobacter ruber* and *Vibrio extorquens*[7]. An aldehyde dehydrogenase with a broad substrate specifity has been reported to occur in *Pseudomonas* AM1, *Protaminobacter ruber*, *Vibrio extorquens* and *Pseudomonas methanica*[7]. This enzyme oxidizes formaldehyde in vitro with dichlorophenolindophenol as an artificial electron acceptor. Formaldehyde may also be oxidized as a result of the dual substrate specificity of methanol dehydrogenase for formaldehyde in an ammonium-dependent reaction[3].

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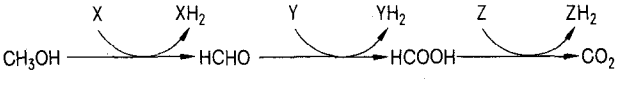

Fig. 1. Pathway of methanol oxidation in C_1-utilizing microorganisms. X, Y and Z represent the different electron acceptors involved in the oxidation of methanol, formaldehyde and formate, respectively.

In the majority of methanol bacteria studied so far, an NAD-dependent formate dehydrogenase has been found to catalyze the oxidation of formate to CO_2. In methanol-utilizing yeasts also, the oxidation of formate to CO_2 is catalyzed by an NAD-dependent formate dehydrogenase[1]. An NAD-linked formate dehydrogenase could not be detected in the methanol-grown actinomycete *Streptomyces sp.* No. 239[8]. In this organism, an enzyme which can use PMS/DCPIP and cytochrome C as artificial electron acceptors, seems to be responsible for the oxidation of the formate generated from methanol.

The oxidation of formaldehyde to CO_2 does not necessarily involve a two-step process catalyzed by formaldehyde- and formate-dehydrogenases. In some microorganisms, formaldehyde- and formate-dehydrogenases could not be detected in cell-free extracts. Instead other more elaborate enzyme systems were found to operate. In the less restricted methylotrophs studied by COLBY and ZATMAN[9], the oxidation of formaldehyde to CO_2 is mediated by a dissimilatory hexulose-phosphate cycle (Figure 2). The overall stoichiometry of the cycle can be given as

$$HCHO + 2NAD(P)^+ + H_2O \rightarrow CO_2 + 2NAD(P)H + 2H^+.$$

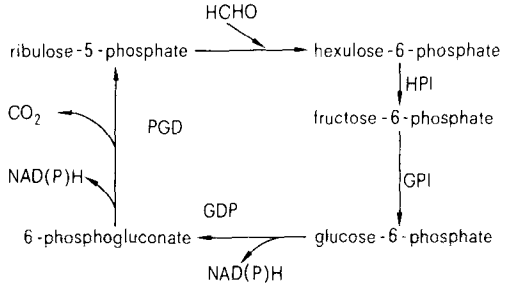

Fig. 2. Dissimilatory hexulose-phosphate cycle for the oxidation of formaldehyde. HPS, hexulose-phosphate synthase; HPI, hexulose-phosphate isomerase; GPI, glucose-phosphate isomerase; GDP, glucose-6-phosphate dehydrogenase; PGD, phosphogluconate dehydrogenase.

This dissimilatory hexulose-phosphate cycle may contribute to the oxidation of formaldehyde in *Pseudomonas methanica* and *Methylococcus capsulatus*[10]. In these organisms, however, formaldehyde- and formate-dehydrogenase are also present[11].

Assimilation of methanol

At present in methanol-utilizing microorganisms, 3 different pathways are known which effect the synthesis of C_3-compounds from the C_1 unit methanol: the Calvin cycle, the ribulosemonophosphate cycle and the serine pathway. Recently the Calvin cycle has also been reported to function as a major route of carbon assimilation in the chemolithotrophic bacterium, *Micrococcus denitrificans*, and in the photoorganotrophic bacteria *Rhodopseudomonas acidophila* and *Rhodopseudomonas gelatinosa* during growth on methanol or formate[12].

The pathway first shown to effect the net synthesis of C_3-compounds from C_1-units is the Calvin cycle. The cycle involves two key enzymes which are specific for organisms which use CO_2 as their sole carbon source. The first enzyme, ribulose-5-phosphate kinase, catalyzes the phosphorylation of ribulose-5-phosphate to ribulose-1,5-diphosphate. The latter compound is the substrate for the other key enzyme, ribulose-1,5-diphosphate carboxylase (carboxydismutase), which catalyzes the initial fixation of CO_2. The product of the condensation reaction is 3-phosphoglycerate which is subsequently converted to glyceraldehyde-3-phosphate. The basic stoichiometry of the Calvin cycle can be given as:

$3CO_2 + 9ATP + 6NAD(P)H + 6H^+ + 5H_2O \rightarrow$ glyceraldehyde-3-phosphate $+ 9ADP + 6NAD(P)^+ + 8P_i$

A pathway more common to C_1-utiliziers than the Calvin cycle is the ribulose-monophosphate cycle of formaldehyde fixation. In contrast to the Calvin cycle, formaldehyde instead of CO_2 is fixed in this cycle. Two characteristic enzymes are involved; hexulose-phosphate synthase catalyzes the condensation of formaldehyde with ribulose-5-phosphate to D-arabino-3-hexulose-6-phosphate[13] and hexulose-phosphate isomerase catalyzes the conversion of hexulose-6-phosphate into fructose-6-phosphate[13]. Three variants have been proposed for this cycle which differ either in the formation of C_3-compounds from fructose-6-phosphate or in the mechanism of rearrangement reactions[9,10]. The overall stoichiometry of the 3 different variants can be given as follows:

a) $3HCHO + ATP \rightarrow$ glyceraldehyde-3-phosphate $+$ ADP. Fructose-1,6-diphosphate (FDP) is an intermediate in the formation of glyceraldehyde-3-phosphate.

b) $3HCHO + NAD(P)^+ \rightarrow$ pyruvate $+ NAD(P)H + H^+$. Glyceraldehyde-3-phosphate required for the conversion of C_6- to C_5-molecules is formed in the Entner-Doudoroff pathway.

c) $3HCHO + 2\,ATP \rightarrow$ glyceraldehyde-3-phosphate $+ 2ADP + P_i$. Rearrangement reactions do not involve transaldolase but are established via transketolase reactions and sedoheptulose diphosphatase.

Until a few years ago, it was thought that the operation of the pathway was restricted to obligate methylotrophs, but recently the pathway has also been reported to function as a major path of carbon assimilation during growth on C_1-compounds in facul-

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tative methylotrophs which can also grow on other organic substrates[9]. The ribulose-monophosphate cycle of formaldehyde fixation has also been reported to operate during growth of the fungus *Trichoderma lignorum* on methanol[14]. Short labeling experiments with whole cells strongly indicated that a pathway similar to the ribulose-monophosphate cycle of formaldehyde fixation occurs in yeasts growing on methanol[15]. Preliminary observations on the fixation of radioactive formaldehyde by cell free extracts of *Candida* species have indicated that a hexulose-phosphate synthase which is strictly dependent on ATP for activity may be present[15,16]. In contrast to the two pathways discussed above, the serine pathway does not involve pentose-phosphates in the condensation reaction but is based on the condensation of C_1-units with C_2- and C_3-compounds. Two C_1-units at the oxidation level of formaldehyde enter the cycle via a condensation reaction with glycerin catalyzed by serine transhydroxymethylase. The third C_1-unit required for the net synthesis of 1 molecule of 3-phosphoglycerate enters as CO_2 via a condensation reaction with phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase. Two variants have been suggested for the serine pathway which differ in the way in which malate (or another C_4-carboxylic acid) is converted into two molecules of glycine[17]. In the icl^+-variant (where icl stands for isocitrate lyase), malate is activated to malyl-CoA which is subsequently converted to glyoxylate and acetyl-CoA. Both glyoxylate and acetyl-CoA are further converted to glycine, acetyl-CoA via the glyoxylate cycle in which isocitrate lyase plays a key role. The overall stoichiometry of the serine pathway with the isocitrate lyase can be given as follows:

$$3HCHO \rightarrow \text{pyruvate}$$

In the alternative variant icl^- of the serine pathway, the synthesis of a C_4-compound is accomplished in a way probably identical to that of the icl^+-variant. However, it differs from this variant in that one of the key enzymes of the glyoxylate cycle, isocitrate lyase, is absent[17]. Furthermore, the enzyme which activates malate to malyl-CoA is absent in some bacteria which assimilate C_1-compounds via this variant.

The serine pathway has been shown to occur in different methanol-utilizing bacteria[18] and also in a methanol-utilizing streptomyces in a methanol-utilizing streptomyces species[8]. Furthermore, the pathway has been found in fungi capable of growth on methanol, formaldehyde and formate[19].

In the three different pathways discussed above, C_3-compounds are synthesized from C_1-units of different oxidation levels (formaldehyde and/or CO_2). This has an important bearing on the overall efficiency of the different pathways on terms of ATP requirement. From a bioenergetic point of view, the assimilation of reduced C_1-compounds via CO_2 is an expensive process since this involves the oxidation of formaldehyde to CO_2 (which is strongly exergonic) followed by reduction of CO_2 to the oxidation level of cell material, which is about equivalent to that of formaldehyde. It is therefore not surprizing that among the assimilation pathways found in C_1-utilizers, the Calvin cycle is energetically most unfavourable, and that the ribulose-monophosphate pathways of formaldehyde fixation are more economical than the serine pathway.

Serine transhydroxymethylase

Serine transhydroxymethylase (STHM) catalyzes the interconversion of serine and glycine. In most microorganisms, its major functions are to provide glycine for protein and purine synthesis and N^5,N^{10}-methylene-tetrahydrofolate for the one carbon pool[20]. This enzyme has a second function in microorganisms which use reduced C_1-compounds as sole sources of carbon and energy, and which use the serine pathway for formaldehyde assimilation. It converts glycine to serine, incorporating a one-carbon unit derived from the oxidation of the substrate used for growth[21].

In facultative methylotrophs, the regulation of STHM is a difficult problem since it must not only be regulated for amino acid biosynthesis, but also for carbon assimilation. From a facultative methylotrophic bacterium, two activities have been purified[22]. One enzyme predominates when the organism is grown on methane or methanol as the sole carbon and energy source, whereas the second enzyme is the major isoenzyme found, when succinate is used as the sole carbon and energy source. The enzyme from methanol-grown cells is activated by glyoxylate, the enzyme from succinate-grown cells is not activated by glyoxylate. Adenine, glyoxylate, or trimethoprim in the growth medium causes an increased level of serine transhydroxymethylase in both methanol- and succinate-grown cells by stimulating the synthesis of the glyoxylate-activated enzyme.

Production of L-*Serine*

Since it is known that methanol is assimilated by some groups of microorganisms via the serine pathway, it is attractive to investigate the production of

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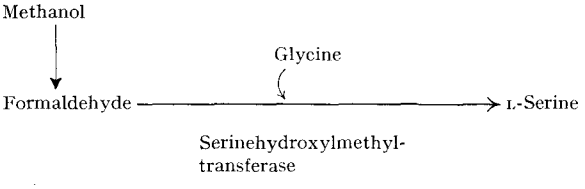
L-serine by these microorganisms. A facultative methanol-utilizing bacterium, *Pseudomonas* 3ab, which utilizes the serine pathway of formaldehyde fixation was used for this study[23]. Since the organism produced only small amounts of L-serine (about 30–40 mg/l) when it was cultivated on mineral salt medium with methanol, pyruvate, lactate or malate as carbon sources; glycine and methanol were added to methanol-grown cells at the end of the exponential growth phase. A remarkable increase in L-serine production was observed when the pH was adjusted to alkaline values, while growth was inhibited under these conditions. The maximum yield of L-serine was obtained when glycine and methanol were added and the pH of culture medium was changed to 8.5. Though *Pseudomonas* 3ab is unable to grow on L-serine or glycine it is very active in decomposing these amino acids. The degradation of L-serine and glycine has been shown be pH-dependent with a minimum at pH 8.5–9.0. This result may explain the pH-optimum at 8.5 for production of L-serine.

There is some evidence that the carbon skeleton of L-serine is derived chiefly from glycine and methanol; therefore L-serine is probably synthesized from glycine and formaldehyde by the serine hydroxymethyltransferase, the first enzyme of the serine pathway as shown in the following scheme:



Theoretical growth yields on methanol

Since there is a wide interest in the possible utilization of C_1-compounds as a substrate for the production of single cell protein, calculations of growth yields are of importance. In the calculation of growth yields of microorganisms on methanol, both the ATP yield of the dissimilation and the energy requirement of the assimilation pathways of this compound have to be considered[24]. The calculations are facilitated by the fact that assimilation of C_1-compounds by either the serine pathway or the ribulosephosphate pathway of formaldehyde fixation proceeds via 3-phosphoglycerate as a key intermediate in the synthesis of cell material. The calculation of the energy requirement for the assimilation of reduced C_1 compounds into cell material can be divided into two parts: 1. Estimation of the energy and reducing power requirement for the synthesis of 3-phosphoglycerate from C_1-units, 2. Estimation of the energy and reducing power requirement for the synthesis of all cell constituents from 3-phosphoglycerate.

When Y_{ATP} on 3-phosphoglycerate was taken at 10.5, a maximal cell yield of organisms of the composition $C_4H_8O_2N$ on methanol was found to be 0.73 g cells/g substrate calculated on the basis of the ribulosephosphate pathway of formaldehyde fixation. Yields calculated on the basis of the serine pathway are on average 20% lower[24].

Methanol fermentation

In recent years considerable attention has been given to methanol as a carbon source for the production of single-cell protein (SCP). While usually batch and continuous fermentations are used, there are a number of reasons for studying a technique in which the substrate methanol is added to a batch process under controlled conditions (extended culture). One reason is that in an extended culture significantly higher cell densities can be obtained than in a batch process. Controlled addition of substrate permits prolonged maintenance of a constant environment with respect to carbon substrate[25].

When *Candida boidinii* was grown in a medium containing different concentrations of methanol (between 0.1% and 0.8%) (w/v) an increase in growth activity up to 0.2% (w/v) methanol was observed, then a decline due to substrate inhibition occurred[25]. Although some observations on the inhibitory nature of the methanol substrate are presented in the literature[26,27], no attempt has been made to correlate this effect by means of a mathematical model. The inhibition function is that proposed by HALDANE[28] for the inhibition of enzyme at high substrate concentrations which can be expressed as:

$$\mu = \frac{\mu'_{max}}{1 + Ks/S + S/K_i}$$

where μ = specific growth rate, time^{-1}; μ_{max} = maximum specific growth rate in the absence of inhibition, time^{-1}; S = substrate concentration, mass/volume; K_S = lower concentration of substrate at which the specific growth rate is one-half of μ_{max}, mass/volume; K_i = inhibition constant, numerically equal to the higher concentration of substrate at which the specific growth rate is one-half of μ_{max}, mass/volume.

In order to identify the parameters in this equation, time courses of specific growth rates were determined by differentiating the experimental growth curves. From this calculation, the maximum specific growth velocity was predicted as a function of methanol concentration. The results indicate a close agreement between experiments and the theoretical equation[25].

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