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Microbial Oxidation of Gaseous Hydrocarbons: Production of Methyl Ketones from Their Corresponding Secondary Alcohols by Methane- and Methanol-Grown Microbes

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Cultures of methane- or methanol-utilizing microbes, including obligate (both types I and II) and facultative methylotrophic bacteria, obligate methanol utilizers, and methanol-grown yeasts were isolated from lake water of Warinanco Park, Linden, N.J., and lake and soil samples of Bayway Refinery, Linden, N.J. Resting-cell suspensions of these, and of other known C1-utilizing microbes, oxidized secondary alcohols to their corresponding methyl ketones. The product methyl ketones accumulated extracellularly. Succinate-grown cells of facultative methylotrophs did not oxidize secondary alcohols. Among the secondary alcohols, 2-butanol was oxidized at the highest rate. The optimal conditions for in vivo methyl ketone formation were compared among five different types of C1-utilizing microbes. Some enzymatic degradation of 2-butanone was observed. The product, 2-butanone, did not inhibit the oxidation of 2-butanol. The rate of the 2-butanone production was linear for the first 4 h of incubation for all five cultures tested. A yeast culture had the highest production rate. The optimum temperature for the production of 2-butanone was 35°C for all the bacteria tested. The yeast culture had a higher temperature optimum (40°C), and there was a reasonably high 2-butanone production rate even at 45°C. Metal-chelating agents inhibit the production of 2-butanone, suggesting the involvement of metal(s) in the oxidation of secondary alcohols. Secondary alcohol dehydrogenase activity was found in the cell-free soluble extract of sonically disrupted cells. The cell-free system requires a cofactor, specifically nicotinamide adenine dinucleotide, for its activity. This is the first report of a nicotinamide adenine dinucleotide-dependent, secondary alcohol-specific enzyme.

Methyl ketone formation is well known in mammals (7), in bacteria (10), and in fungi (3). In these cases, the ketone is formed by decarboxylation of a β-keto acid and has, therefore, one less carbon atom than the precursor. On the other hand, bacterial formation of methyl ketones from *n*-alkanes, demonstrated first by Leadbetter and Foster (11), represents a unique alpha-oxidation, with no change in the carbon skeleton. However, in this latter report it was stated that the ketone formation was by cooxidation in the presence of the growth substrate and indicated that no activity was found with the resting cells.

Phenazine methosulfate (PMS)-dependent methanol dehydrogenase has been reported from many methylotrophic bacteria (1, 2, 16-18, 22). This enzyme oxidizes primary alcohols from C1 to C10, but does not oxidize secondary alcohols. Nicotinamide adenine dinucleotide (NAD)-dependent alcohol dehydrogenases have been reported from liver and from yeast (3). These alcohol dehydrogenases oxidize primary alcohols and acetaldehyde, but have no activity on methanol. In addition, the alcohol dehydrogenases from yeast and liver also oxidize some secondary alcohols at a very low rate. NAD phosphate (P)-dependent alcohol dehydrogenases were also reported in *Pseudomonas* (15, 20), *Escherichia coli* (8), and *Leuconostoc* (9). However, these enzymes were active only toward long-chain primary alcohols (8, 9, 20), or long-chain hydroxy fatty acids, with some activity on medium-chain-length secondary alcohols containing a hydroxy group at the center of the carbon chain (15).

In our studies on the microbial oxidation of gaseous hydrocarbons, we found that resting cell suspensions of methane- and methanol-grown methylotrophs oxidized *n*-propane and *n*-butane to their corresponding methyl ketones. In addi-

tion, we demonstrated for the first time the conversion of secondary alcohols to their corresponding methyl ketones by resting cell suspensions of C1-utilizing microbes. This paper describes the isolation of 23 new C1-utilizing microbes from lake water of Warinanco Park, Linden, N.J., and lake and soil samples of Bayway Refinery, Linden, N.J. It also describes the formation of 2-butanone from 2-butanol by resting-cell suspensions of these new cultures as well as from known C1 utilizers, including obligate (both types I and II) and facultative methylotrophs, obligate methanol utilizers, and methanol-grown yeasts. Subsequent studies compared environmental factors that influence methyl ketone production among five distinct gorups of C1 utilizers. Secondary alcohol dehydrogenase (SADH) activity was also demonstrated in cell-free systems.

MATERIALS AND METHODS

Bacterial strains. C1-utilizing cultures were isolated from lake water of Warinanco Park, Linden, N.J., and lake and soil samples of Bayway Refinery, Linden, N.J. (Table 1). These methylotrophs were identified by the classification given by Whittenbury et al. (21) and Patt et al. (19). *Methylosinus trichosporium* OB3b, *Methylosinus sporium* 5, *Methylocystis parvus* OBBP, *Methylomonas methanica* S_1, *Methylomonas albus* BG 8, and *Methylobacter capsulatus* Y were kindly provided by R. Whittenbury (School of Biological Sciences, University of Warwick, Coventry, United Kingdom). *Methylobacterium organophilum* XX ATCC 24886 was kindly provided by R. S. Hanson (University of Wisconsin, Madison). Other organisms were from either the Northern Regional Research Laboratories (Peoria, Ill.) or the American Type Culture Collection (Rockville, Md.). The organisms were maintained on mineral salt agar plates in a desiccator jar under an atmosphere of gaseous hydrocarbon and air (1:1) at 30°C. Organisms were grown at 30°C in 300-ml flasks containing 50 ml of mineral salt medium (17) with gaseous hydrocarbons (gaseous hydrocarbon and air, 1:1, vol/vol) as the sole carbon and energy source. When alcohols (at 0.3%) were used, the gaseous phase of the flask was air.

Chemicals. Gaseous hydrocarbons were obtained from Matheson Gas Products, Inc. (East Rutherford, N.J.). All alcohols were obtained from MC/B Manufacturing Co. (Norwood, Ohio). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Activity assay. When whole cells were used, the harvested cells from shake-flask cultures were washed twice with 0.05 M phosphate buffer, pH 7.0. The final pellet was resuspended in fresh buffer to obtain an optical density at 660 nm of 0.5. One-half milliliter of this washed cell suspension (containing a given amount of dry cell mass) was placed in a 10-ml vial. Ten microliters of liquid substrate was added, and the vial was sealed with a rubber cap to minimize evaporation. The reaction mixture was incubated at 30°C on a water bath rotary shaker (New Brunswick Scientific Co., Edison, N.J.) at 300 rpm. A 3-μl sample was removed with a syringe and was assayed with flame ionization gas chromatography by using a stainless-steel column (20 feet by ⅛ inch [ca. 609.6 cm by 3.17 mm]) packed with 10% Carbowax 20M on 80/100 Chromosorb W (Supelco, Inc., Bellefonte, Pa.). The column temperature was maintained isothermally at 130°C, and the carried gas flow was 35 ml of helium per min. The methyl ketones were identified by retention time comparisons and cochromatography with authentic standards. The amount of methyl ketones accumulated was determined from the peak area by using a standard curve which had been constructed with authentic samples. Duplicate measurements were performed for each assay. A typical gas chromatogram is shown in Fig. 1.

For the cell-free system, the washed cells were disrupted with a Wave Energy Ultrasonic Oscillator, model W201 (Wave Energy System, Inc., Newtown, Pa.) and centrifuged at 20,000 × *g* for 30 min. The clear supernatant was used for the enzyme assay. The enzyme activity was measured with a fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn., model MPF 44A) by following the formation of reduced NAD (EX 340 nm, Em 460 nm). The formation of reduced NAD was also followed with an absorption spectrophotometer at 340 nm. The assay system (3 ml) contained (micromoles): potassium phosphate buffer (pH 7.0), 150; NAD 1; a given amount of enzyme preparation; and secondary alcohol, 10. The reaction was started by the addition of substrate. One unit of enzyme activity represents the reduction of 1 nmol of NAD per min. Protein concentrations were determined by the method of Lowry et al. (12).

RESULTS

Resting-cell suspensions of methane- and methanol-grown microbial cells oxidized secondary alcohols to their corresponding methyl ketones. After the incubation, the reaction mixture was centrifuged to remove the cells. The product

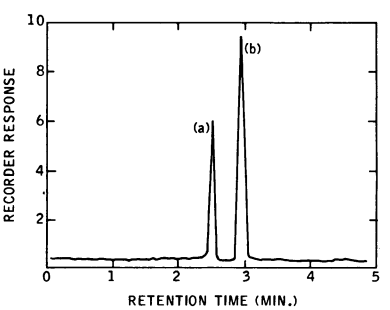

FIG. 1. *Gas-liquid chromatography of the products of oxidation of 2-butanol by Pseudomonas sp. ATCC 21439. The reaction and column conditions were as described in the text. Resting-cell suspensions of strain ATCC 21439 grown on methanol were incubated with 10 μl of butanol-2 at 30°C. After 1 h of incubation, 3 μl was injected into the column. (a) Product 2-butanone; (b) substrate 2-butanol.*

methyl ketones were found to be accumulated extracellularly. Control experiments with heat-killed cells indicated that the methyl ketones were produced enzymatically. A comparison of the activity for converting 2-butanol to 2-butanone by C1-utilizing microbes is listed in Table 1. SADH activity was found in all of the C1 utilizers tested.

TABLE 1. *Conversion of 2-butanol to 2-butanone by cell suspensions of C1-utilizing microbes*[a]

Microbes		Conversion rate (μmol/h per mg of protein)
Obligate methane utilizers (methane grown)		
Type II membrane structure		
Methylosinus trichosporium	OB3b	4.8
Methylosinus sp.	CRL 15	4.5
Methylosinus sporium	5	3.0
Methylosinus sp.	CRL 16	2.5
Methylocystis parvus	OBBP	1.1
Methylocystis sp.	CRL 18	1.0
Type I membrane structure		
Methylomonas methanica	S_1	0.4
Methylomonas albus	BG8	3.5
Methylomonas sp.	CRL 4	2.5
Methylomonas sp.	CRL 8	3.0
Methylomonas sp.	CRL 10	0.5
Methylomonas sp.	CRL 17	2.0
Methylomonas sp.	CRL 20	2.0
Methylomonas sp.	CRL 21	2.8
Methylomonas sp.	CRL 22	1.5
Methylomonas sp.	CRL M6P	1.4
Methylobacter sp.	CRL M6	1.4
Methylobacter sp.	CRL 23	1.0
Methylobacter sp.	CRL M1Y	3.0
Methylobacter sp.	CRL 19	1.8
Methylobacter sp.	CRL 5	2.0
Methylococcus capsulatus	CRL M1	5.0
M. capsulatus	Y	0.8
Methylococcus sp.	CRL 25	0.9
Methylococcus sp.	CRL 24	2.5
Facultative methane utilizers (methane grown)		
Methylobacterium organophilum	CRL 26	2.5
	XX	2.5
Obligate methanol utilizers		
Pseudomonas sp. CRL 75 ATCC 21439		5.4
Methylomonas methylovora ATCC 21852		2.5
Facultative methanol utilizers (methanol grown)		
Pseudomonas sp. CRL 74 ATCC 21438		3.2
Pseudomonas Ms. ATCC 25262		3.5
Yeasts (methanol grown)		
Candida boidinii NRRL Y-2332		6.0
Candida utilis ATCC 26387		6.8
Hansenula polymorpha ATCC 26012		5.8
Hansenula anomala NRRL Y-336		5.5
Pichia pastoris NRRL Y-55		5.8
Pichia sp. CRL 72		4.0

[a] Strains with CRL numbers are newly isolated cultures in our lab.

SADH activity was also found in cell suspensions of the methanol-grown or methyl amine-grown microbes listed in Table 1. However, SADH is not a constitutive enzyme; the enzyme activity was not found in succinate-grown facultative C1 utilizers.

Optimal conditions for the production of 2-butanone by C1-grown microbes. Optimal conditions for the production of 2-butanone were compared among five distinct types of methane or methanol utilizers: *M. trichosporium* OB3b (type II, obligate), *Methylococcus capsulatus* CRL MI (type I, obligate), *M. organophilum* CRL 26 (facultative), *Hansenula polymorpha* ATCC 26012, and *Pseudomonas* sp. ATCC 21439 (obligate methanol utilizer).

Time course. The production of 2-butanone from 2-butanol reached a maximum after 14 h of incubation in batch experiments in all the microbes tested. The amount of 2-butanone had not declined after 30 h of incubation. The rate of 2-butanone production was linear for the first 4 h (Fig. 2). Therefore, the production of 2-butanone was measured within this interval whenever the effect of a variable was tested.

pH. The effect of pH on the production of 2-butanone was studied with tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.05 M) for pH values of 8.0 to 10.0 and with 0.05 M potassium phosphate buffer for values from 5.0 to 8.0. A pH of around 8.0 was found to be the optimum for 2-butanone formation in all the five distinct type microbes tested (Fig. 3). Strains ATCC 21439 and CRL 26 showed high activity at both pH 8 and 9. In the case of strain OB3b, the 2-butanone formation was significantly lower at pH 9 than that at pH 8. Yeast

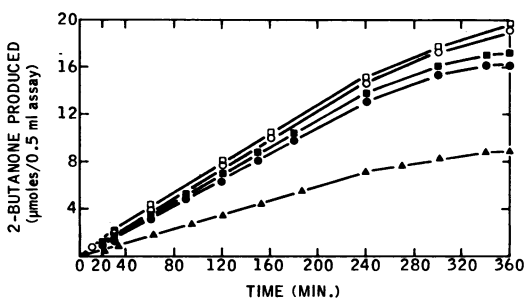

FIG. 2. *Time course of 2-butanone production by resting-cell suspensions of methanol-grown cultures from five distinct types of C1-utilizing microbes. Resting-cell suspensions (0.7 mg of protein in 0.5 ml) were incubated with 10 μl of 2-butanol at 30°C for various time. A 3-μl sample was assayed at a given time with gas chromatography. Symbols: ●, Strain OB3b; ■, strain CRL M1; ▲, strain CRL 26; ○, strain ATCC 21439; and □, strain ATCC 26012.*

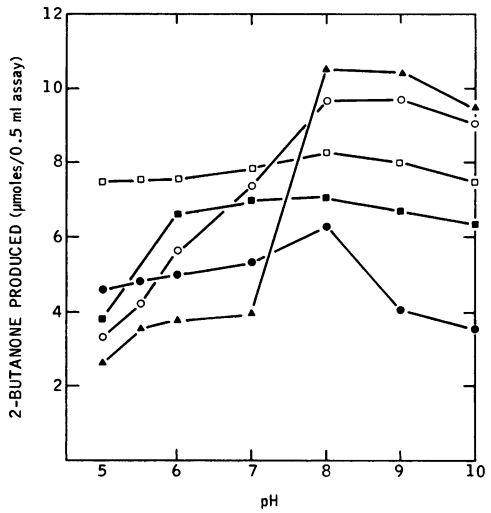

FIG. 3. *Effect of pH on the production of 2-butanone by resting-cell suspensions of methanol-grown cultures from five distinct types of C1-utilizing microbes. Reaction conditions were the same as those described in the legend to Fig. 2. Product 2-butanone was assayed after 2 h of incubation. Tris(hydroxymethyl)aminomethane-hydrochloride buffers (0.05 M) were used for pH values from 8.0 to 10.0 and potassium phosphate buffers (0.05 M) were used for values from 5.0 to 8.0. Symbols: ●, Strain OB3b (0.55 mg); ■, strain CRL M1 (0.7 mg); ▲, strain CRL 26 (0.8 mg); ○, strain ATCC 21439 (0.7 mg); □, strain ATCC 26012 (0.66 mg).*

cells appeared less affected by pH in the production of 2-butanone. Authentic samples of 2-butanol and 2-butanone, final concentrations of 8 μmol/0.5 ml, were added to heat-killed cell suspensions of strain OB3b in 0.05 M buffer at pH 5.0, 7.0, and 10.0 to test for nonenzymatic oxidation and degradation of 2-butanol and 2-butanone, respectively. The concentrations of 2-butanol and 2-butanone in these heat-killed cell suspensions were not decreased after 16 h of incubation, indicating that nonenzymatic oxidation or hydrolysis of 2-butanol or 2-butanone did not occur under the assay conditions.

Temperature. The temperature optimum for the production of 2-butanone by cell suspensions was about 35°C except for the yeast culture, which had an optimum of about 40°C (Fig. 4).

Substrate concentration. Various concentrations of 2-butanol were added to cell suspensions of yeast and of strain ATCC 21439. The production of 2-butanone was assayed after 35 min of incubation. The amount of 2-butanone produced was dependent on the amount of substrate initially added. A 2-butanol concentration of about 50 μmol per 0.5 ml supported maximum 2-butanone production (Fig. 5).

Cell concentration. The cell concentration also influenced the rate of 2-butanone production. The amount of 2-butanone accumulated after 2 h of incubation increased linearly as the cell concentration was increased up to about 1.2 mg/0.5 ml for yeast and for strain CRL M1 and about 1.7 mg/0.5 ml for strains CRL 26, OB3b, and ATCC 21439 (Fig. 6).

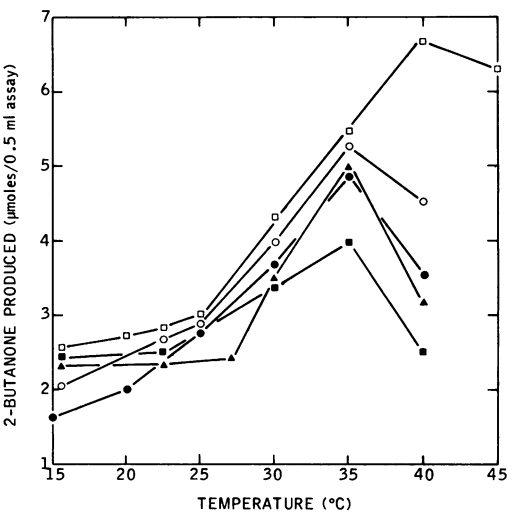

FIG. 4. *Effect of temperature on the production of 2-butanone by resting-cell suspensions of methanol-grown cultures from five distinct types of C1-utilizing microbes. Reaction conditions were the same as those described in the legend to Fig. 2. Product 2-butanone was assayed after 2 h of incubation at various temperatures. Symbols: ●, strain OB3b (0.37 mg of protein); ■, strain CRL M1 (0.34 mg); ▲, strain CRL 26 (0.7 mg); ○, strain ATCC 21439 (0.37 mg); □, strain ATCC 26012 (0.26 mg).*

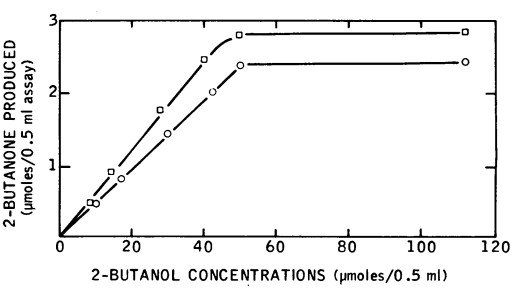

FIG. 5. *Effect of 2-butanol concentration on the production of 2-butanone by resting-cell suspensions of strains ATCC 21439 and 26012 grown on methanol. Reaction conditions were the same as those described in the legend to Fig. 2 except that various amounts of 2-butanol were added. Product 2-butanone was assayed after 35 min of incubation. Cell concentrations used were 0.92 mg per 0.5 ml. Symbols: ○, Strain ATCC 21439; □, strain ATCC 26012.*

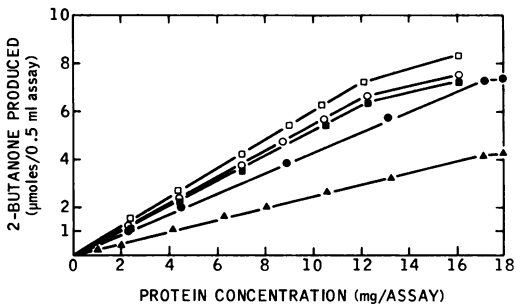

FIG. 6. *Effect of cell concentrations on the production of 2-butanone by resting-cell suspensions of methanol-grown cultures from five distinct types of C1-utilizing microbes. Reaction conditions were the same as those described in the legend to Fig. 2 except that various amounts of cells were used. Product 2-butanone was assayed after 1 h of incubation. Symbols: ●, Strain OB3b; ■, strain CRL M1; ▲, strain CRL 26; ○, strain ATCC 21439; and □, strain ATCC 26012.*

Product inhibition and further oxidation. Examination of the time course of 2-butanone production revealed that the rate decreased after 4 h of incubation, suggesting, among other possibilities, either product inhibition or further oxidation of 2-butanone. To test these possibilities, 8 μmol of 2-butanone was added to viable or heat-killed cell suspensions and incubated under the standard conditions. No decline was observed in 2-butanone concentration in all the heat-killed cell suspensions, but 2-butanone slowly disappeared in the presence of viable cells of all five strains. When 2-butanol (5 μl/0.5 ml of reaction mixture) was added to viable cell suspensions along with the exogenously supplied 2-butanone, a net increase in 2-butanone production was detected (Fig. 7). The reaction rates were identical to those shown in Fig. 2 and were not affected by the presence of the exogenously supplied 2-butanone. These data indicate that there is no product inhibition in the production of 2-butanone. A small amount of further oxidation of 2-butanone by viable cell suspensions was observed. Therefore, the decrease in 2-butanone production rate after 4 h of incubation may be due to the depletion of other requirement(s), e.g., a cofactor(s).

Inhibition studies. The production of 2-butanone from 2-butanol by cell suspensions of all five strains was inhibited by metal-chelating agents such as 1,10-phenanthroline and α,α-dipyridyl. However, the activity was not inhibited by sodium cyanide or thiourea (Table 2). This suggests the involvement of metal(s), possibly not a heme type, in the oxidation of secondary alcohols.

Substrate specificity. The sustrate specificity for the oxidation of secondary alcohols by the five strains of C1 utilizers was studied. Among the secondary alcohols, 2-propanol and 2-butanol were oxidized at higher rates; 2-pentanol, 2-hexanol, and 2-heptanol were oxidized at a much slower rate (Table 3). The oxidation products of these secondary alcohols were the corresponding methyl ketones, as determined by

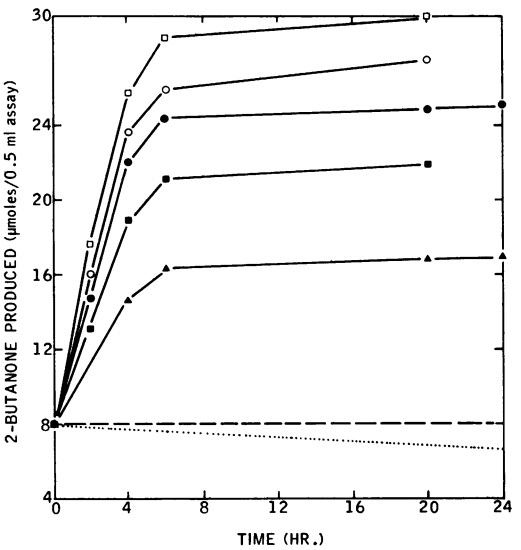

FIG. 7. *Product inhibition and degradation of 2-butanone by resting-cell suspensions of methanol-grown cultures from five different C1-utilizing microbes. Reaction conditions were the same as those described in the legend to Fig. 2. Heat-killed cells were prepared by boiling the cell suspension for 5 min. 2-Butanone (8 μmol) and 2-butanol (10 μl) were added to the vials unless otherwise stated before the incubation. Symbols: - - -, heat-killed cells (either culture); .....,viable cells (either culture) plus 2-butanone only; ●, strain OB3b (0.68 mg of protein); ■, strain CRL M1 (0.5 mg of protein); ▲, strain CRL 26 (0.64 mg of protein); ○, strain ATCC 21439 (0.7 mg of protein); □, strain ATCC 26012 (0.8 mg of protein).*

TABLE 2. *Effect of metal-chelating agents and other inhibitors on the production of 2-butanone by cell suspensions of methanol-grown Methylococcus capsulatus CRL M1*

Metal-chelating agents[a]	Inhibition (%)
Sodium cyanide	0
Sodium azide	10
Ethylenediaminetetraacetic acid	70
1,10-Phenanthroline	95
α,α-Dipyridyl	75
Thiourea	0

[a] Concentration of 1 mM.

GC retention time comparisons with authentic standards.

Cell-free system. Cell-free soluble extracts from sonically disrupted cells also oxidized 2-butanol to 2-butanone (Table 4). However, the cell-free system required the addition of a cofactor, NAD, for its activity. Other cofactors tested (including NADP reduced form), NADP, PMS, glutathione, flavine adenine dinucleotide, potassium ferricyanide, and dichlorophenol indophenol) were not effective. The stoichiometry for the consumption of 2-butanol, the reduction of NAD, and the formation of 2-butanone was obtained (Table 5). Primary alcohols (methanol to *n*-decanol) and tertiary alcohols (*tert*-butanol, *tert*-pentanol) were not substrates of the enzyme. This is the first report of an NAD-dependent secondary alcohol-specific alcohol dehydrogenase.

TABLE 3. *Oxidation of secondary alcohols by cell suspensions of C1 utilizers grown on methanol*

C1 utilizers	Oxidation rate (μmol/h per mg of protein)			
	2-Propanol to acetone	2-Butanol to 2-butanone	2-Pentanol to 2-pentanone	2-Hexanol to 2-hexanone
Methylosinus trichosporium OB3b	0.30	4.8	2.7	0.09
Methylococcus capsulatus CRL M1	2.0	5.0	0.24	0.08
Methylobacterium organophilum CRL 26	0.72	2.5	1.0	0.09
Hansenula polymorpha ATCC 26012	5.9	5.8	1.4	0.72
Pseudomonas sp. ATCC 21439	3.5	5.4	0.05	0.03

TABLE 4. *Oxidation of 2-butanol to 2-butanone by cel-free soluble extracts of C1-utilizing microbes*

Microbes	Growth substrate	Conversion rate (nmol/min per mg of protein)
Obligate methylotrophs		
Type II membrane structure		
Methylosinus trichosporium OB3b	CH_4	4.5
	CH_3OH	2.4
Type I membrane structure		
Methylococcus capsulatus CRL M1	CH_4	3.2
	CH_3OH	2.0
Facultative methylotroph		
Methylobacterium organophilium CRL 26	CH_4	1.8
	CH_3OH	2.5
Obligate methanol utilizer		
Pseudomonas sp. CRL 75 ATCC 21439	CH_3OH	25.0
Yeast		
Hansenula polymorpha ATCC 26012	CH_3OH	23.2

TABLE 5. *Stoichiometry of the production of 2-butanone from 2-butanol by cell-free extracts of strain ATCC 21439*[a]

Expt	2-Butanol consumed (nmol)	NAD consumed (nmol) XX	2-Butanone X produced (nmol)
1	260	270	250
2	530	540	520

[a] The reaction mixtures (1.0 mg of protein in 3 ml) were incubated at 30°C for 10 min (experiment 1) and for 20 min (experiment 2) in the presence of 1.0 μmol of NAD and 10 μmol of 2-butanol. X, Determined gas chromatographically; XX, determined fluorescence spectrophotometrically, and endogenous consumption of NAD was corrected.

DISCUSSION

We have confirmed the report of Lukins and Foster (13) that *n*-alkanes are cooxidized to their corresponding methyl ketones by methylotrophs in the presence of methane. We have also demonstrated that resting-cell suspensions of methane-grown cells oxidized *n*-alkanes to their methyl ketones in the absence of growth substrate. In addition, we demonstrated for the first time the conversion of secondary alcohols to their corresponding methyl ketones by resting-cell suspension of either alkane- or alcohol-grown cells. Succinate-grown cells do not have SADH activity, suggesting that either alkane or alcohol is required for inducing the enzyme. The reason why methylotrophs possess a secondary alcohol dehydrogenase is not well understood. However, possessing this enzyme is of great advantage to the organism as its growth yield, when growing on gaseous alkanes as the sole source of carbon and energy, could be exclusively NADPH dependent. Secondary alcohols are intermediates in the oxidation of *n*-alkanes by either *Pseudomonas* or *Mycobacterium* (13). In addition, at least one methane monooxygenase is a nonspecific system which oxidizes *n*-alkanes to both their primary and secondary alcohols (4). Therefore, it is not a complete surprise to see secondary alcohol dehydrogenase activity in the methylotrophic bacteria. One thing unclarified by these studies is the ability of the obligate methylotrophs to oxidize extensively substrates which they are incapable of utilizing for growth. The metabolism of the obligate methylotrophs is uniquely dependent on a one-carbon compound (formaldehyde) for the biosynthesis of certain essential cellular constitutents. This

compound can be obtained from methane and methanol, but is unobtainable from the non-growth-supporting compounds.

The taxonomic characteristics of the newly isolated C1-utilizing microbes will be published elsewhere. Cell suspensions of these new cultures as well as known C1 utilizers grown on either methane or methanol oxidized secondary alcohols to their corresponding methyl ketones. Five cultures selected from distinct genera were compared for their optimal conditions in the production of 2-butanone. These cultures were: *M. trichosporium* OB3b (a type II obligate methane utilizer); *M. organophilum* CRL 26 (a facultative methane utilizer); *H. polymorpha* ATCC 26012; and *Pseudomonas* sp. ATCC 21439 (an obligate methanol utilizer). The rate of 2-butanone production was linear for the first 4 h of incubation for all five cultures tested. The yeast culture had the highest production rate. The optimum temperature for the production of 2-butanone was 35°C for all the bacteria tested. The yeast culture had a higher temperature optimum (40°C; Fig. 4), and a reasonably high 2-butanone production rate was also observed at 45°C for this yeast. The production of 2-butanone was affected by substrate concentration and cell concentration. The inhibition by metal-chelating agents of the production of 2-butanone suggests the invovlement of metal(s). No product (2-butanone) inhibition was observed in any of the cell suspensions from all the five cultures tested.

We have found that cell-free soluble extracts from sonically disrupted cells also oxidize 2-butanol to 2-butanone. The cell-free system requires addition of a cofactor, specifically NAD, for its activity. One of the explanations for the rate decreases in 2-butanone production after 4 h of incubation (Fig. 2 and 7), therefore, may be the depletion of NAD in the resting cell suspensions.

NAD-dependent alcohol dehydrogenase and PMS-dependent methanol dehydrogenase are well-characterized enzymes. Both of these dehydrogenases have a broad specificity toward primary alcohols. Recently, Mehta (14) reported an NAD-linked alcohol dehydrogenase from a yeast grown on methanol. This primary alcohol dehydrogenase also oxidizes 2-propanol. In addition, the report stated that this alcohol dehydrogenase was very unstable and that it lost all of its enzyme activity within 24 h after fourfold purification. Results from our preliminary studies, however, indicate that our secondary alcohol dehydrogenase is a secondary alcohol-specific enzyme with its highest activity on 2-propanol and 2-butanol and has no activity towards primary alcohols. The purification and properties of this NAD-linked secondary alcohol-specific SADH from various C1-utilizing microbes will be described in another paper (9a).

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