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Oxidation of Secondary Alcohols to Methyl Ketones by Yeasts

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Cell suspensions of yeasts, *Candida utilis* ATCC 26387, *Hansenula polymorpha* ATCC 26012, *Pichia* sp. NRRL-Y-11328, *Torulopsis* sp. strain A_1, and *Kloeckera* sp. strain A_2, grown on various C-1 compounds (methanol, methylamine, methylformate), ethanol, and propylamine catalyzed the oxidation of secondary alcohols to the corresponding methyl ketones. Thus, isopropanol, 2-butanol, 2-pentanol, and 2-hexanol were converted to acetone, 2-butanone, 2-pentanone, and 2-hexanone, respectively. Cell-free extracts derived from methanol-grown yeasts catalyzed an oxidized nicotinamide adenine dinucleotide-dependent oxidation of secondary alcohols to the corresponding methyl ketones, Primary alcohols were not oxidized. The effect of various environmental factors on the production of methyl ketones from secondary alcohols by methanol-grown *Pichia* sp. was investigated.

Since Ogata et al. (10) first reported the assimilation of methanol by a yeast, many methanol-utilizing strains have been isolated from natural sources or found in stock culture collections (6, 9, 11-13). Interest in the cultivation of microorganisms on cheap and abundantly available compounds such as methanol has increased greatly as a result of the potential importance of microbial protein as a food or fodder material. Many engineering problems associated with the production of microbial protein from methanol-grown yeasts have been discussed (1, 4, 6, 9, 11-13).

Oxidation of methanol and other primary alcohols in yeasts has been shown to be catalyzed by an alcohol oxidase (3, 14, 15). Alcohol oxidase contained a flavine adenine dinucleotide as a prosthetic group (15). Secondary alcohols are not oxidized by this alcohol oxidase.

Recently, we discovered that cell suspensions of methanol-grown yeasts catalyzed the oxidation of various secondary alcohols. In this report, we describe the oxidation of 2-propanol, 2-butanol, 2-pentanol, and 2-hexanol to the corresponding methyl ketones acetone, 2-butanone, 2-pentanone, and 2-hexanone, respectively, by cell suspensions of yeasts grown on various C-1 compounds and alcohols. We have also investigated the effect of various environmental factors on the production of methyl ketones from the oxidation of secondary alcohols by cell suspensions of the yeast *Pichia* sp.

MATERIALS AND METHODS

Organisms. *Candida utilis* ATCC 26387 and *Hansenula polymorpha* ATCC 26012 were obtained from the American Type Culture Collection, Rockville, Md. *Pichia* sp. NRRL-Y-11328, *Kloeckera* sp. strain A_2, and *Torulopsis* sp. strain A_1 were isolated from soil by the enrichment culture technique with methanol as the sole source of carbon and energy. The organisms were maintained on mineral salts (2) agar plates containing yeast extracts (0.1%) and methanol (0.4%, vol/vol).

Preparation of cell suspensions. Yeasts were grown at 30°C in 2.8-liter flasks containing 700 ml of mineral salts medium (2) with yeast extracts (0.1%) and methanol (0.4%, vol/vol). Cells were harvested during exponential growth by centrifugation at 12,000 × *g* for 15 min. The cell pellet was washed twice with a 50 mM phosphate buffer, pH 7.0. The final pellet was resuspended in the same buffer. Cell suspensions of yeasts grown on ethanol, methylamine, and methylformate were prepared as described above with 0.4% (vol/vol) ethanol, 10 mM methylamine, and 10 mM methylformate as the sole source of carbon and energy.

Ketone production. A 1-ml portion of each washed cell suspension of yeasts grown on various carbon sources was put into 10-ml vials at 4°C. Ten microliters of secondary alcohol (isopropanol, 2-butanol, 2-pentanol, and 2-hexanol) was added, and the vials were sealed with a cap. The vials were then incubated at 30°C on a rotary water bath shaker at 200 rpm. Control vials containing cell suspensions of yeasts without substrate and boiled cell suspensions of yeasts with substrate were also included.

Assay of ketone product. The ketone product obtained from oxidation of secondary alcohols by cell suspensions of yeasts was estimated by flame ionization gas chromatography by using a stainless steel column (12 feet by ⅛ inch [ca. 366 by 0.32 cm]) packed with 10% Carbowax 20 M on 80/100 Chromasorb W column (Perkin Elmer Corp., Norwalk, Conn.). The column temperature was maintained isothermally at 130°C, and the carrier gas flow was 30 ml of helium per min. The various ketone products (acetone, 2-

butanone, 2-pentanone, and 2-hexanone) were identified by gas chromatography retention time comparisons and co-chromatography with an authentic standard. A 1-μl sample was removed with a syringe and injected at 10-min time intervals for up to 2 h. The amount of methyl ketones accumulated was determined from the peak area with a standard curve which has been constructed with authentic standards. The protein content of cell suspensions was determined by the method of Lowry et al. (7).

Preparation of crude extracts. Cell suspensions (2 g, wet weight) of packed cells in 10 ml of 50 mM sodium phosphate buffer, pH 7.0, were disrupted intermittently for 5 min with a Megason ultrasonic disintegrator at 4°C. The sonicated cell suspensions at 4°C were centrifuged for 15 min at 30,000 × *g*. The supernatant liquid was termed crude extracts.

Enzyme assay. Secondary alcohol dehydrogenase activity was measured spectrophotometrically at 340 nm with oxidized nicotinamide adenine dinucleotide (NAD^+) as an electron acceptor. The reaction mixture, in a total volume of 3.0 ml, contained 50 mM phosphate buffer, pH 8.0, 5 μmol of NAD^+, crude extracts, and substrate. The reactions were started by addition of 100 μl of 0.1 M substrate, and the rate of NAD^+ reduction was measured. Protein concentration was determined by the method of Lowry et al. (7).

RESULTS

Oxidation of secondary alcohols by cell suspensions of yeasts. Cell suspensions of *Candida utilis* ATCC 26387, *Hansenula polymorpha* ATCC 26012, *Pichia* sp. NRRL-Y-11328, *Torulopsis* sp. strain A_1, and *Kloeckera* sp. strain A_2 grown on methanol, ethanol, methylamine, and methylformate catalyzed the oxidation of various secondary alcohols. The product of oxidation of secondary alcohols was detected by gas chromatography retention time comparison and co-chromatography with an authentic standard. Thus, cell suspensions of yeasts catalyze the oxidation of isopropanol, 2-butanol, 2-pentanol, and 2-hexanol to the corresponding methyl ketones acetone, 2-butanone, 2-pentanone, and 2-hexanone, respectively (Table 1). The products of oxidation of secondary

TABLE 1. *Oxidation of secondary alcohols to ketones by cell suspensions of yeasts*[a]

Organism	Growth substrate	Conversion rate (μmoles/h per mg of protein)			
		Isopropanol to acetone	2-Butanol to 2-butanone	2-Pentanol to 2-pentanone	2-Hexanol to 2-hexanone
C. utilis ATCC 26387	Methanol	6.2	6.8	1.5	0.8
	Ethanol	5.2	5.2	1.0	0.72
	Methylamine	5.0	5.0	1.2	0.61
	Methylformate	5.6	6.2	1.3	0.75
	Propylamine	4.2	4.2	0.9	0.52
H. polymorpha ATCC 26012	Methanol	5.9	5.8	1.4	0.72
	Ethanol	5.0	4.8	1.1	0.54
	Methylamine	5.2	4.5	1.2	0.62
	Methylformate	5.6	5.2	1.3	0.70
	Propylamine	4.1	4.0	0.82	0.48
Pichia sp. NRRL-Y-11328	Methanol	5.2	6.8	1.2	0.50
	Ethanol	4.5	6.2	1.0	0.28
	Methylamine	4.2	5.1	0.72	0.31
	Methylformate	4.9	6.9	0.98	0.48
	Propylamine	3.2	2.1	0.60	0.21
Torulopsis sp. strain A_1	Methanol	4.5	4.9	1.0	0.21
	Ethanol	4.2	4.7	1.2	0.20
	Methylamine	4.3	4.5	0.9	0.12
	Methylformate	4.5	4.9	1.1	0.25
	Propylamine	3.2	3.8	0.62	0.10
Kloeckera sp. strain A_2	Methanol	4.8	5.9	1.2	0.25
	Ethanol	4.5	5.7	1.0	0.12
	Methylamine	4.0	5.4	1.0	0.10
	Methylformate	4.9	5.9	1.2	0.28
	Propylamine	4.0	4.2	0.92	0.11

[a] The products of oxidation were identified by gas chromatography retention time comparison and co-chromatography with an authentic standard. A 1-μl sample was injected at 10-min time intervals up to 2 h. Analysis also revealed that no further oxidation of products (methyl ketones) occurred.

alcohols were accumulated extracellularly, and no further oxidation of products (methylketones) was revealed by gas chromatographic analysis.

The influence of various environmental factors on the production of methyl ketones from oxidation of secondary alcohols by cell suspensions of *Pichia* sp. NRRL-Y-11328 was examined.

Time course of ketone production. The production of acetone and 2-butanone from the oxidation of isopropanol and 2-butanol, respectively, by cell suspensions of methanol-grown *Pichia* sp. was examined at 30°C as described in Materials and Methods. The rate of production of acetone and 2-butanone was linear with time during 60 min of incubation as shown in Fig. 1.

Effect of cell concentration on ketone production. The effect of cell concentration on the production of acetone and 2-butanone from oxidation of isopropanol and 2-butanol, respectively, by cell suspensions of methanol-grown *Pichia* sp. was examined. The rate of production of acetone and 2-butanone was directly dependent upon the cell concentration up to 1 to 8 mg of cell protein per ml. At cell concentrations above 8 mg/ml, a decrease in the rate of production of ketones was observed (Fig. 2).

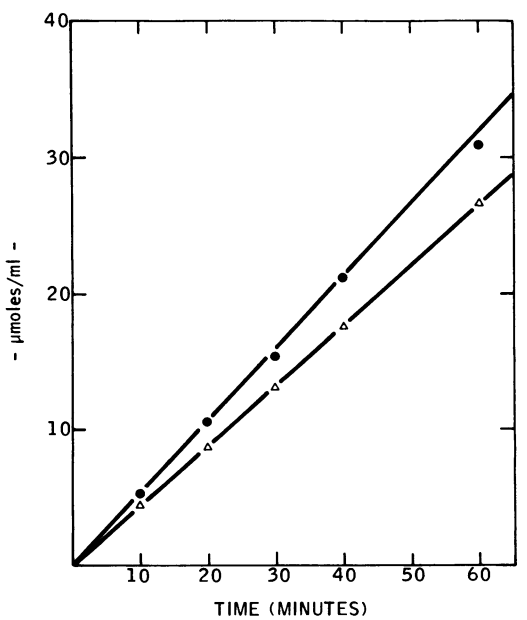

FIG. 1. *Time course of the production of acetone and 2-butanone from oxidation of isopropanol and 2-butanol, respectively, by cell suspensions (4.8 mg of protein per ml) of Pichia sp. The products were identified and estimated by gas chromatography as described in the text. Symbols: ●, 2-butanone; △, acetone.*

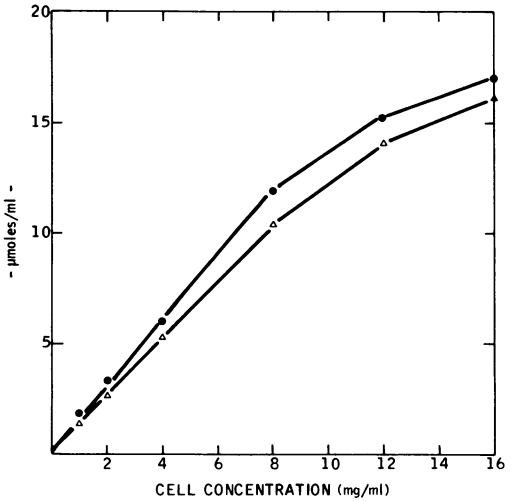

FIG. 2. *Effect of cell mass (protein) concentration on the production of acetone and 2-butanone from oxidation of isopropanol and 2-butanol, respectively, by cell-suspensions of Pichia sp. The products were identified and estimated by gas chromatography as described in the text after a 15-min incubation of the reaction mixture at 30°C. Symbols: ●, 2-butanone; △, acetone.*

Effect of temperature on ketones production. The optimum temperature for the production of acetone and 2-butanone from oxidation of isopropanol and 2-butanol by cell suspension of methanol-grown *Pichia* sp. was found to be 45°C (Fig. 3). Upon increasing temperature to 50°C, the rate of production of acetone and 2-butanone was significantly decreased.

Effect of pH on ketone production. The production of acetone and 2-butanone from the oxidation of isopropanol and 2-butanol, respectively, by cell suspensions of methanol-grown *Pichia* sp. was not significantly affected in the pH range from 5 to 9 (Fig. 4). The optimum pH for ketone production was 8.0.

Oxidation of secondary alcohols by cell-free extracts of yeasts. Cell-free extracts from *C. utilis* ATCC 26387, *H. polymorpha* ATCC 26012, and *Pichia* sp. NRRL-Y-11328, *Torulopsis* sp. strain A_1, and *Kloeckera* sp. strain A_2 grown on methanol catalyzed NAD^+-dependent oxidation of isopropanol, 2-butanol, 2-pentanol, and 2-hexanol. The products of oxidation of secondary alcohols were identified as the corresponding ketones by gas chromatography. The specific activity of NAD^+-dependent secondary alcohol dehydrogenase with 2-butanol as a substrate is shown in the Table 2. $NADP^+$, 2,6-dichlorophenol indophenol, phenazine methosulfate, or potassium ferricyanide could not act as electron acceptor. Primary alcohols (C-1 to

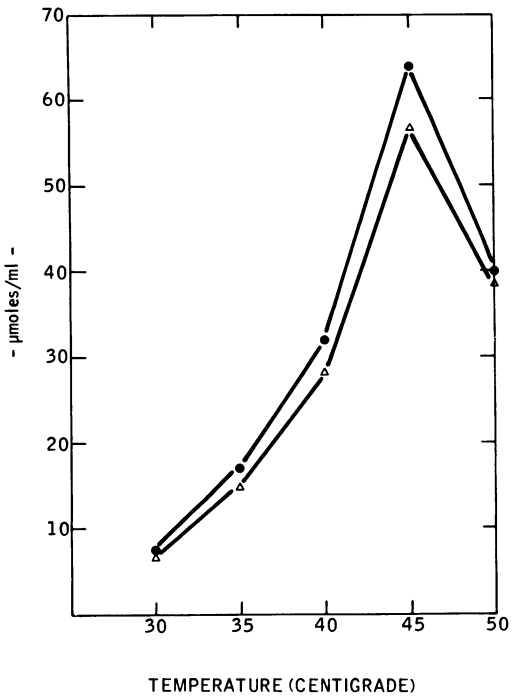

FIG. 3. *Effect of temperature on the production of acetone and 2-butanone from the oxidation of isopropanol and 2-butanol, respectively, by cell suspensions (4.4 mg of protein per ml) of Pichia sp. The products were identified and estimated by gas chromatography as described in the text after 15-min incubation of the reaction mixture. Symbols: ●, 2-butanone; △, acetone.*

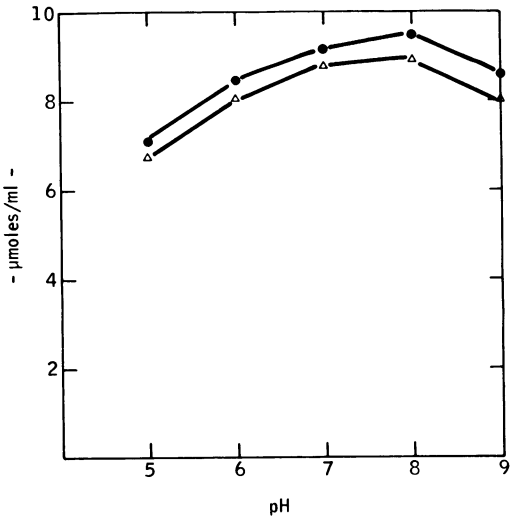

FIG. 4. *Effect of pH on the production of acetone and 2-butanone from oxidation of isopropanol and 2-butanol, respectively, by cell suspensions (5.2 mg of protein per ml) of Pichia sp. The products were estimated and identified by gas chromatography as described in the text after a 15-min incubation of the reaction mixture at 30°C. Symbols: ●, 2-butanone; △, acetone.*

TABLE 2. *Oxidation of secondary alcohols by cell-free extracts of yeasts*

Organism	Sp act[a]
C. utilis ATCC 26387	78
H. polymorpha ATCC 26012	85
Pichia sp. NRRL-Y-11328	100
Torulopsis sp. strain A_1	62
Kloeckera sp. strain A_2	90

[a] Secondary alcohol dehydrogenase activity was estimated spectrophotometrically as described in the text with secondary butanol as a substrate. Specific activity was expressed as nanomoles of NAD^+ reduced per minute per milligram of protein.

C-6 tested) were not oxidized by NAD^+-dependent secondary alcohol dehydrogenase.

DISCUSSION

Leadbetter and Foster (5) reported that cell suspensions of *Pseudomonas methanica* grown on methane do not oxidize other *n*-alkanes (e.g., ethane, propane, butane). However, by employing a co-oxidation technique with *P. methanica* growing at the expense of methane, they reported the production of a series of homologous oxidation products from cosubstrate gases. Thus, ethanol, acetaldehyde, and acetic acid were produced from ethane; *n*-propanol, propionic acid, and acetone were produced from *n*-propane. Subsequently, Lukins and Foster (8) reported the formation of methyl ketones from the oxidation of *n*-alkanes by cell suspensions of propane-grown *Mycobacterium smegmatis*.

Recently, Thompson et al. (16) reported the production of acetone during the oxidation of ethane by cell suspensions of the methane-utilizing bacterium, *Methylosinus trichosporium*. The acetone production occurred by a different mechanism (via 3-hydroxybutyrate and acetoacetate).

This is the first report on the oxidation of secondary alcohols to the corresponding methyl ketones by cell suspensions of yeasts grown on various C-1 compounds (methanol, methylamine), methylformate, ethanol, and propylamine. Cell extracts of the yeasts examined contained an NAD^+-dependent secondary alcohol dehydrogenase which catalyzed the oxidation of secondary alcohols to the corresponding methyl ketones.

Primary alcohols were not oxidized by secondary alcohol dehydrogenase. Oxidation of primary alcohols by yeasts has been shown to be catalyzed by a flavine-containing alcohol oxidase (15). Secondary alcohols were not oxidized by alcohol oxidase.

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