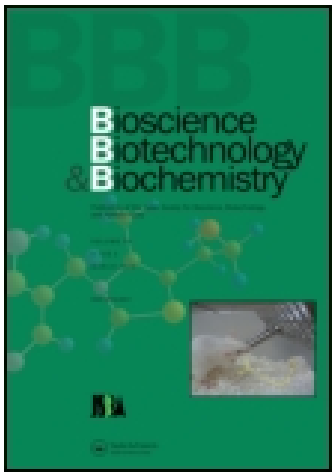

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Selective Oxidation of 2-Methylnaphthalene to 2-Methyl-1-naphthol by *Rhodococcus* sp. M192

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About 6000 isolates of microorganisms assimilating methylketones (C_3–C_6) were tested for their selective oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol. Strain M192 was the highest 2-methyl-1-naphthol producer and was classified as the genus *Rhodococcus*. The optimal conditions for the site-specific oxidation were studied using resting *Rhodococcus* sp. M192. The 2-methyl-1-naphthol productivity was specifically increased using methylethylketone as a carbon source, 1-propanol as a solvent to dissolve the substrate, and ethylxanthate or diethyldithiocarbamate as an inhibitor of 2-naphthoic acid (side-product) production. In the presence of these compounds, 2-methylnaphthalene was specifically oxidized at the 1-position without the conversion to 2-naphthoic acid. The productivity of 2-methyl-1-naphthol was about 90 μM from 1 mM 2-methylnaphthalene.

Key words: menadione; 2-methylnaphthalene; 2-methyl-1-naphthol; site-specific oxidation; monooxygenase

Since menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) is reportedly the most active synthetic homolog of vitamin K,[1] several methods of menadione synthesis have been developed.[2–4] However, a practical and proven basis for preparing large quantities of menadione remains elusive. Menadione synthesis by chromic acid oxidation of 2-methylnaphthalene has been the commercial method.[5] However, this method causes pollution problems through industrial waste containing chromate. Furthermore, in addition to menadione (in about 40% yield), 6-methyl-1,4-naphthoquinone is also produced as a side-product and this complicates the purification of menadione.

We studied the microbial conversion of 2-methylnaphthalene to menadione, and isolated bacteria oxidizing 2-methylnaphthalene to 2-methyl-1-naphthol using an intermediate of the butane oxidation pathway, such as 2-butanol or methylethylketone, as a carbon source for the screening medium.[6] The 2-methyl-1-naphthol can be easily oxidized to menadione by a chemical process, so that a site-specific oxidation at the 1-position of 2-methylnaphthalene is essential for the microbial conversion of 2-methylnaphthalene to menadione. However, a side product appeared in the oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol.

In this study, we identified the side product and isolated alkane (C_3–C_6)-assimilating microorganisms. Among them, microorganisms oxidizing 2-methylnaphthalene to 2-methyl-1-naphthol were selected and factors affecting the site-specific oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol were investigated.

Materials and Methods

Isolation of methylketone-assimilator. A soil sample (about 10 mg) was inoculated into 5 ml of medium I (pH 7.0), containing 0.5% (w/v) methylketone (methylethylketone, methylpropylketone, methylbutylketone, or acetone), 0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgCl_2$, and trace metals and vitamins as described previously,[6] and cultivated at 28 C for 1–3 days under reciprocal shaking in a test tube (18 × 180 mm). Cells in 50 μl of the culture were cultivated under the same conditions. The culture was spread on medium I agar plate (2%) and incubated at 28 C for 1–3 days. Methyleketone-assimilators were isolated from the plate.

The bacterium M192 was identified by The National Collections of Industrial and Marine Bacteria Limited, Scotland, UK.

Resting cell reaction. The same reaction mixture containing 1 mM 2-methylnaphthalene as described[6] was used for the oxidation of 2-methylnaphthalene by resting cells. In the first screening of bacteria oxidizing 2-methylnaphthalene to 2-methyl-1-naphthol, resting cells from 5 ml of medium I culture ($OD_{610\,nm}$, 1.0) were suspended in 1 ml of the reaction mixture in a test tube (18 × 180 mm) and shaken reciprocally (140 rpm) at 28 C. For the second screening or analysis of reaction conditions, cells were reciprocally (110 rpm) cultivated in 100 ml of medium II (pH 7.0), containing a 1.0% carbon source (methylethylketone was used in the second screening), 1.0% $NaNO_3$, 0.2% NH_4Cl, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.2% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, and the same metals and vitamins in medium I, in a 500-ml shaking flask at 28 C. The cultivated cells were suspended ($OD_{610\,nm}$, 10) in 100 ml of the reaction mixture in 500-ml shaking flasks and shaken reciprocally (110 rpm) at 28 C.

Extraction and analysis of products. After reaction, 1 ml of the reaction mixture was shaken with 1 ml of 1-butanol for 15 min and then centrifuged. The butanol phase was analyzed by HPLC under the same conditions as described.[6] To identify the compounds produced in the reaction mixture, a photodiode array spectrophotometer SPDM-6A (Shimadzu) was used instead of the UV-spectrophotometer SPD-10A in the HPLC system.

Chemicals. 2-Methylnaphthalene and menadione were purchased from Wako Chemical Co. 2-Methyl-1-naphthol was purchased from Aldrich Chemical Co., Ind. 2-Naphthoic acid was purchased from Nacalai tesque Co., Ltd. All other chemicals were commercial preparations.

Results

Isolation of methylketone-assimilators

Microorganisms (5992, mainly consisting of bacteria) were isolated from soil samples using each of the methylketones (C_3–C_6) as the sole carbon source in medium I.

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Among them, 93 methylethylketone-assimilating bacteria oxidized 2-methylnaphthalene (1 mM) to 2-methyl-1-naphthol (>5 μM) in the first screening. None of the microorganisms oxidized 2-methylnaphthalene to 2-methyl-1-naphthol without a side product (Fig. 1), which was identified as 2-naphthoic acid by HPLC analysis with several naphthalene derivatives. The retention time (1.5 min) and the UV spectrum of the side product {UV λ_{max} nm (ε); 235 (50,100), 280 (6,300), 335 (1,300)} were identical to those of 2-naphthoic acid.

The ratio of the amount of 2-methyl-1-naphthol to that of 2-methyl-1-naphthol and 2-naphthoic acid was measured as site specificity in the bacteria. Nineteen strains oxidized 2-methylnaphthalene with over 10% site specificity and produced more than 20 μM 2-methyl-1-naphthol (closed circles in Fig. 1). In the second screening from 19 strains, strain M192 produced 64 μM 2-methyl-1-naphthol (6.4% in yield), 6 μM menadione (0.6% in yield), and 15 μM 2-naphthoic acid (1.5% in yield) and its site specificity was the highest. Strain M192 was used in the following study.

Strain M192 was classified into the genus *Rhodococcus* in *Nocardiaceae*. The principal properties were as follows: Gram stain, positive; motility, negative; colony morphology (48 h), salmon pink/organge, opaque, round, regular, entire, shiny, smooth, convex, 0.5 mm diameter; growth, positive at 37°C and negative at 45°C; catalase reaction, positive; fermentation in glucose, negative.

Effects of the carbon source of culture medium on 2-methyl-1-naphthol production

The optimal conditions for 2-methyl-1-naphthol production by *Rhodococcus* sp. M192 were investigated. *Rhodococcus* sp. M192 did not grow on 1-butanol, lactose, glycerol, methanol, 1,4-butanediol, or methyl-*n*-butylketone. As shown in Table I, cells grown on the 14 compounds produced 2-methyl-1-naphthol and 2-naphthoic acid in the resting cell reaction. Methylethylketone and ethanol positively affected 2-methyl-1-naphthol productivity and cell growth. The productivity of 2-naphthoic acid with methylethylketone was lower than that with ethanol. Although 1-propanol also had good effects on the 2-methyl-1-naphthol production and the selective oxidation at 1-position, 1-propanol was not appropriate as the carbon source due to the low growth rate. Thus, methylethylketone was used as the carbon source for cultivation in the following study.

Table I. Effects of Carbon Source on Production of 2-Methyl-1-naphthol and 2-Naphthoic Acid

Carbon source (1%)	Cultivation time[a] (day)	2-Methyl-1-naphthol produced (μM)	2-Naphthoic acid produced (μM)
Ethanol	2	43	306
Methylethylketone	2	48	108
2-Butanol	8	29	493
2-Propanol	8	22	719
1-Propanol	6	38	98
Glucose	1	0	840
Fructose	6	4	824
Mannitol	1	0	792
Citrate	4	0	632
Succinate	6	0	841
Malate	4	0	801
Sorbitol	1	0	779
2,3-Butanediol	2	9	762
Methyl-*n*-propylketone	8	32	459

[a] Cells were harvested after the full growth on each carbon source. Strain M192 did not grow on 1-butanol, lactose, glycerol, methanol, 1,4-butanediol, and methyl-*n*-butylketone.

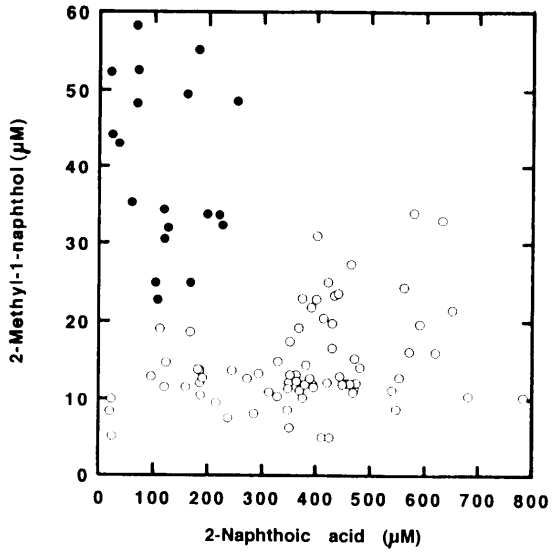

Fig. 1. 2-Methyl-1-naphthol and 2-Naphthoic Acid Productivities of Methylketone-assimilators.

Cultivation and the first screening proceeded as described under Materials and Methods. The productivities of 2-methyl-1-naphthol and 2-naphthoic acid of 93 strains, among 5,992 isolates, with 2-methyl-1-naphthol productivity of over 5 μM were plotted. Nineteen strains (closed circles) produced over 20 μM of 2-methyl-1-naphthol and over a 10% ratio of 2-methyl-1-naphthol to the total oxidation products. These strains were used for the second screening. The other strains (open circles) produced less than 20 μM of 2-methyl-1-naphthol and/or less than 10% of the ratio.

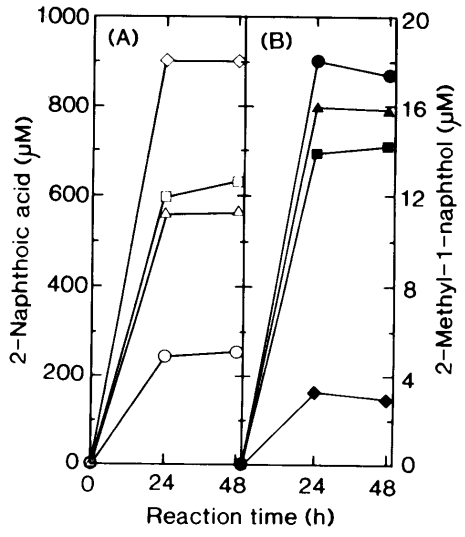

Fig. 2. Stimulation of 2-Methyl-1-naphthol Productivity of the Resting Cell of *Rhodococcus* sp. M192 by Methylethylketone.

Cells grown in medium II containing glucose as a carbon source were exposed to 1% methylethylketone at 28°C for 0 h (rhombuses), 1 h (triangles), 2 h (squares), or 4 h (circles). The cells were then applied to the resting cell reaction with 1 mM 2-methylnaphthalene dissolved in ethanol (final concentration of 1%) at 28°C. The amounts of 2-naphthoic acid (A) and 2-methyl-1-naphthol (B) were measured as described under Materials and Methods.

Stimulation of methylethylketone on the 2-methyl-1-naphthol productivity

The effect of methylethylketone on the oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol was tested. Cells grown on glucose oxidized 2-methylnaphthalene only to 2-naphthoic acid and did not produce 2-methyl-1-naphthol (Table I). The cells cultivated on glucose ($OD_{610\,nm}$, 1.0) were exposed to 1% methylethylketone in 25 mM potassium phosphate buffer, pH 7.0 at 28 °C and the production of 2-methyl-1-naphthol was tested in resting cells after washing. As shown in Fig. 2, the cells exposed for longer periods to methylethylketone produced more 2-methyl-1-naphthol and less 2-naphthoic acid.

Effects of solvents on the 2-methyl-1-naphthol productivity and the site specificity

Ethanol has been used to dissolve 2-methylnaphthalene in the reaction mixture.[6] To test the effects of other solvents on the oxidation, 2-methylnaphthalene was dissolved in various organic solvents and the ratio of the two products was measured (Table II). As a result, 1-propanol had the best effect on increasing 2-methyl-1-naphthol and decreasing 2-naphthoic acid production. Then, 1-propanol was used in the following study.

Screening of an inhibitor for the production of 2-naphthoic acid

The enzyme oxidizing 2-methylnaphthalene to 2-methyl-1-naphthol was considered to be different from that oxidizing 2-methylnaphthalene to 2-naphthoic acid, since the ratio of both products varied. By inhibiting the enzyme oxidizing 2-methylnaphthalene to 2-naphthoic acid, higher productivity of 2-methyl-1-naphthol against 2-naphthoic acid was predicted. A potent inhibitor for the oxidation to naphthoic acid was screened against 17 metal-binding compounds, 8 heme-binding compounds, 24 metal ions, and 6 uncouplers and inhibitors of electron transport. Table III shows a list of inhibitors that increased the ratio of 2-methyl-1-naphthol to total products. The oxidation at the methyl group was specifically inhibited by 1 mM diethyldithiocarbamate (metal binding compound) or 1 mM ethylxanthate (heme protein binding compound).

Table II. Effects of Solvent Dissolving 2-Methylnaphthalene on Production of 2-Methyl-1-naphthol and 2-Naphthoic Acid

Solvent[a]	2-Methyl-1-naphthol produced (μM)	2-Naphthoic acid produced (μM)
2-Propanol	1	720
2-Butanol	31	523
Acetone	16	243
Methylethylketone	52	555
Methyl-*n*-propylketone	14	164
Methyl-*n*-butylketone	10	1
2-Methyl-2-butanone	1	635
Ethanol	34	336
1-Propanol	61	89
1-Butanol	36	161

[a] 2-Methylnaphthalene was dissolved in each solvent and added into the reaction mixture. The solvent concentration in the reaction mixture was 1% (v/v).

Effects of aeration in the resting cell reaction on the 2-methyl-1-naphthol productivity

The effects of aeration on the 2-methyl-1-naphthol production were tested in 100 or 400 ml of the reaction mixture in a 500-ml shaking flask sealed with a vented cap under reciprocal shaking. The amounts of 2-methyl-1-naphthol produced in 100 ml and 400 ml of the reaction mixture were 71 μM and 88 μM, respectively. In the following study, 400 ml of the reaction mixture was used.

Effects of cell concentration on the 2-methyl-1-naphthol productivity

The effects of the cell concentration on 2-methyl-1-naphthol productivity were tested with or without the two inhibitors for 2-naphthoic acid production (Fig. 3). In the presence of the inhibitor (Fig. 3B, diethyldithiocarbamate or Fig. 3C, ethylxanthate), 2-naphthoic acid was undetectable at any cell concentration. The optimal cell concentration for 2-methyl-1-naphthol production under these conditions was 20 at $OD_{610\,nm}$. In the absence of inhibitor (Fig. 3A), 2-naphthoic acid was produced (about 20–40 μM) at all of cell concentrations examined. In the absence of inhibitor, the optimal cell concentration was 10 at $OD_{610\,nm}$. These results showed that the cell concentration was important for 2-methyl-1-naphthol production. The maximal 2-methyl-1-naphthol production, 90 μM, was obtained at 20 of cell concentration, when 1 mM ethylxanthate was the inhibitor in the reaction mixture.

Discussion

Ammonia monooxygenase in *Nitrosomonas europaea* oxidizes benzene to phenol, phenol to hydroquinone,[7] methane to methanol,[8] and ethylene to ethylene oxide.[9] At least two different enzymes or two catalytic sites are thought to be responsible for the oxidations.[7-9] In the oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol and 2-naphthoic acid, at least two enzymes of *Rhodococcus* sp. M192 are responsible, since the ratio of 2-methyl-1-naphthol to 2-naphthoic acid varied with the carbon source

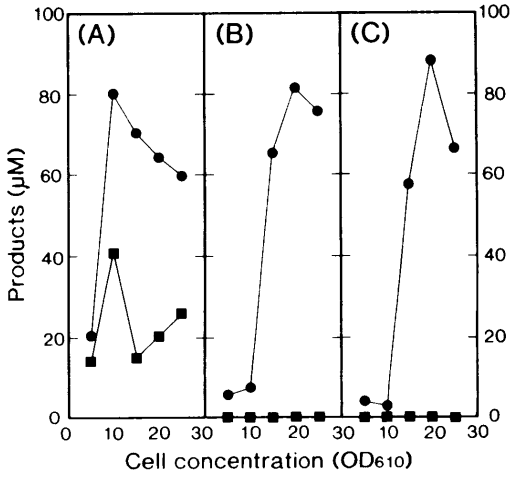

Fig. 3. Effects of the Cell Concentration on Production of 2-Methyl-1-naphthol and 2-Naphthoic Acid in the Presence or Absence of Inhibitor.

Cells were suspended to the indicated density in the reaction mixture. The resting cell reaction proceeded in 400 ml of reaction mixture in the presence of 1 mM diethyldithiocarbamate (B), 1 mM ethylxanthate (C), or the absence of inhibitor (A). Amounts of 2-methyl-1-naphthol (circles) and 2-naphthoic acid (squares) produced were measured as described under Materials and Methods.

Table III. Effects of Inhibitors on Production of 2-Methyl-1-naphthol and 2-Naphthoic Acid

Inhibitor (conc.)	2-Methyl-1-naphthol produced (μM)	2-Naphthoic acid produced (μM)	Site-specificity[a] (mol%)
None	34	182	16
Diethyldithiocarbamate (10^{-3} M)	65	5	93
Ethylxanthate (10^{-3} M)	59	16	79
Dicyclohexyl carbodiimide (10^{-4} M)	43	30	59
p-Chloromercuribenzoic acid (10^{-5} M)	38	47	45
Phenylmercuriacetate (10^{-5} M)	48	34	59

[a] The ratio of 2-methyl-1-naphthol to the total amount of 2-methyl-1-naphthol and 2-naphthoic acid produced (a site-specificity).

for cultivation. This interpretation was supported by the finding that only 2-naphthoic acid production was inhibited in the presence of an inhibitor, diethyldithiocarbamate or ethylxanthate (Table III).

The enzyme oxidizing 2-methylnaphthalene to 2-methyl-1-naphthol can be induced. The enzyme oxidizing 2-methylnaphthalene to 2-naphthoic acid is, however, constitutively produced, since the enzyme for 2-naphthoic acid is always produced (Table I). However, the level varied somewhat with the carbon sources.

The production of 2-methyl-1-naphthol was stimulated by methylethylketone (Fig. 2), which is an intermediate of the butane-assimilation pathway including diterminal oxidation by monooxygenase,[10-12] and methylethylketone induces the monooxygenase of a butane-assimilator.[13] The oxidation of 2-methylnaphthalene to 2-methyl-1-naphthol suggested the involvement of a butane monooxygenase or of a similar regulation to the monooxygenase of the butane-assimilator.

Ethanol as the solvent, gave a higher amount of the side product, 2-naphthoic acid. On the other hand, 1-propanol, in which the 2-methylnaphthalene was dissolved, increased the production of 2-methyl-1-naphthol and decreased that of 2-naphthoic acid. Monooxygenase is involved in the oxidation pathway of butane, and the oxidation requires a hydrogen donor, such as NADH, so the solvent might be a source from which to regenerate the NADH supplying hydrogen and/or an inducer of oxygenase. The higher site specificity with 1-propanol might be due to the decreased effect on 2-naphthoic acid productivity and to the ability to regenerate NADH supplying hydrogen.

Butane-assimilating bacteria oxidize phenol to hydroquinone.[13] Bacteria assimilating intermediates of the butane oxidation pathway, especially methylethylketone, could also oxidize 2-methylnaphthalene to 2-methyl-1-naphthol.[6] In this study, methylethylketone (C_4) and other methylketones in the oxidation pathways of other alkanes (C_3, C_5, and C_6) were used as the carbon source of screening medium. However, other methylketone-assimilators had less 2-methyl-1-naphthol productivity than the methylethylketone-assimilators. This indicated that an enzyme in the butane oxidation pathway specifically co-oxidizes 2-methylnaphthalene to 2-methyl-1-naphthol.

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