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Isolation and initial characterization of bacteria growing on tetralin

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Abstract

Eight strains of bacteria utilizing tetralin as sole source of carbon and energy have been obtained. Four strains have been selected from culture collections. The others were isolated from hydrocarbon-polluted areas. The newly isolated strains belong to the genera *Acinetobacter, Arthrobacter* and *Moraxella.* Most of the selected strains were able to grow on other aromatic hydrocarbons, but none of them grew on cyclohexane. Tetralin-utilizing organisms were difficult to isolate and cultivate, because tetralin was toxic to the cells at concentrations above 15 μl/l. Consequently tetralin was supplied either *via* the vapour phase or an organic solvent/water two-phase system was employed.

Abbreviations: FC 40 – fluorocompound 40, DBP – dibutylphthalate, DEP – diethylphthalate, DOP – dioctylphthalate

Introduction

The production of fine chemicals is an area with many opportunities for biotechnology. The initial step in the design of such a biotechnological process is the selection of a suitable biocatalyst for the production of the desired compound. Bioproduction of 5,6,7,8-tetrahydro-1-naphthol (Fig. 1) for the fragrance industry from 1,2,3,4-tetrahydronaphthalene (tetralin) may eventually lead to an interesting process. Detection and selection of a suitable biocatalyst for the tetrahydro-1-naphthol reaction seems most feasible by isolating tetralin-degrading microorganisms. The rationale for selecting tetralin-degrading organisms is based on several known biodegradative routes in the metabolism of aromatics involving the formation of *cis*-dihydro-diols through a dioxygenase type of reaction. *cis*-Dihydrodiols may chemically rearrange to hydroxy-aromatics.

Many microorganisms have been isolated that can grow on various aromatic and alicyclic compounds, and a great deal of knowledge exists on the microbial metabolism of such hydrocarbons (Dagley 1985; Trudgill 1984). Tetralin is a bicyclic molecule, that consists of an aromatic and an alicyclic moiety (Fig. 1). It may be attacked either at the aromatic or at the alicyclic ring. So far, organisms studied degrade or transform tetralin by an initial hydroxylation of the alicyclic ring (Jamison et al. 1971; Ganapathy et al. 1966; Holland et al. 1987).

Isolation of microorganisms that can utilize tetralin as sole source of carbon and energy apparently is a difficult task. Several pure cultures of hydrocarbon utilizers were unable to grow on tetralin (Ladd 1956; Tsuchii 1977). Tetralin utilization,

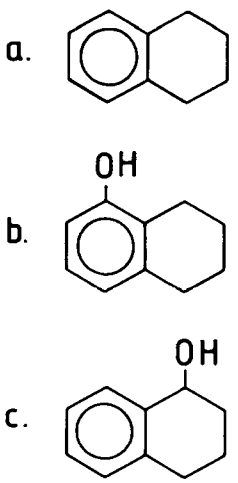

Fig. 1. Structural formulas of (a) 1,2,3,4-tetrahydronaphthalene (Tetralin), (b) 5,6,7,8-tetrahydro-1-naphthol, and (c) 1,2,3,4-tetrahydro-1-naphthol.

however, was observed with mixed cultures (Strawinski & Stone 1940), and with pure cultures when mixed substrates were supplied (Soli & Bens 1972, 1973; Kappeler & Wuhrmann 1978). Only in one instance has the isolation of a bacterium been reported that can grow, though poorly, on tetralin as sole source of carbon and energy (Schreiber & Winkler 1983). This bacterium, a *Pseudomonas* sp., starts with an initial hydroxylation of the alicyclic ring and accumulates 1,2,3,4-tetrahydro-1-naphthol and 1,2,3,4-tetrahydronaphthalone.

We now report the isolation of eight bacteria that can grow on tetralin and show that the toxicity of tetralin for these organisms explains the difficulties met by others in isolating tetralin-degrading organisms.

Materials and methods

Microorganisms

Microorganisms tested for tetralin utilization were from different collections. Bacteria from our culture collection were: *Xanthobacter* 124X (van den Tweel et al. 1986b), *Nocardia* S3 and *Rhodococcus* S5 (Hartmans et al. 1990) isolated on styrene; *Pseudomonas* strain 50 isolated on benzene (van den Tweel et al. 1988); *Mycobacterium* E3 isolated on ethene (Habets-Crützen et al. 1984); *Alcaligenes* OBB65 isolated on 1,3-dichlorobenzene (de Bont et al. 1986); *Pseudomonas putida* LW4 isolated on D-phenylglycine (van den Tweel et al. 1986a). In addition unidentified strains isolated from soil on benzene (EM1, EM3, EM4, EM6); on toluene (KZ4 and RA15); on ethylbenzene (EB1 and EB2); on naphthalene (N1 and N3); and on cyclohexane (C2) were also tested.

Microorganisms obtained from the culture collection of the Department of Microbiology of the Agricultural University were: *Arthrobacter* A177 and *Corynebacterium* C125 isolated on o-xylene (Schraa et al. 1987); *Pseudomonas* A2 isolated on mesitylene (1,3,5-trimethylbenzene); *Pseudomonas* P47 isolated on D-phenylglycine; *Pseudomonas* P53 isolated on o-cresol; *Pseudomonas putida* P60 isolated on phenol; the yeast *Trichosporon cutaneum* isolated on phenol; and an unidentified bacterium, strain 102, isolated on lindane.

A laboratory strain of *Aspergillus nidulans* able to grow on various substituted aromatic compounds was obtained from the collection of the Department of Genetics (Agricultural University Wageningen). *Nocardia corallina* (*Rhodococcus* sp.) V49 was obtained from the American Type Culture Collection, ATCC 19070 (Jamison et al. 1971). Organisms isolated on tetralin during the present investigation were *Arthrobacter* T2, *Acinetobacter* T5, *Arthrobacter* T6 and *Moraxella* T7.

Maintenance of microorganisms

Microorganisms were kept on slants of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. Tetralin-utilizing strains were also kept on slants of a mineral salts medium, containing in 1 l of demineralized water: K_2HPO_4, 1.55 g; $NaH_2PO_4 \cdot 2H_2O$, 0.85 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2 \cdot 6H_20$, 0.075 g and 0.2 ml of a trace elements solution (Vishniac and Santer 1957). Slants were placed in a 5–1 desiccator containing a flask with 50 μl tetralin.

Selection and isolation of tetralin-utilizing strains

Pure cultures were tested for tetralin utilization by supplying 10 ml mineral salts medium in 100-ml serum bottles with 5 µl tetralin. Alternatively, tetralin was supplied via the vapour phase (Coty 1967) using 300-ml Erlenmeyer flasks containing 50 ml mineral salts medium. A test tube was placed in the flask. After sterilization and inoculation (0.1 mg wet weight/ml), tetralin (10 µl) was pipetted into the tube and flasks were sealed with Teflon Mininert valves (Pierce Europe, Oud Beijerland, Holland). The tetralin concentration in the water under the applied conditions was 16 µl/l, as determined spectrophotometrically (Schreiber 1981) using standard but sterile incubations. In a third approach, tetralin was supplied by adding tetralin-containing fluorocompound 40 (FC 40) to the mineral salts medium, resulting in a solvent/water (10:90) two-phase system with approximately 15 µl/l tetralin in the water phase. The partitioning coefficient for tetralin over the FC 40/water system was 8. Enrichment of tetralin-degrading microorganisms was in 300-ml Erlenmeyer flasks that were fitted with Teflon Mininert valves. Inocula of 1 gram of soil were introduced in the flasks with 50 ml of mineral salts medium, and tetralin was added using the three techniques described above. Control flasks did not contain tetralin. The flasks were incubated at 30° C on a rotary incubator (200 rpm). Carbon dioxide production was measured daily and once growth was observed, 0.1 ml of a suitable dilution was plated onto agar plates with mineral salts medium. The agar plates were incubated in a 5-l desiccator containing a flask with 50 µl tetralin. Control plates were incubated in a 5-l desiccator in the absence of tetralin. Colonies that developed on tetralin plates were isolated and checked for purity by plating on yeast extract/glucose agar plates. These pure cultures were subsequently tested for tetralin utilization by plating on mineral salts medium and incubation in desiccators in the presence and absence of tetralin.

Identification of bacteria

Bacteria isolated during this investigation were characterized according to Bergey's Manual of Determinative Bacteriology (eighth edition). Additional information was obtained from identification kits, API 20 NE and API 20 B (Analytical Profile Index, Montalieu Vercieu, France).

Culture conditions

In growth experiments, volatile hydrocarbons were provided indirectly to the mineral salts medium *via* the vapour phase as described for tetralin. Growth was assessed by monitoring the culture fluid turbidity together with the production of carbon dioxide from the supplied substrates. Cultivation of cells to be used in incubation experiments was in 5-l Erlenmeyer flasks, containing 1 l mineral medium supplied with 10 g l^{-1} of sodium-acetate and 0.5 g l^{-1} yeast extract or with 50 µl/l hydrocarbon supplied in a separate reservoir.

Preparation of washed cell suspensions

The cells were harvested in the mid-exponential growth phase by centrifugation (16,000 × g for 10 min at 15° C), washed twice with potassium phosphate buffer pH 7.0 (50 mM) and resuspended in the same buffer (2 times the cell volume).

Whole cell incubations

Incubation experiments with washed cells were performed with suspensions of freshly harvested cells. Toxicity of tetralin was monitored in incubations in 1-l serum bottles. Cells (0.1 g wet weight) were resuspended in 100 ml phosphate buffer (50 mM; pH 7.0) with various amounts of tetralin in the absence or presence of an organic solvent. Carbon dioxide production was determined after 7 days of incubation.

Cells for incubations with organic solvents were resuspended in 9 ml mineral medium supplement-

ed with acetate (1.0% w/v), and yeast extract (0.1% w/v), and 1 ml organic solvent was added. Incubations were in 100-ml serum bottles for two weeks at 30° C in a rotary incubator (200 rpm). Carbon dioxide production was then determined by head space analysis.

Analytical techniques

Carbon dioxide concentrations were determined by injecting 0.1 ml head space samples on a Packard 427 gaschromatograph (Packard/Becker, Delft, The Netherlands) fitted with a Porapack Q column (Chrompack, Middelburg, The Netherlands).

Tetralin concentrations in both aqueous and organic phases were determined spectrophotometrically at 274 nm (Schreiber, 1981) using a UV-Vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Chemicals

1,2,3,4-tetrahydronaphthalene was purchased from Janssen Chimica (Beerse, Belgium). Fluorocompound 40 was obtained from 3M (St. Paul, MN, USA). All other chemicals were of commercially available analytical grade.

Results

Utilization of tetralin by microorganisms from culture collections

Twenty eight strains (see materials and methods) tested for tetralin utilization were selected on the basis of their ability to utilize hydrocarbons. Tetralin (500 μl/l) was added to the water phase, and turbidity of the medium, as well as production of carbon dioxide from tetralin, was monitored. None of the organisms utilized tetralin supplied in this manner. Subsequently the substrate was supplied indirectly to the water phase *via* the vapour phase and under these conditions four strains utilized tetralin (Table 1). In another attempt to lower the substrate concentration in the direct environment of the microorganism, a two-phase system with the inert fluorocompound 40 (FC 40) was used. In this system three organisms used tetralin as carbon and energy source (Table 1).

Isolation of microorganisms

Initial attempts to isolate tetralin-utilizing microorganisms were not successful. Various soil samples, sludge from industrial waste water treatment facilities, and mud from the river Rhine were used as inoculum, and tetralin was added to the enrichment medium both at 250 μl/l and 100 μl/l. Growth was not observed in incubations containing 250 μl/l but it did occur in several instances at 100 μl/l.

Table 1. Utilization of tetralin as sole source of carbon and energy by bacteria selected from culture collections.

Organism[a]		Isolation substrate	Growth[b]			Reference
			I	II	III	
Arthrobacter	A177	o-Xylene	−	+	+	Schraa et al. 1987
Corynebacterium	C125	o-Xylene	−	+	+	Schraa et al. 1987
Nocardia	S3	Styrene	−	+	+	Hartmans et al. 1990
Pseudomonas	A2	Mesitylene	−	+	−	

[a] Twenty four other organisms tested did not utilize tetralin as sole source of carbon and energy.

[b] Incubations with tetralin added in the water phase (I), via the vapour phase (II) or in a two-phase system with FC 40 (III). Growth is indicated by (+), while (−) means no growth as compared to a control incubation without substrate added.

Purification of these mixed cultures, however, was unsuccessful.

In a new attempt, cultures were set up with tetralin added *via* the vapour phase. Growth occurred in several incubations and two organisms were isolated by plating on solid mineral medium and by supplying tetralin *via* the vapour phase. From an enrichment set up with soil from a petrol station a Gram-positive, strictly aerobic non-motile coccoid organism was obtained (strain T2). During growth in complex media, the cells changed from predominantly rod-shaped to coccoid. Based on these characteristics and further tests on the utilization of various substrates for growth (Seiler 1983), this bacterium was classified as an *Arthrobacter* sp. From an enrichment set up with activated sludge from an industrial waste water treatment facility a strictly aerobic, non-motile Gram-negative organism was obtained (strain T5). Cells changed from rod-shaped during the logarithmic phase to coccoid in the stationary phase. The organism hydrolyzed gelatin and esculin; it was oxidase and urease negative. On the basis of these characteristics and on the results from the API 20 NE test the organism was identified as an *Acinetobacter* sp. (Baumann et al. 1968).

The isolation of microorganisms was also attempted using an organic solvent/water two-phase system with an inoculum taken from a Diesel spillage at a farmyard. Different tetralin concentrations up to 150 μl/l were supplied in the presence and absence of FC 40. After two weeks of incubation, growth was observed in enrichments without solvent at tetralin concentrations up to 7 μl/l. However, growth occurred at 70 μl/l tetralin in the presence of solvent (10% v/v). After purification, two bacteria were obtained which grew on tetralin. One bacterium, referred to as strain T6, was purified from an enrichment culture with 15 μl/l tetralin in the presence of 10% FC 40. This Gram-positive organism that changed from rod-shaped in fresh cultures to coccoid in older cultures was tentatively classified as an *Arthrobacter* sp. based on the results from an API 20 B test. From an enrichment culture with 35 μl/l tetralin in the presence of 10% FC 40, a Gram-negative, non-motile, coccoid organism was isolated. This organism was referred to as strain T7. The results from the API 20 NE test indicated that this organism belonged to the *Moraxella* group. On the basis of the positive oxidase reaction this bacterium was tentatively classified as a member of the genus *Moraxella* (Baumann et al. 1967).

Growth characteristics

The eight tetralin-utilizing organisms obtained were further characterized. Mean generation times for the selected bacteria for growth on tetralin ranged from approximately 20 hours to more than 70 hours (Table 2). The organisms were tested for their ability to grow on various aromatic hydrocarbons. Strain T5 did not grow on any other aromatic compound tested but all other bacteria were able to use various other aromatic compounds (Table 2). None of the organisms grew on cyclohexane.

Toxicity of tetralin in strain T2

Maximal tetralin concentrations allowing growth were determined and it appeared that for all strains the substrate was toxic at concentrations of 15 μl/l and higher. *Arthrobacter* T2 was studied in more detail, since it had a relatively short doubling time. It is clear that the tetralin concentration in the aqueous phase should be minimized to prevent damaging effects on the cells. One method to lower the substrate concentration in the aqueous phase is the addition of a water-immiscible organic solvent. In Fig. 2 the beneficial effect of organic solvents is shown for dibutylphthalate (partition coefficient for tetralin 60) and the inert solvent fluorocompound 40 (partition coefficient for tetralin 8).

General aspects of tetralin toxicity

It was also tested whether tetralin is toxic for bacteria that are not able to metabolize this compound. Following the procedure described by Inoue and Horikoshi (1989) for toluene resistant organisms, it was attempted to obtain tetralin resistant orga-

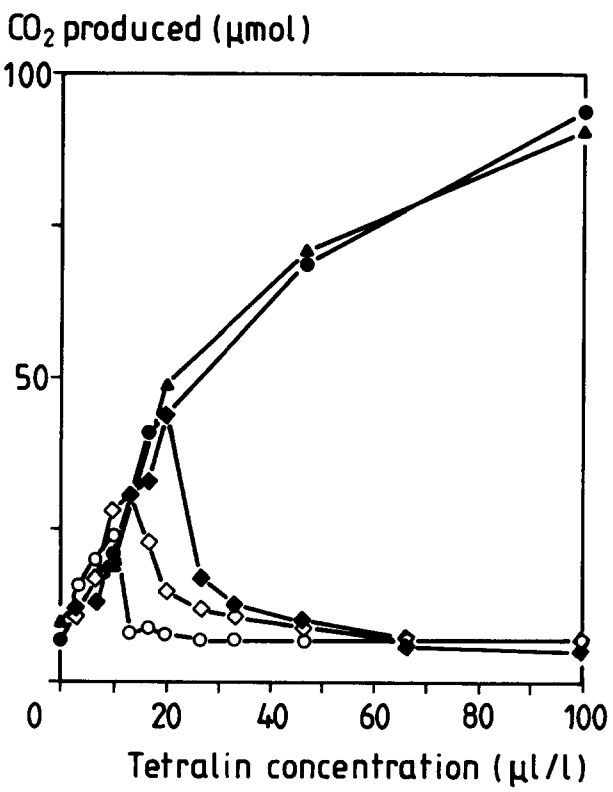

Fig. 2. Effects of organic solvents on carbon dioxide production from various tetralin concentrations by *Arthrobacter* T 2. CO_2 production was monitored after 7 days of incubation. Mineral salts medium in the absence of a solvent (○), and in the presence of 10% FC 40 (◇), 20% FC 40 (◆), 10% DBP (●), and 20% DBP (▲).

nisms growing in a yeast extract/glucose medium. Incubating soil samples in yeast extract/glucose medium in the presence of tetralin (1% and 10% v/v) did not result in any growth after a two week incubation period, and it was concluded that no such tetralin resistant organisms were present in these samples. Also, pure cultures that were tolerant of various organic solvents (Table 3) were tested for their resistance to tetralin. As shown in Table 3, none of the organisms tested was able to grow in the presence of 10% (v/v) tetralin, although they were resistant to various other organic solvents (10% v/v).

Discussion

Tetralin degradation by microorganisms has mainly been reported for mixed cultures or for pure cultures not growing on tetralin, but carrying out a co-oxidative reaction. Hydrocarbon utilization by mixed cultures is not uncommon as demonstrated for instance for cyclohexane utilization (Beam & Perry 1974). Cyclohexane, however, is also utilized by pure cultures (Stirling et al. 1977, Anderson et al. 1980, Trower et al. 1985). Degradation of hydrocarbons by pure cultures through co-oxidation (Perry 1979) has been reported for several compounds including cyclohexane, and methyl-substituted aromatic compounds. For tetralin, both mixed cultures growing on the compound as well as

Table 2. Growth of tetralin-utilizing bacteria on aromatic hydrocarbons.

Organism	Doubling time on tetralin	Benzene	1,2-Diethyl-benzene	Ethyl-benzene	Mesity-lene	Naph-thalene	Styrene	Toluene	o-Xylene
Arthrobacter A177	20	−	−	+	−	−	−	−	+
Corynebacterium C125	20	+	+	+	−	+	−	+	+
Nocardia S3	56	+	−	+	−	+	+	+	−
Pseudomonas A2	52	−	−	−	+	−	−	−	+
Acinetobacter T5	24	−	−	−	−	−	−	−	−
Arthrobacter T2	18	−	−	+	−	+	+	−	+
Arthrobacter T6	>72	+	−	+	−	+	+	−	−
Moraxella T7	23	+	−	−	−	−	+	+	−

The tested compounds were applied *via* the vapour phase. (+) indicates growth, (−) indicates no growth relative to a control incubation without substrate added.

co-oxidative systems have been described. Strawinski & Stone (1940) established a mixed culture of motile Gram-negative rods growing on, amongst others, tetralin. Cooxidative degradation of tetralin has been observed in complex substrate mixtures (Soli & Bens 1973, Kappeler & Wuhrmann 1978), and under defined conditions with one cosubstrate available (Jamison et al. 1971; Schreiber & Winkler 1983). Microorganisms isolated on hydrocarbons were not able to use tetralin as sole source of carbon and energy (Ladd 1956; Tsuchii et al. 1977). Only one report is available on tetralin utilization by a pure culture. Schreiber & Winkler (1983) isolated *Pseudomonas stutzeri* AS 39 which grew poorly on tetralin-vapour after precultivating it on naphthalene.

During this investigation, eight bacteria were obtained that grow on tetralin. Both Gram-negative and Gram-positive strains were encountered, indicating tetralin utilization is not restricted to specific bacteria. However, growth rates on tetralin were low in all cases (Table 2), also when compared with growth rates of these organisms on other hydrocarbons. The observed low growth rates may be one of the reasons for the problems encountered in isolating microorganisms on tetralin. But toxicity of tetralin is also very important in the isolation and cultivation of the bacteria. Our results show that it is quite well possible to establish mixed cultures growing on tetralin. Purification of these enrichment cultures caused problems since tetralin inhibition occurs at concentrations above 15 μl/l. Attempts to obtain pure cultures from the enrichments were only successful when tetralin was supplied in low concentrations. As shown in Fig. 3, the beneficial effect of a two-phase system is directly related to the partition coefficient of the substrate over the aqueous phase and the organic solvent applied as well as to the relative amount of solvent in the system.

In expressing toxicity of solvents, the logP-value is a very useful parameter (Laane et al. 1987, Rezessy-Szabo et al. 1987). This logP-value is defined as the logarithm of the partition coefficient of a certain solvent in a standard octanol/water two-phase system (Rekker 1977). Laane et al. (1987) and Rezessy-Szabo et al. (1987) both found that solvents with a logP-value higher than 4 were generally not harmful to growing bacterial cells, and solvents with a logP-value higher than 3 did not fully inhibit biological activity. Tetralin has a logP-value of 3.86 (Rekker 1977) and therefore was not

Table 3. Effect of various organic solvents (10% v/v) on the growth of pure cultures in yeast extract/glucose medium.

Organism	Solvent							
		FC 40	DOP	Hexadecane	DBP	Tetralin	Hexane	DEP
	logP	11.4	9.6	7.8	5.6	3.9	3.5	2.3
Arthrobacter A177		+	+	+	+	−	+	−
Corynebacterium C125		+	+	+	+	−	+	−
Nocardia S3		+	+	+	+	−	+	−
Pseudomonas A2		+	+	+	+	−	+	+
Acinetobacter T5		+	+	+	+	−	+	+
Arthrobacter T2		+	+	+	+	−	+	−
Arthrobacter T6		+	+	+	+	−	+	−
Moraxella T7		+	+	+	+	−	+	+
Mycobacterium E3		+	+	+	−	−	−	−
Nocardia corallina V49		+	+	+	+	−	+	−
Pseudomonas P58		+	+	+	+	−	+	+
Xanthobacter 124X		+	+	+	+	−	−	−

Serum bottles containing 8.5 ml of yeast extract/glucose medium and 1 ml of an organic solvent were inoculated with 0.5 ml of a pre-culture (1 mg wet weight/ml) of the microorganism to be tested. Growth was assessed by monitoring the culture fluid turbidity and by measuring the amount of carbon dioxide produced. Growth is indicated by (+), (−) means no growth as compared to a control without substrate added.

expected to be as toxic as observed. This toxicity is not directly coupled to metabolism of tetralin since bacteria not able to metabolize the compound were also affected by it. When tetralin was applied as a second phase (10% v/v) to cultures growing on glucose, bacteria were no longer metabolically active whereas other organic solvents, with a lower logP-value, did not affect these organisms. Similar observations have been made by Furuhashi et al. (1986) when applying tetralin in an organic solvent/water two-phase system for the biocatalytic production of epoxyoctane. Moreover, it was not possible to isolate bacteria with an increased tetralin resistance following the protocol described for toluene (logP-value 2.5) by Inoue & Horikoshi (1989). These observations indicate that tetralin is very toxic to microbial cells, and that the basis of this toxicity can not solely be explained on basis of the logP theory.

The catabolic pathways of the different organisms isolated will be studied subsequently to determine if one or more of the isolates initially attack tetralin at the aromatic ring. Two of the selected bacteria are known to attack substituted aromatics on the phenyl group and not on the more activated benzylic atom(s) of the substituent (Schraa et al. 1987, Hartmans et al. 1990). It therefore seems reasonable to expect that the aim, production of 5,6,7,8-tetrahydro-1-naphthol using one of these organisms eventually may be met.

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