STUDIES ON THE MICROBIAL DEGRADATION OF HOMOCHOLINE

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Ph.D. Dissertation

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2010)
DEDICATION

I would like to dedicate this humble thesis to:

My beloved wife Khalda and my sweet son Elmujtaba

My family: Ali, Zeinab, Buthina, Fatihia, Mohamed Ahmed, Hajer, Nawal, Eghbal, Entisar and Ekhlass

For their generous love and support
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-AB</td>
<td>β-Alanine betaine</td>
</tr>
<tr>
<td>Basal-HC</td>
<td>Basal-homocholine medium</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl trimethyl ammonium bromide</td>
</tr>
<tr>
<td>DCIP</td>
<td>2,6-dichlorophenol-indophenol</td>
</tr>
<tr>
<td>DMA-Propanol</td>
<td>3-N-dimethylamino-1-propanol</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenyldrazine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FAB-MS</td>
<td>Fast atom bombardment – mass spectrometry</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GMC</td>
<td>Glucose-methanol-choline oxidoreductases</td>
</tr>
<tr>
<td>MLA</td>
<td>Methyllycaconitine</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>nQACs</td>
<td>Naturally occurring quaternary ammonium compounds</td>
</tr>
<tr>
<td>NTB</td>
<td>Nitro tetrazolium blue</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>sQACs</td>
<td>Synthetic quaternary ammonium compounds</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylamine</td>
</tr>
<tr>
<td>TMABA</td>
<td>4-N-trimethylamino-1-butyraldehyde</td>
</tr>
<tr>
<td>TMA-Butanol</td>
<td>4-N-trimethylamino-1-butanol</td>
</tr>
<tr>
<td>TMAP</td>
<td>3-N-trimethylamino-1-propanol (homocholine)</td>
</tr>
<tr>
<td>TMAPaldehyde</td>
<td>Trimethylaminopropionaldehyde</td>
</tr>
<tr>
<td>QACs</td>
<td>Quaternary ammonium compounds</td>
</tr>
</tbody>
</table>
CHAPTER 1
GENERAL INTRODUCTION

1.1 INTRODUCTION
Quaternary ammonium compounds (QACs) are organic compounds that contain four functional groups attached covalently to a positively charged central nitrogen atom (R₄N⁺). These functional groups (R) include either long chain alkyl, benzyl or methyl groups. The term quaternary ammonium compounds refer to either chemically synthesized long-chain quaternary ammonium compounds (sQACs) or naturally occurring quaternary ammonium compounds (nQACs). Long-chain sQACs have been important since their bactericidal properties were recognized in the 1930s. Since then, they are extensively used in domestic and industrial applications as surfactants, emulsifiers, fabric softeners, disinfectants and corrosion inhibitors (Garcia et al., 2004; Patrauchan and Oriel, 2003). Such extensive use of sQACs in various branches of industry has resulted in increased presence of these compounds in wastewater, waterways, lakes, sludges and soils (Gerike et al., 1978). These compounds are toxic to aquatic plant and animal organisms. With their surface-active properties, they facilitate solubility of many dangerous micropollutants, such as pesticides, of which toxins can easily penetrate living organisms (Grabinska-Sota 2004). On the other hand, nQACs are widely distributed in the biosphere: there are more than 100 reported examples including well-known representatives such as choline, glycine betaine and β-alanine.
betaine (Anthoni et al., 1991) which have different biological functions, such as adaptation of organisms to environmental stress and transportation of chemical groups in many metabolic processes (Anthoni et al., 1991). Choline is an essential nutrient that is widely distributed in foods, principally in form of phosphatidylcholine. Choline or its metabolites are required for synthesis of phospholipids in cell membranes, methyl group metabolism and cholinergic neurotransmission and transmembrane signaling, as well as for lipid-cholesterol transport and metabolism (Fig. 1.1). This quaternary amine is present in tissues predominantly as one of the choline-phospholipids such as phosphatidylcholine and sphingomyelin (Zeisel et al., 1994). The osmoprotective role of glycine betaine, which derived from choline via an oxidation reaction, is evident in a number of diverse microbial systems, including enteric bacteria (Andresen et al., 1988), soil bacteria (Smith et al., 1988), halophilic bacteria (Galinski et al., 1982), cyanobacteria (Mackay et al., 1984) and methanogenic archaebacteria (Robertson et al., 1990). Besides being an osmoprotectant, glycine betaine also has a role in general metabolism where one methyl group of glycine betaine is incorporated into methionine in mammals (Finkelstein et al., 1972, Millian et al., 1998) and microorganisms (White et al., 1971), or into cobalamin (Vitamin B₁₂) in microorganisms (White et al., 1971). Recently, glycine betaine was proven to be an effective cryoprotectant for a wide range of prokaryotic organisms during freeze-drying, liquid-drying and liquid nitrogen freezing (Cleland et al., 2004). L-Carnitine a trimethylated amino acid that is similar in structure to choline is highly polar zwitterionic quaternary amine carboxylic acid and present in some prokaryotes and all eukaryotes (Ramsay et al., 2001). In prokaryotic cells, L-carnitine serves either as a nutrient, such as a carbon and nitrogen source (Kleber, 1997), or as an osmoprotectant (Jung et al., 1990; Robert et al., 2000). However, in eukaryotic cells, it serves exclusively as a carrier of acyl moieties through
various subcellular compartments (Ramsay et al., 1993). Moreover, it has an important role in the transport of activated long-chain fatty acids across the inner mitochondrial membrane (Bremer, 1983). 3-N-Trimethylamino-1-propanol (homocholine) is an analogue of choline, in which the amino alcohol group is lengthened by one CH₂-group, has been shown to resemble choline in many aspects of cholinergic metabolisms (Boksa and Collier, 1980). It has been reported that when homocholine transported into the rat brain synaptosome, it acetylated and released as acetylhomocholine from a superior cervical ganglion and minces of mouse forebrain by a calcium-dependent process during depolarization (Collier et al., 1977; Carroll and Aspry, 1980). Moreover, it was found to be effective in preventing fat infiltration both in fat and cholesterol fatty livers (Channon et al., 1937). From the choline-like structure, one would expect that homocholine is degraded similarly and at a rate comparable to that of choline. To date, no microorganism degrading homocholine as a sole source of carbon and nitrogen has been isolated and consequently, this catabolic pathway remains unclear.

![Diagram of Metabolic Fate of Choline](Degani et al., 2006)

**Fig. 1.1** Metabolic fate of choline (Degani et al., 2006)
1.2 SYNTHETIC LONG CHAIN QUATERNARY AMMONIUM COMPOUNDS (sQACs)

Synthetic quaternary ammonium compounds (sQACs) containing a long chain alkyl group or a benzyl group are cationic surfactants (Table 1.1) that play an important role in many industrial fields due to their versatile physico-chemical properties. Such widespread uses as disinfectants, fabric softening agents, foam depressants, and antistatic agents lead to massive discharge into the environment with its associated concerns (Wee and Kennedy, 1982).

Potential risks have been reported in many previous studies that repeated exposure to long-chain sQACs can induce microbial resistance against antibiotics in many pathogenic microorganisms (McDonnell and Russell, 1999). In addition, discharge of long-chain sQACs can disturb the purifying activities of natural aquatic systems or public wastewater treatment plants because of their toxicity to microbial life (Laopaiboon et al., 2002; Tubbing and Admiraal, 1991). The possibility to eliminate sQACs-related pollution effectively has been investigated in many ways. As one of the most economical means, biological treatment found to be an effective way to remove sQACs. In particular, aerobic treatment processes can provide rapid biodegradation via a consortium of bacteria (Scott and Jones, 2000).
Table 1.1 Structural features of synthetic sQACs and their industrial usage

<table>
<thead>
<tr>
<th>QAC Group</th>
<th>Molecular Structure</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkyl trimethyl ammonium</td>
<td>( R-\overset{N^+}{\text{CH}_3} )</td>
<td>Organoclays, phase transfer catalyst, oilfield applications</td>
</tr>
<tr>
<td>Di‐alkyl dimethyl ammonium</td>
<td>( R-\overset{N^+}{\text{CH}_3} )</td>
<td>Biocides, wood preservatives, oilfield applications</td>
</tr>
<tr>
<td>Alkyl benzyl dimethyl ammonium</td>
<td>( R-\overset{N^+}{\text{CH}_3} )</td>
<td>Biocides, cosmetics, wood preservation, phase-transfer catalyst, organoclayes</td>
</tr>
<tr>
<td>Alkyl pyridinium</td>
<td>( \text{Ph} )</td>
<td>Phase transfer catalyst, pesticides</td>
</tr>
<tr>
<td>Diethyl ether dimethyl ammonium (Esterquat)</td>
<td>( \text{CH}_3\text{CH}_2\text{OOCR} )</td>
<td>Active ingredient in fabric softeners</td>
</tr>
</tbody>
</table>

1.3 NATURALLY OCCURRING QUATERNARY AMMONIUM COMPOUNDS (nQACs)

Naturally occurring quaternary ammonium compounds constitute a class of metabolites with more than 100 reported examples, such as choline, glycine betaine, \( \beta \)-alanine betaine, \( \gamma \)-butyrobetaine and L-carnitine (Anthoni et al., 1991). They form a structurally heterogeneous class of compounds with a unifying character of a polar and fully methyl substituted nitrogen atom, creating a permanent positive charge on the N moiety.
(Rhodes and Hanson, 1993). Their occurrence apparently reflects several independent evolutionary patterns ranging from highly specialized functions for each nQAC to a general strategy for adaptation of the organisms to fluctuating or stressing environmental conditions (Anthoni et al., 1991). Moreover, it has been suggested that nQACs may be involved in transporting various chemical moieties within plants, acts as macromolecular components, and have role in overcoming water and salt stresses (Blunden and Gordon, 1986). In addition, the presence or absence of these compounds could be used as taxonomic indicator as well as they could used in the pharmacology to prevent artherosclerosis (Hoppe, 1979), to reduce plasma cholesterol levels (Kaneda and Abe, 1984), smooth muscle relaxant (Baker and Murphy, 1976) and to inhibit mammalian neuromuscular transmission (Hosein and McLennan, 1959).

1.3.1 Choline

Choline is an essential nutrient that is widely distributed in foods, principally in the form of phosphatidylcholine. Organ meats, eggs, soybeans, nuts, fish and broccoli are particularly good sources (Howe et al., 2004). The only source of choline other than diet is de novo biosynthesis of phosphatidylcholine from phosphatidyl-ethanolamine (Zeisel, 1990). Choline is required to make the phospholipids such as phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen and sphingomyelin-essential components of all cell membranes. It is a precursor for the biosynthesis of the neurotransmitter acetylcholine and in biological methylation reactions. Choline phospholipids in cell membranes play a role in generating various second messengers during signal transduction (Zeisel, 1990; Zeisel and Blusztajn, 1994). Choline can be acetylated, phosphorylated and oxidized (Fig. 1.2). Only a small fraction of dietary choline is acetylated by choline acetyltransferase (EC.2.3.1.6) (Zeisel, 1990; Zeisel and
This enzyme is highly concentrated in the terminals of cholinergic neurons, but also present in non-nervous tissue such as the placenta. Phosphorylation of choline is the first step in the major pathway for phosphotidylcholine synthesis, catalyzed by choline kinase (EC. 2.7.1.32), using Mg$^{2+}$ and ATP. This enzyme is widely distributed in mammalian tissues, including liver, brain, kidney and lung.

Choline deficiency results in liver dysfunction, fatty liver, decreased in growth, infertility, Alzheimer’s disease, abnormal kidney function, decreased red blood cell synthesis, high blood pressure and liver cancer (Zeisel, 1990; Zeisel and Canty, 1993; Zeisel and Blusztajn, 1994).

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**1.3.2 Homocholine**

Homocholine (3-N-trimethylamino-1-propanol) an analogue of choline, in which the amino alcohol group is lengthened by one CH$_2$-group, has been shown to resemble choline structurally and in many aspects of cholinergic metabolisms (Boksa and Collier,
1980). After it is transported into the rat brain synaptosome, it is acetylated and released as acetylhomocholine from a superior cervical ganglion and minces of mouse forebrain by a calcium-dependent process during depolarization (Collier et al., 1977; Carroll and Aspy, 1980). Homocholine, however, differs dramatically from choline in a very important aspect. That is not acetylated by solubilized cholineacetyltransferase, but is acetylated by intact rat brain synaptosomes (Collier et al., 1977), possibly because homocholine is acetylated by cholineacetyltransferase that is associated with vesicular and/or neuronal membranes (Carroll and Aspy, 1980). Nelson et al. (1980) assumed that the extracellular precursors of choline and homocholine may be directly accumulated by a crude vesicular fraction of mouse forebrain, independently of the cytoplasm, and may be utilized to replace the loss in acetylcholine. Additionally, they suggested that the extracellular products, acetylcholine and acetylhomocholine, may not be capable of replacing acetylcholine lost from a crude vesicular fraction. Moreover, acetylhomocholine is released from brain slices both spontaneously and in response to stimulation via mechanism similar to those that released acetylcholine, but some differences in specificity of the acetylcholine storage and/or release processes might be present (Nelson et al., 1980). Previous studies on acetylhomocholine synthesis and release have partly classified these false transmitter criteria by showing that homocholine is acetylated in tissues containing cholinergic nerve terminals (Collier et al., 1977), that acetylhomocholine is released by a calcium-dependent mechanisms during nerve stimulation (Collier et al., 1977), and that acetylhomocholine has actions qualitatively similar to but quantitatively different from those of acetylcholine on both muscarinic and nicotinic receptors (Hunt and Renshaw, 1934; Curtis and Ryall, 1966; Barrass et al., 1970). In pharmacology, choline analogues such as homocholine, methylcholine and ethylcholine are used to study the chemical specificity of the various
processes involved in neurotransmission and in particular it has been used to investigate the subcellular origin of the transmitter released by stimulation.

1.3.3 Glycine Betaine

Glycine betaine is found in microorganisms, plants and animals, and is a significant component of many foods including wheat, shellfish, spinach and sugar beets (Craig, 2004). Glycine betaine is compatible osmolyte that increases the water retention of cells, replaces inorganic salts and protect intracellular enzymes against osmotically induced or temperature induced inactivation. In human, glycine betaine is catabolized via a series of enzyme reactions that occur mainly in the mitochondria of liver and kidney cells. Glycine betaine also acts as a catabolic source of methyl groups via transmethylation for use in many biochemical pathways. The formation of methionine from homocysteine can occur either via glycine betaine or 5-methyltetrahydrofolate. The conversion of homocysteine to methionine is important to conserve methionine, detoxify homocysteine and produce S-adenosylmethionine (Millian et al., 1998). Glycine betaine is synthesized by a two-step oxidation of choline or by methylation reaction from glycine. Two N-methyltransferases genes from halotolerant cyanobacterium *Aphanothece halophytica* have been isolated (Waditee et al., 2003).

1.3.4 β-Alanine Betaine

Many plants, bacteria, and marine algae accumulate quaternary ammonium compounds (QACs) in response to abiotic stresses such as drought and salinity (Rhodes and Hanson, 1993; Gorham, 1995). Zwitterionic compounds such as glycine betaine, β-alanine betaine and proline betaine, are known to be the most effective osmoprotectants.
In most members of the highly stress-tolerant plant family *Plumbaginaceae*, β-alanine betaine was reported to replace glycine betaine (Hanson *et al.*, 1991, 1994). β-alanine betaine synthesis is not constrained by choline availability, because it is derived by the N-methylation of the non-protein amino acid, β-alanine (Fig. 1.3). Unlike glycine betaine synthesis, β-alanine betaine synthesis does not require oxygen, and hence it was suggested to be suitable for osmoprotection under saline and hypoxic conditions (Hanson *et al.*, 1994; Rathinasabapathi, 2000). Accordingly, β-alanine betaine was distributed among species of the *Plumbaginaceae*, adapted to a wide range of adverse stress environments including saline and hypoxic conditions (Hanson *et al.*, 1994). Although β-alanine betaine accumulation has been intensely studied for its role in osmoprotection (Hanson *et al.*, 1994; Rathinasabapathi *et al.*, 2000, 2001), early work on this compound in marine algae also suggested that it had a cholesterol-reducing effect in animal feeding experiments (Abe and Kaneda, 1973).

![The synthetic pathway to β-alanine betaine in plant (Duhaze *et al.*, 2003)](image-url)
1.3.5 L-Carnitine

A trimethylated amino acid roughly similar in structure to choline, L-carnitine is a highly polar zwitterionic quaternary amine carboxylic acid present in some prokaryotes and all eukaryotes (Ramsay et al., 2001). In prokaryotic cells, L-carnitine serves either as a nutrient, such as a carbon and nitrogen source (Kleber, 1997), or as an osmoprotectant (Jung et al., 1990; Robert et al., 2000). However, in eukaryotic cells, it serves exclusively as a carrier of acyl moieties through various subcellular compartments (Ramsay et al., 1993). It has an important role in the transport of activated long-chain fatty acids across the inner mitochondrial membrane (Bremer, 1983). Cytosolic long-chain fatty acids, which are present as CoA esters, are transesterified to L-carnitine at the mitochondrial outer membrane (Fig. 1.4).

![Diagram of L-carnitine's function in fatty acid metabolism](image-url)

**Fig. 1.4** Function of L-carnitine in fatty acid metabolism (Nelson and Cox, 2004)
In this reaction, the acyl moiety of the long chain fatty acids is transferred from CoA to the hydroxyl group of carnitine. The resulting long chain acylcarnitine esters are transported over the inner mitochondrial membrane via a specific carrier, carnitine-acylcarnitine translocase. In the mitochondrial matrix, the enzyme carnitine acetyltransferase is able to reconvert short and medium chain acyl-CoAs into acylcarnitines using intramitochondrial carnitine. Through this mechanism of reversible acylation, carnitine is able to modulate the intracellular concentrations of free CoA and acyl-CoA. Furthermore, it is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO₂ and H₂O in the Krebs cycle (Jakobs and Wanders, 1995).

In humans, approximately 75% of body L-carnitine sources comes from the diet and 25% from de novo biosynthesis (Tein et al., 1996). In general, L-carnitine is low in foods of plant origin and high in animal foods (Rebouche, 1992). L-Carnitine ultimately is synthesized from lysine and methionine. L-Lysine provides the carbon chain and nitrogen atom of carnitine, and L-methionine provides the methyl group. In the pathway of L-carnitine in mammals, ε-N-trimethyl-L-lysine is synthesized only as a post-translational modification of protein synthesis and is released for L-carnitine biosynthesis by normal processes of protein turnover. A number of proteins contain one or more ε-N-trimethyl-L-lysine residues, including histones, actin, myosin, and calmodulin. The majority of ε-N-trimethyl-L-lysine synthesized in the body probably is formed in skeletal muscle (Rebouche, 1992).
1.4 MICROBIAL DEGRADATION OF QUATERNARY AMMONIUM COMPOUNDS

1.4.1 Biodegradation of Long Chain QAC by Microorganisms

The susceptibility of cationic surfactants to biodegradation was first reported in the 1950s. Gerike (1982) confirmed the study done before using activated sludge and cetyl trimethyl ammonium bromide (CTAB), dodecyl benzyl dimethyl ammonium chloride and didecyl dimethyl ammonium chloride.

Studies on the biodegradation pathways have been conducted for various sQACs. The utilization of tetramethyl ammonium chloride by *Pseudomonas* sp. was described by Hampton and Zatman (1973). According to their work, the oxidation of this compound is initiated by splitting the C-N bond, therefore producing methanal and trimethylamine. The intermediate trimethylamine is oxidized to trimethylamine N-oxide (Large *et al.*, 1972) or transformed to methanal and dimethylamine (Colby and Zatman, 1973; Meiberg and Harder, 1978). The trimethylamine N-oxide is further converted to dimethylamine (Large, 1971; Myers and Zatman, 1971), followed by the generation of methanal and methylamine (Colby and Zatman, 1973). Methylamine is then converted to methanal and ammonium. Based on an investigation of the utilization of alkyl trimethyl ammonium bromides by microorganisms derived from sewage and soil, Dean-Raymond and Alexander (1977) demonstrated that the first step of degradation occurs with hydroxylation of the terminal carbon of the alkyl group and the resulting carboxylic acid undergoes β-oxidation. Another study dealing with hexadecyl trimethyl ammonium chloride suggested a central fission of $C_{alkyl}$-N bond as the first step of degradation (Van Ginkel *et al.*, 1992). Not only hexadecyl trimethyl ammonium chloride, but also
hexadecanal and hexadecanoate produced as metabolites, were all utilized by *Pseudomonas* species. It is believed that the central $C_{\text{alkyl}}$-N bond fission is first carried out by an oxygen dependent dehydrogenase as suggested for alkyl trimethyl ammonium compounds (Van Ginkel et al., 1992) and then intermediates are formed consecutively by $N$-demethylation reactions.

In contrast to the previous findings, a recent study proposed the degradation of dodecyl trimethyl ammonium chloride by *Pseudomonas* sp. strain 7-6, isolated from a wastewater treatment plant, via dual pathways, which besides an initial attack on the $C_{\text{alkyl}}$-N bond also initiates degradation via cleavage of a $C_{\text{methyl}}$-N bond, hence producing dodecyl dimethylamine as an intermediate product (Takenaka et al., 2007).

Overall, general degradation pathways of cationic surfactants shown in Fig. 1.5 indicated that biodegradation is mostly commenced with cleavage of the $C_{\text{alkyl}}$-N bond irrespective of the type of sQACs and the degradation of the produced alkanals proceeds via $\beta$-oxidation for complete mineralization (Van Ginkel, 2004).

On the other hand, because of the surfactants character and the limited enzymatic ability of individual microorganisms, only a few known surfactants, alkane sulphonates, alkyl sulphates, and alkyl amines, are completely degraded by a single microorganism (Van Ginkel, 1996). For this reason, consortia of microorganisms are highly efficient, as well as necessary for the complete degradation of surfactants (Scott and Jones, 2000; Van Ginkel, 1996). The lack of the isolation of microbes degrading quaternary ammonium surfactant to completion may be due to the fact that in all those studies nitrogen was provided in form of ammonium in addition to the nitrogen-containing
surfactants. In this way, no selective pressure to use nitrogen deriving from the surfactant itself was imposed during enrichment and isolation.

Fig. 5 General degradation pathway of cationic surfactants.

The alkanals formed through the oxidation of the α-carbon of the alkyl chain are further metabolized via β-oxidation (Van Ginkel, 2004).
1.4.2 Biodegradation of Naturally Occurring QAC by Microorganisms

1.4.2.1 Bacterial metabolism of choline

Choline is found ubiquitously in nature and the ability to degrade choline is widespread amongst microorganisms. The aerobic degradation of choline was firstly reported in *Achromobacter cholinophagum* that metabolized choline via betaine as first oxidation products (Shieh 1964). Subsequently, Kortstee (1970) showed that many microorganisms of the genera *Agrobacterium*, *Arthrobacter*, *Micrococcus*, *Pseudomonas*, *Rhizobium* and *Streptomyces* were found to grow with choline as a sole source of carbon and nitrogen. He also found that all the tested choline-utilizing bacteria were able to grow with betaine, *N*, *N*-dimethylglycine or sarcosine as a sole carbon and nitrogen source.

In microorganisms, the degradation of choline proceeds by stepwise oxidation of the ethanol-group yielding betaine aldehyde and glycine betaine (Fig. 1.6). Thereafter, glycine betaine is degraded by subsequent splitting off the methyl-groups, leading to the metabolites dimethylglycine, sarcosine and finally glycine (Shieh, 1964; Kortstee, 1970; Mori, 1981). In plants such as spinach (Rathinasabapathi *et al*., 1997), sugar beet and amaranth (Russell *et al*., 1998), the first step reaction is catalyzed by choline monooxygenase (EC.1.14.15.7), a ferredoxin-dependent soluble Rieske-type protein, whereas in animals and microorganisms, it is catalyzed by either membrane-bound choline dehydrogenase (EC.1.1.99.1) (Nagasawa *et al*., 1976; Landfald and Strom, 1986; Russell and scope, 1994; Tsuge *et al*., 1980) or soluble flavoprotein, choline oxidase (EC.1.1.3.17) (Ikuta *et al*., 1977; Yamada *et al*., 1977; Rozwadowski *et al*., 1991). Choline oxidase catalyzes the four-electron oxidation of choline to glycine betaine via betaine aldehyde as intermediate (Ikuta *et al*., 1977). Based on amino acid sequence
comparisons, the enzyme can be grouped in the glucose-methanol-choline flavin-dependent (GMC) oxidoreductase enzyme superfamily, which comprises enzymes like glucose oxidase, cholesterol oxidase, or cellobiose dehydrogenase that utilizes FAD as a cofactor for catalysis and non-activated alcohols as a substrate. The highly GC rich codA gene encoding for choline oxidase was cloned from genomic DNA of *Arthrobacter globiformis* and expressed to high yield in *Escherichia coli* (Fan et al., 2004). The resulting recombinant enzyme was highly purified and showed to be a dimer of identical subunits containing covalently bound FAD.

In the second step, the oxidation of betaine aldehyde is catalyzed by a soluble, NAD(P)⁺-dependent betaine aldehyde dehydrogenase (EC.1.2.1.8) that has been purified to homogeneity from *Pseudomonas aeruginosa* (Nagasawa et al., 1976), *E. coli* (Falkenberg et al., 1990), *Xanthomonas translucens* (Mori et al., 1992), *Arthrobacter globiformis* (Mori et al., 2001) and *Cylindrocarpon didymum* (Mori et al., 1980).

Betaine-homocysteine methyltransferase is an enzyme catalyzing methyl transfer reaction from glycine betaine to homocysteine, forming dimethylglycine and methionine, respectively. Betaine-homocysteine methyltransferase (EC.1.2.1.8) has been purified to homogeneity from rat (Lee et al., 1992), pig (Garrow, 1996), human (Skiba et al., 1982) and cyanobacteria (Waditee and Incharoensakdi, 2001). It is a zinc metalloenzyme consisting of a hexamer of 45 kDa subunits and is active in the livers and kidneys of humans and pigs, but only in the livers of rats (Mckeever et al., 1991; Millian and Garrow, 1998). Following the betaine-homocysteine methyltransferase reaction, the last two reactions of choline oxidation convert dimethylglycine to glycine via sarcosine as an intermediate. The oxidative demethylation of dimethylglycine to
form sarcosine is catalyzed by dimethylglycine oxidase (Mori et al., 1980) or membrane bound dimethylglycine dehydrogenase (Porter et al., 1985). Subsequently, the formation of glycine from sarcosine is catalyzed by the mitochondrial enzyme sarcosine dehydrogenase (Porter et al., 1985) or sarcosine oxidase (Frisell, 1971; Mori et al., 1980).

![Choline oxidation pathway](image)

**Fig. 1.6** Choline oxidation pathway (Hassan, 2008)
1.4.2.2 Bacterial metabolism of 4-N-trimethylamino-1-butanol

4-N-trimethylamino-1-butanol (TMA-Butanol) is an analogue of choline, in which the amino alcohol group is lengthened by two CH₂-groups, has been shown to resemble choline structurally. In the screening for TMA-Butanol degrading microorganisms from soil, many bacterial strains were isolated. One of these strains, *Pseudomonas* sp. 13CM was found to degrade TMA-Butanol rapidly and showed high and stable NAD⁺-dependent alcohol dehydrogenase activity was selected as a TMA-Butanol degrading strain (Hassan, 2008). In *Pseudomonas* sp. 13CM, and similar to choline, TMA-Butanol was oxidized to 4-N-trimethylamino-1-butyraldehyde (TMABaldehyde) by a NAD⁺-dependent TMA-Butanol dehydrogenase (Fig. 1.7), and consequently TMABaldehyde was oxidized to γ-butyrobetaine by a NAD⁺-dependent TMABaldehyde dehydrogenase (Hassan et al., 2007 & 2008). Kinetic studies on substrates and substrate analogs of both enzymes showed that positively charged trimethylammonium or trimethylammonium groups of the substrates have critical effect on the catalytic activity of the enzymes (Hassan, 2008). Similarly, Gadda et al., (2004) reported that the trimethylammonium moiety is critically important for the binding of ligands at the active site of choline oxidase.
1.4.2.3 Bacterial metabolism of L-carnitine

Different Pseudomonas and Agrobacterium species are able to grow aerobically using L-carnitine as a sole source of carbon and nitrogen. Under aerobic conditions, oxidation of L-carnitine is catalyzed by NAD⁺-dependent L-carnitine dehydrogenase (EC. 1.1.1.108) (Goulas, 1988; Mori et al., 1988, 1994). The L-carnitine dehydrogenase gene from Xanthomonas translucens was cloned and expressed in E. coli (Mori et al., 1988). 3-Dehydrocarnitine formed by the L-carnitine dehydrogenase could be degraded to glycine betaine by the addition of NAD⁺, ATP, and CoA (Lindstedt et al., 1967). A NAD⁺-dependent D-carnitine dehydrogenase (EC.1.1.1.254) has been purified and characterized from various Agrobacterium species, which were able to utilize D-carnitine as a sole source of carbon and nitrogen (Hanschmann et al., 1994; Setyahadi et al.)

Fig. 1. 7 Degradation pathway of 4-N-trimethylamino-1-butanol in Pseudomonas sp. 13CM (Hassan, 2008)
During growth on D-carnitine, a carnitine racemase and L-carnitine dehydrogenase were induced in *Pseudomonas* sp. AK1 (Monnich et al., 1995).

Enterobacteriacea such as *E. coli*, *Salmonella typhimurium* and *Proteus vulgaris* are able to convert L-carnitine via crotonobetaine to γ-butyrobetaine in the presence of carbon and nitrogen sources under anaerobic conditions (Seim et al., 1982). A two-step pathway, including L-carnitine dehydratase (EC. 4.2.1.89) and a crotonobetaine reductase (EC. 1.3.99), was demonstrated in *E. coli* (Eichler et al., 1994) and *Proteus* sp. (Engemann et al., 2005). The isolation and characterization of the *cai* operon encoding the structural genes of the anaerobic L-carnitine pathway in *E. coli* and *Proteus* sp. were reported (Bernal et al., 2007; Eichler et al., 1994; Engemann et al., 2005).

*Acinetobacter calcoaceticus* 69/N is able to metabolize L-carnitine, L-O-acylcarnitines and γ-butyrobetaine as a sole carbon source. *A. calcoaceticus* is able to split the C-N bond of L- and D-carnitine to yield trimethylamine (Englard et al., 1983) and a monooxygenase catalyzed cleavage has been postulated (Ditullio et al., 1994).

1.5 RESEARCH OBJECTIVES

Based on the above-mentioned literature review, research on the biodegradation of homocholine by soil microorganisms is not yet carried out and consequently, the biodegradation pathway of this compound remains unclear. Furthermore, from the choline-like structure, one would expect that homocholine could be degraded similarly and at a rate comparable to that of choline. Consequently, an osmoprotectant β-alanine betaine could be an intermediate product in the degradation pathway of homocholine. Therefore, the study of homocholine degradation pathways and the enzymes involved in this degradation pathway is of considerable interest. From an application standpoint, the
recent findings that many marine bacterial and plant species accumulate β-alanine betaine in response to salt stress or water deficit (Hanson et al., 1994; Rathinasabapathi et al., 2000) have spurred considerable interest and investigation on β-alanine betaine biosynthesis, with the goal of genetically engineering water and osmotic stress resistance in beneficial bacteria and crop plants (Rathinasabapathi et al., 2000).

Based on the aforementioned assumptions and concepts, the overall objective of this thesis was to obtain information to help in understanding homocholine degradation ability by soil bacteria as well as to elucidate the degradation pathway of this compound by the isolated strains.

The specific aims of the study were:

1- Screening of microorganisms that able to grow with homocholine as a sole source of carbon and nitrogen.

2- Isolation, characterization and identification of microorganisms capable of degrading homocholine.

3- Postulation of the degradation pathway of homocholine by the isolated strains.

4- Screening for the enzymatic activities in the degradation pathway of homocholine by the isolated strains.
CHAPTER 2
SCREENING, ISOLATION AND IDENTIFICATION OF HOMOCHOLINE DEGRADING MICROORGANISMS

2.1 INTRODUCTION
Homocholine (3-N-trimethylamino-1-propanol) an analogue of choline, in which the amino alcohol group is lengthened by one CH₂-group, has been shown to resemble choline in many aspects of cholinergic metabolisms (Boksa and Collier, 1980). It is transported into rat brain synaptosome, and is acetylated and released as acetylhomocholine from a superior cervical ganglion and minces of mouse forebrain by a calcium-dependent process during depolarization (Collier et al., 1977; Carroll and Aspyr, 1980). It is also effective in preventing fat infiltration both in fat and cholesterol fatty livers (Channon et al., 1937). It is well known that choline is an essential nutrient that is widely distributed in foods, principally in form of phosphatidylcholine. It is also a precursor of membrane and lipoprotein phospholipids and the neurotransmitter acetylcholine; thus it is important for the integrity of cell membranes, lipid metabolism and cholinergic nerve function (Zeisel et al., 1994; Zeisel 200). From the simple and choline-like structure, one would expect that homocholine is degraded in a similar way and rate comparable to that of choline. To date, no microorganism degrading homocholine as a sole source of carbon and nitrogen has been isolated and consequently
the catabolic pathway is not yet elucidated. Considering the importance of this compound and the design and development of similar compounds, it is important to know the microbial strategies and the biochemical pathway of its degradation.

2.2 MATERIALS AND METHODS

2.2.1 Materials

3-N-dimethylamino-1-propanol (DMA-Propanol), 3-N-dimethylaminopropionic acid (DMA-Propionic acid) and 3-aminopropionaldehyde diethylacetal were purchased from Tokyo Kasai (Tokyo, Japan). Yeast extract was from Nacalai Tesque (Kyoto, Japan). Peptone was from Nihon Seiyaku (Tokyo, Japan). Gram staining kit and methyl iodide were purchased from Wako Chemicals (Tokyo, Japan). Primers used in this work were obtained from Sigma Genosys (Sigma, Japan). Unless otherwise specified, all other reagents used in this study were of analytical grade.

2.2.2 General Methods

General weight measurements were made using IB-200H electronic balance (Shimadzu corp. Kyoto, Japan) and smaller quantities and measurements for the preparation of standards were made using Mettler AE240 analytical balance (Mettler, Toledo, AG, Switzerland). pH was determined using an Horiba F-22 pH meter (Horiba Ltd, Kyoto, Japan). Cultures and extracts were centrifuged using either a medium size centrifuge Himac CF16RX2 or big size centrifuges Himac SCR 18B and RC 20B (Hitachi Koki Co Ltd., Tokyo, Japan). Inoculated liquid media were incubated either in at 25°C using a NR-300 double shaker (Taitec corporation, Cupertino, CA, USA) or at 30°C and low temperature in LTI-601SD EYALA low temperature incubator equipped with NR-30
double shaker (EYALA, Tokyo, Japan). Incubation of plates occurred in Taitec M260F temperature controlled incubate box. Media were autoclaved at 121°C for 20 minutes using BS-235 high pressure steam sterilizer (Tomy Seiko Co., Ltd., Tokyo, Japan). Filter sterilization of solutions was carried out using 0.22 μm disposable filters MILLEX®-GA (Millipore, Molshiem, France). Samples were vortex using a Vortex Genie 2 (Scientific Industries, Inc., USA). Benzoylation of samples and standards was carried out at 90°C using Eyala MG-200 hot plate (Tokyo Rikakika Co., Ltd., Japan). PCR Thermal Cycler Dice model TP 600 of TaKaRa was used for PCR reactions (TaKaRa Bio Inc., Shiga, Japan).

2.2.3 Chemical Synthesis

3-N-trimethylamino-1-propanol iodide (homocholine) was prepared by N-methylation of DMA-Propanol following the method of Hassan et al. (2007). Briefly, Methyl iodide (160 mmol) was added dropwise to a solution of DMA-Propanol (100 mmol) in diethyl ether (200 ml) at room temperature. After stirring for 1 h, the mixture was filtered. The precipitate was dried in a vacuum dessicator for overnight and afforded white powder (85 %). The structure and purity of homocholine were confirmed according to its 1H NMR spectrum (ECP 500 MHz, NMR spectrometer; JEOL). Approximately 5 mg homocholine was dissolved in D2O (Merck, Co. Inc., Darmstadt, Germany) and analyzed using 1H NMR. The strong signal of NMR spectrum at 2.9 ppm (Fig 2.1) reflected the presence of trimethyl group with 99% purity.

3-N-trimethylaminopropionaldehyde was prepared from 3-aminopropionaldehyde diethylacetal by methylation with methyl iodide. Large excess amount of
methyl iodide (42 ml) was added to a suspension of 3-aminopropionaldehyde diethylacetal (5.0 g, 34.0 mmol) and K$_2$CO$_3$ (11.7 g, 35 mmol) in 50 ml ethanol at room temperature. After stirring at 40°C for 2 h, the mixture was cooled, and evaporated in vacuo. The residue was dissolved in 50 ml water and then extracted with CH$_2$Cl$_2$ (50 ml x 3 times). Combined organic extracts were dried over sodium sulfate, filtrated and concentrated. The residue was crystallized from methanol-ethylacetate to give colorless solid (8.5 g, 77%) of 3-N-trimethylaminopropionaldehyde diethylacetal iodide. Thereafter, it was hydrolyzed by 0.1 M HCl for 3 h at room temperature to produce TMAPaldehyde iodide as a 30 mM solution. The structure of TMAPaldehyde iodide was determined by $^1$H-NMR spectroscopy and capillary electrophoresis.

Fig. 2. $^1$H NMR spectrum of chemically synthesized 3-N-trimethylamino-1-propanol (Homocholine)
2.2.4 Screening and Isolation of Homocholine Degrading Bacteria

Enrichment cultures of soil samples from different location in Tottori University and around Tottori City, Tottori, Japan, were used to obtain homocholine degrading strains. Approximately 100 mg of each soil sample was inoculated into 5 ml basal medium (basal-homocholine media; Table 2.1) containing 5 g/L homocholine as sole source of carbon and nitrogen; 2 g/L KH2PO4; 2 g/L K2HPO4; 0.5 g/L MgSO4·7H2O; 0.5 g/L yeast extract; and 1 g/L polypeptone (pH 7.0). Cultivation was done at 30°C for 2 to 7 days in reciprocal shaker at 144 strokes/min. Subsequently, 200 μl of the culture was transferred to fresh basal-homocholine (basal-HC) media for another 2 days incubation.

After enrichment culture, an appropriate amount (1 ml) of culture solution was taken and serial 10-fold dilutions were prepared with physiological saline. Then, 100-μl samples of the 10^7 to 10^9 dilutions were plated on agar medium of basal-HC (Table 2.2) and/or meat extract (Table 2.3) and then incubated at 30°C for 2 days. After successive transfers to new plates, individual, distinguishable colonies were selected and stroked into slant media. Single colonies were reinoculated in basal-HC liquid media, and the cell growth was estimated by measuring the turbidity at 660 nm with Novaspec II spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). A suitable control was set using basal media without substrate (homocholine). The highly growth isolates were either stroked into basal-HC slant media and stored at 5°C in a cold room or transferred to 50% glycerol and stored in a refrigerator at -80°C. Highly growth isolates were further screened for the removal of homocholine from the growth media using TLC plates and capillary electrophoresis as described bellow.
Table 2.1 Basal-homocholine (basal-HC) liquid medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA-Propanol iodide (Homocholine)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Table 2.2 Basal-homocholine (basal-HC) agar medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA-Propanol iodide (Homocholine)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
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<tr>
<td>Total volume</td>
<td>1000 mL</td>
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</tbody>
</table>

Table 2.3 Nutrient broth (meat extract) media

<table>
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<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Meat extract</td>
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</tr>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
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<td>NaCl</td>
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</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

The pH of all media was adjusted to pH 7.0 with either 1M NaOH or 1M HCl.

2.2.5 Morphological, Biochemical and Physiological Characterization

The morphological characterization of the cells was examined using optical microscope (Olympus BX 41; Olympus Corp., Tokyo, Japan) under light and phase contrast conditions, and by using a transmission electron microscope (JEOL 100 CX II Tokyo, Japan).
Japan). For observation of cell morphology by transmission electron microscopy (TEM), cells were grown on basal-HC liquid media to both exponential and stationary phases, collected and suspended in physiological saline solution. A small drop of the suspension was placed on carbon-coated copper grid (Nisshin EM Co. Ltd, Tokyo, Japan) and negatively stained with 0.1% uranyl acetate. After air-drying, the grids were examined by the electron microscope. The Gram staining was tested using a Gram staining kit (Wako Pure Chemical Industries, Ltd. Tokyo, Japan) according to the manufacture's instructions, and using KOH method (Buck 1982). Gram stained bacterial cells were observed using optical microscope under both light and phase contrast conditions.

Catalase activity was determined by bubble formation in a 3% hydrogen peroxide (H₂O₂) solution. A colony was picked from the agar plate and mixed with the solution of 3% H₂O₂. Frothing indicated the catalase activity. Motility was checked by water hanging drop method. Utilization of different carbon sources and selected metabolic activities were investigated by using API 20 NE commercially available test kit (Biomerieux SA, Geneva, Switzerland) following the manufacture's instructions. The API stripes tests were visually read after 24h and 48h. Utilization of selected carbon compounds and combined carbon and nitrogen sources were tested by measuring turbidity in liquid culture (5 ml) at pH 7.0 using basal medium supplemented with 5 g/l (w/v) of the compound of choice. The compounds used were: choline, homocholine, 4-N-trimethylamino-1-butanol, glycine betaine, β-alanine betaine, γ-butyrobetaine, dimethyl glycine, dimethylamino-1-propionic acid, sarcosine, L-carnitine, D-carnitine, propionate, acrylate, 3-hydroxypropionate, malonate, trimethylamine, dimethylamine and monomethylamine. All tests were preformed with cells taken from the colonies pre-
grown on basal-HC medium. Cultures were incubated at 25 °C on a shaker for 48 h, and all tests were done in triplicates.

2.2.6 Sequencing of 16S rRNA Gene

2.2.6.1 Polymerase chain reaction (PCR)

To identify the isolated bacteria, 16S rRNA gene fragments were amplified by colony PCR with four primers sets (Table 2.4). Bacterial isolates were stroked on basal-HC and/or meat extract agar plates at 25°C for 48 h and single colonies were suspended in 25 μl of PCR reaction mixtures.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotides sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>314 F</td>
<td>5’-CCTACGGGAGGCAGCAG-3’</td>
</tr>
<tr>
<td>907R</td>
<td>5’-CCGTCAATTCCTTTTGAGTT-3’</td>
</tr>
<tr>
<td>6F</td>
<td>5’-GGAGAGTTAGATCCTGGTCAG-3’</td>
</tr>
<tr>
<td>1510R</td>
<td>5’-GTGCTGCAAGGTTACCTTGTTACGACT-3’</td>
</tr>
<tr>
<td>16f27</td>
<td>5’-AGAGTTTGATCTTGGCTCAG-3’</td>
</tr>
<tr>
<td>16r1488</td>
<td>5’-CGGTTACCTTGTTAGGACTTCACC-3’</td>
</tr>
<tr>
<td>Ps short F</td>
<td>5’-CTACGGGAGGCAGCAGTG-3’</td>
</tr>
<tr>
<td>Ps short R</td>
<td>5’-TCGGTAACGTCAAACAGCAAAGT-3’</td>
</tr>
</tbody>
</table>

Amplification using primer set 341 F and 709 R

The amplification reaction was composed of 20 pM of each respective primer (341F and 907R), 200 uM of dNTPs, 10× reaction buffer, 1.5 mM MgCl₂ and 2.5 U r-Taq DNA polymerase (Roche Applied Science) in a final volume of 25 μl. The temperature/time profile used was an initial denaturation of 2 min at 95°C followed by
40 cycles of denaturation for 45 sec at 95°C, annealing for 30 sec at 50°C and elongation for 1.45 min at 72°C and finally an extension for 5 min at 72°C.

**Amplification using primer set 6 F and 1510 R**

The amplification reaction was composed of 20 pM of each primer, 200 uM of dNTPs, 10× reaction buffer, 4 µl of GC-rich solution and 2.5 U Pwo Super Yield DNA Polymerase (Roche Applied Science) in a final volume of 20 µl. The temperature/time profile used for primer sets was an initial denaturation of 2 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 55°C and elongation for 1.45 min at 72°C and finally an extension for 7 min at 72°C.

**Amplification using primer set 16f27 and 16r1488**

The amplification reaction was composed of 20 pM of each primer, 200 uM of dNTPs, 10× Sp. Taq reaction buffer, 5× Tuning buffer (10%) and 2.5 U Pwo Super Yield DNA Polymerase (Roche Applied Science) in a final volume of 20 µl. The temperature/time profile used the primer sets was an initial denaturation of 2 min at 95°C followed by 35 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 55°C and elongation for 1.45 min at 72°C and finally an extension for 7 min at 72°C.

**Amplification using primer set Ps short F and Ps short R**

The amplification reaction was composed of 20 pM of each primer, 200 uM of dNTPs, 10× Sp. Taq reaction buffer, 5× Tuning buffer (10%) and 2.5 U Pwo Super Yield DNA Polymerase (Roche Applied Science) in a final volume of 20 µl. The temperature/time
profile used the primer sets was an initial denaturation of 2 min at 95°C followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 20 sec at 60°C and elongation for 45 sec at 72°C and finally an extension for 7 min at 72°C.

2.2.6.2 Agarose gel electrophoresis of DNA and visualization

Agarose gel electrophoresis was used to detect PCR products. Gels were composed of 1% w/v agarose in 1.0 x Tris-Acetate EDTA (TAE) buffer and run at 100V for 30 minutes in the same buffer using a Mupid mini-gel electrophoresis apparatus (Advance Corporation, Tokyo, Japan). DNA samples were run alongside Lambda DNA molecular weight marker to estimate product size. Gels were stained with ethidium bromide. DNA was visualized using a UV Transilluminator. Photographs of agarose gels were taken using the Pharmacia Image master VDS system (Pharmacia Biotechnology, Uppsala, Sweden).

2.2.6.3 PCR product purification and sequencing

The PCR products obtained were purified using NucleoSpin Extract II kit (Macherey-Nagel, Duren, Germany) according to the instruction manual, and sequenced using Big Dye Terminator cycle sequencing kit (Applied biosystem) following the instruction manual. The sequences were analyzed at research institute for biological sciences (Okayama, Japan) with automated DNA sequencer ABI PRISM 3130 XL (Applied Biosystems, USA) following the manufacture’s manual.
2.2.7 Phylogenetic Analysis

In order to phylogenetically identify 16S rRNA sequences, one must compare the sequences in a database of known species. Worldwide, there are a number of publicly available sequence databases that build massive sequence databases compiled from submissions from individual laboratories and large batch sequencing projects. The 16S rRNA sequence files received in ABI format from the sequencing facility were first opened and viewed in Codon Code Aligner software. Sequence chromatograms were examined for overlapping nucleotide sequences and clarity of resolution. Once the sequence was determined to be of good quality, it was saved in the FASTA format. The sequence was then copied into BLASTN, nucleotide database, and searched in the DDJB using both FASTA and BLASTN algorithms (Bosshard et al., 2004) for closest relatives. Once identified, the sequence was then compared with its closest match and phylogenetic affiliations were made based on these matches. Sequences from closest relatives to each isolate were downloaded for use in phylogenetic tree construction.

2.2.8 Creation of Phylogenetic Trees

The Clustal W, ver. 1.83 tool within DDJB database was used to create phylogenetic trees to produce graphical interpretations of phylogenetic affiliations and relatedness of the positive homocholine degrading isolates. Sequences of interest and close relatives were uploaded into the program and aligned, distance matrixes were calculated based on a specific algorithm of calculating evolutionary differences, and phylogenetic trees were created. Neighbor joining trees using the distance matrix algorithm for maximum likelihood are commonly used when comparing organisms of phylogenetically diverse taxonomic hierarchy and were used in this study. The evolutionary tree for the datasets
was constructed by using the neighbour-joining method of Saito and Nei (1987) and viewed with TreeView software. The confidence of the resultant tree topologies were evaluated by performing bootstrap analyses of the neighbour-joining method based on 1000 resamplings.

2.2.9 Analytical Methods

2.2.9.1 Thin layer chromatography (TLC)

This experiment was carried out to detect metabolites in the culture broth of homocholine-cultivated microorganisms in order to check the ability of the isolates to degrade homocholine as well as to identify the degradation pathway of homocholine in the isolates. About 5 µL of the samples and homocholine as standard (contain about 100 nmol) were applied with capillary tube onto 7cm × 10 cm pre-coated cellulose F plates (DC-Fertigplatten). The chromatography was then developed with a mixture of n-butanol: acetic acid: water (4:1:2) as mobile phase to distances of 7~7.5 cm. The developed spots were visualized with either iodine vapour or dragendorff for about 2~5 min. The disappearance of the dragendorff positive spots indicated complete degradation of homocholine by the isolates. Moreover, the appearance of new bands and in comparison with the provided standards, gave initial indication for the degradation pathway of homocholine by the isolated strains.

2.2.9.2 Capillary electrophoresis (CE)

Capillary Electrophoresis has become an accepted method for the separation of inorganic and organic ions. Usually, direct and indirect optical detection methods were used in conventional CE and much better detection limits in the low ppb range were
obtained compared to other detection methods. Due to high sensitivity of CE, it was used in this study to detect the metabolites of homocholine by the isolated strains. The isolates were cultivated on 50 mL basal-HC liquid media at 25°C for a time ranged from 0 to 48 h. At intervals of 6 h, aliquots of 10 ml media were withdrawn and centrifuged at 10,000 x g for 20 min to separate the bacterial cells from the supernatant. Since homocholine has no absorbance at UV/Vis wavelength, 0.1ml of the supernatant was benzoylated with benzoyl chloride (100 mg ml⁻¹ in pyridine) for 15 min at 90°C and analyzed by low pH capillary electrophoresis (Zhang et al., 2001).

Capillary electrophoresis analysis was carried out using Photal CAPI-3300 (Otsuka, Electronics. Co. Ltd, Osaka, Japan) equipped with a fused silica capillary of 75 μm i.d. with a total length of 80 cm (effective length of 68 cm). A new capillary was conditioned with 0.1M NaOH for 5 min followed by 3 min distilled water and 3 min electrolyte buffer (50 mM sodium phosphate buffer, pH 3.0). Samples and relative standards were injected hydrostatically (25 mm, 60 sec). The applied potential was 25 kV, and the peaks were monitored at 200 nm.

2.3 RESULTS AND DISCUSSION

2.3.1 Screening of Homocholine Degrading Microorganisms

Screening of homocholine degrading microorganisms by enrichment techniques resulted in the isolation of 142 pure colonies, which showed growth on the basal-HC medium (Fig. 2.2). Out of them, about 30 strains showed the turbidity at 660 nm of more than 1.0 were selected. These isolates were further screened for their metabolic ability to degrade homocholine and to use it as a sole source of carbon and nitrogen. Following the
decreasing and disappearance of the Dragendorff positive spots on the TLC plates indicated complete removal of homocholine from the culture medium by these isolates. Moreover, the disappearance of homocholine peak from the capillary electrophoresis chromatogram also confirmed the degradation of the compound by the isolates. Among these isolates, 10 strains showed the highest growth and ability to metabolize homocholine were selected for further studies. Those isolates were chosen as most superior homocholine degraders and were identified to the specific level according to general principles of microbial classification. For all isolated strains, *Actinobacteria* and *Proteobacteria* comprised the two main groups of homocholine degrading bacteria. The *Actinobacteria* included mainly *Rhodococcus* and *Arthrobacter* strains. *Proteobacteria* included mainly *γ-Proteobacteria* and more specifically *Pseudomonas*. These results indicated, and similar to choline, that the ability to grow on and to degrade homocholine is widespread amongst microorganisms since a large and diverse number of bacteria were found to grow on homocholine as a sole source of carbon and nitrogen. Although there are very few reports on the existence of homocholine in mammalian brain and/or cytoplasm cells and no direct report on the existence of the compounds in the plants and microorganisms, the ability to degrade homocholine is widespread. Very few reports on the existence of homocholine motif in alkaloids isolated from some plants as well as recent reports identified some homocholine esters in some plants (Barker et al., 2004). These reports shed the light on the existence and importance of this compound in the nature. The results of this study confirmed the fact that repeated exposure to homocholine in the soil would usually increase the adaptive capabilities of the microorganisms and though increase the rate of degradation with a new exposure to a
compound. This means that homocholine is naturally and widely exist in the biosphere although that is rarely reported.

Fig. 2.2 Histogram showing the cell growth of isolated microorganisms. Cell growth was estimated by measuring the turbidity at 660 nm.

2.3.2 Isolation and Characterization of Homocholine Degrading *Arthrobacter* sp. Strain E5

Based on the enrichment of homocholine degrading microorganisms under conditions described in “Materials and methods” section, pure colonies with high growth (turbidity >1) were isolated. Out of the 30 highly growth strains, one bacterium designated as strain E5 showed a good growth and ability to utilize homocholine. This strain also degraded homocholine at a good rate, therefore, was selected for further study.
2.3.2.1 Morphological and physiological characteristics of strain E5

Strain E5 is an aerobic, non-motile and gram-positive bacterium that forms primary branching and long rods of variable length when grown on homocholine agar media during the early growth phase. Then the cells fragmented into irregular short rods and/or cocci, thereby completing the growth cycle as the culture aged (Fig. 2.3). The colonies are yellow in color, convex, round and entire with smooth and regular margins on both homocholine and nutrient agar medium. The colony size ranged from 1.5 ~3.0 mm on homocholine-agar plates at 30°C for 2 days. These morphological characteristics were consistent with strain E5 belonging to the genus *Arthrobacter*. The isolated strain E5 showed catalase activity, but no oxidase activity. The bacterium was able to grow on homocholine media at an optimum temperature of 25-30°C, but did not grow at either 4°C or 41°C. Some other physiochemical properties of strain E5 are summarized in Table 2.5. Phenotypic and physiochemical tests suggest that the isolated strain E5 is belongs to the genus *Arthrobacter*.

*Arthrobacter* is a common genus of gram-positive and obligate aerobic soil bacteria characterized by polymorphism and gram variability. *Arthrobacter* species are among the most frequently isolated, indigenous, aerobic bacterial genera found in harsh conditions for extended periods of time (Mongodin *et al.*, 2006). They are metabolically versatile and therefore can grow on a wide range of substrates, including nicotine (Kodama *et al.*, 1992; Ganas *et al.*, 2007), herbicides and pesticides (Sajjaphan *et al.*, 2004) and other contaminants such as phenol (Karigar *et al.*, 2006), fluorene (Casellas *et al.*, 1997) or 4- chlorophenol (Nordin *et al.*, 2005). Due to their ubiquitous presence in soil, their high resistance against environmental stress factor and their ability to
metabolize a variety of substances, bacteria of this genus are of great interest for potential environmental and industrial applications.

![Morphology of Arthrobacter sp. E5](image)

**Fig. 2.3** Morphology of *Arthrobacter* sp. E5 as seen by light microscopy under (A) optical (B) phase contrast conditions.

The colony morphology in panel (A) and the morphological differentiation in cells shape and size after cultivation for 48 h (B) are typical gram-positive bacteria.

### 2.3.2.2 16S rRNA gene sequence analysis

The world molecular taxonomy was revolutionized, however, in the mid-1980s with the advent of full sequence analysis of molecular chronometers such as rRNA (Rossello-Mora and Amann, 2001). By the mid-1990s sequencing of the small subunit (16S) rRNA genes had become commonplace, considered as a standard tool for microbial taxonomists not only for elucidating phylogenetic relatedness but also as a means of bacterial identification (Kolbert and Persing, 1999; Rossello-Mora and Amann, 2001).

Phylogenetic relationships could be inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known bacteria. To investigate the phylogenetic relationships between strain E5 and *Arthrobacter* species, a 16S rRNA
gene sequence was compared with those of representative members of the genus *Arthrobacter*. The 16S rRNA data supported the results of morphological and phenological analysis. The phylogenetic tree drawn from the partial 16S rRNA sequence (544 nt) of the isolated strain E5 (FJ595954) clearly demonstrated that this strain belongs to the *Arthrobacter nicotinovorans* 16S rRNA subclade (Fig. 2.4). The organism was most closely related to the type strains of *Arthrobacter nicotinovorans* (AY833098) with a homology of 99.8%, to *Arthrobacter* sp. HSL strain (AY714235) with homology of 99.8%, and to *Arthrobacter histidinolovorans* (AF501356) with homology of 99.8%. The high level of similarity observed between the 16S rRNA sequence of the isolated strain E5 and several *Arthrobacter* species suggest that the strain E5 could be a strain of one of those species. However, it is accepted that 16S rRNA sequence comparison may indicate species level identification with a probability but are not considered to be definitive. Thus, strain E5 should be considered as *Arthrobacter* species with a close phylogenetic relationship to *Arthrobacter nicotinovorans*. 
Fig. 2.4 Phylogenetic tree based on 16S rRNA sequence showing the relationship between strain E5 and most closely related species of the genus *Arthrobacter*.

Numbers at nodes indicated level of bootstrap support ≥ 50%, based on a neighbour-joining analysis of 1000 re-sampled datasets. Bar = 0.005 nucleotide substitution per nucleotide position.

### 2.3.2.3 Utilization of several organic compounds by strain E5

For the determination of nutritional and biochemical properties of *Arthrobacter* sp. strain E5, a variety of selected organic compounds were tested (Table 2.5). The strain was found to grow with homocholine, which was used for its isolation and the intermediate metabolite of its degradation pathway namely β-alanine betaine. Dimethylaminopropanol also was utilized for growth by the isolated strain E5. Out of the tested substrates, Cl-compounds such as trimethylamine and monomethylamine did not support the growth, while dimethylamine did. It is remarkable that the isolated strain beside its preference to homocholine it prefer the other C and N-containing compounds that have dimethyl group instead of tri- and monomethyl group. Most of
the substrates tested as carbon sources were utilized for growth except caprate, adipate, malonate, and 3-hydroxypropionate.

**Table 2.5** Morphological and physiological characteristics of *Arthrobacter* sp. strain E5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristics</th>
<th>Parameter</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphogenic sequence</td>
<td>R-C</td>
<td>Assimilation of</td>
<td></td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Entire &amp; convex</td>
<td>D-glucose</td>
<td>+</td>
</tr>
<tr>
<td>Colony color</td>
<td>Yellow</td>
<td>L-arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>D-mannose</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>Non</td>
<td>D-mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>N-Acetyl-glucosamine</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>D-maltose</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>Glucone</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of nitrates to nitrite</td>
<td>-</td>
<td>Caprate</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of nitrates to nitrogen</td>
<td>-</td>
<td>Adipate</td>
<td>-</td>
</tr>
<tr>
<td>Indol production</td>
<td>-</td>
<td>Malate</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>-</td>
<td>Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dehydrolase</td>
<td>-</td>
<td>Phenyl acetate</td>
<td>+</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>+</td>
<td>Growth with (0.5%)</td>
<td></td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>+</td>
<td>Propionate</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Acrylate</td>
<td></td>
<td>Acrylate</td>
<td>+</td>
</tr>
<tr>
<td>Esculin</td>
<td>-</td>
<td>3-Hydroxypropionate</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>Malonate</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>-</td>
<td>Homocholine</td>
<td>+</td>
</tr>
<tr>
<td>37°C</td>
<td>+</td>
<td>β-Alanine betaine</td>
<td>+</td>
</tr>
<tr>
<td>45°C</td>
<td>-</td>
<td>Dimethylaminopropional</td>
<td>±</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>-</td>
<td>Trimethylamine</td>
<td>±</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>+</td>
<td>Dimethylamine</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monomethylamine</td>
<td>±</td>
</tr>
</tbody>
</table>

Characteristic are scored as follow; +, positive, utilized; ±, weakly positive; -, negative; R-C, rod-coccus growth cycle

### 2.3.3 Isolation and Characterization of Homocholine Degrading *Rhodococcus* sp. Strains A2

The genus *Rhodococcus* is comprised of genetically and physiologically diverse bacteria, which have been isolated from various habitats, for example soil and seawater plants (Martinkova et al., 2009). They share with a distantly related *Pseudomonas* species a capacity to degrade a large number of organic compounds including some of the most
difficult compounds that cannot be easily transformed by other organisms (Larkin et al., 2005). The large genome of rhodococci, their redundant and versatile catabolic pathways, their ability to uptake and metabolize various organic compounds such as aliphatic and aromatic hydrocarbons, halogenated compounds, nitriles and various herbicides, beside their ability to produce acrylamide and acrylic acid and to persist in adverse conditions makes them suitable industrial microorganisms for biotransformation and the biodegradation of many environmentally important organic compounds (Martinkova et al., 2009).

2.3.3.1 Enrichment and isolation of strain A2

Enrichment for homocholine degrading microorganisms was preformed in batch culture at 30°C and pH 7.0 using basal medium supplemented with homocholine as a sole source of carbon, energy and nitrogen at a concentration of 5 g/l. This enrichment culture was subsequently tested for growth (turbidity). Initially, a mixed culture was obtained; however, after streaking the mixed culture onto nutrient agar and homocholine agar plates, individual colonies were isolated and, eventually, a pure culture capable of growing in medium with homocholine as a sole source of carbon and nitrogen was obtained. About 30 colonies with high growth (turbidity > 1) were isolated in pure culture, and one of these colonies was analyzed in detailed for its degradation capacity and designed as strain A2.

2.2.3.2 Morphological and physiochemical characterization of strain A2

Strain A2 is an aerobic, non-motile, non-spore forming and Gram-positive bacterium that forms mycelium and long rods of variable length when grown on basal-HC agar
media during the early growth phase. Most cells are form filaments or show elementary branching at early growth phase (Fig. 2.5) and then fragmented into irregular short rods and/or cocci, thereby completing the growth cycle as the culture aged. The colonies are pale pink in color, opaque, convex, round and entire with smooth and regular margins on both basal-HC and nutrient agar media. Colonies are about 1.5-3.0 mm diameter after 6 days when grown on nutrient agar plates. Strain A2 showed catalase activity, but no oxidase activity. The bacterium was able to grow on basal-HC medium at a wide range of temperatures, with an optimum at 25-30°C and grew optimally over a broad pH range 6.0-8.0. Some other physiochemical properties of strain A2 were listed in Table 2.6. Phenotypic and physicochemical tests suggest that strain A2 is belongs to the genus *Rhodococcus*.

### Table 2.6 Morphological and physiological characteristics of *Rhodococcus* sp. strain A2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristic</th>
<th>Parameter</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphogenic sequence</td>
<td>EB-R-C</td>
<td>Glucose fermentation</td>
<td>-</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Entire &amp; convex</td>
<td>Arginine dehydrolase</td>
<td>-</td>
</tr>
<tr>
<td>Colony color</td>
<td>Pale pink</td>
<td>β-galactosidase</td>
<td>+</td>
</tr>
<tr>
<td>Colony size</td>
<td>1.5-3.0 mm</td>
<td>Hydrolysis of</td>
<td>-</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>Esculin</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>Non</td>
<td>Gelatin</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>Growth at</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>4°C</td>
<td>±</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>15°C</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of nitrates to nitrite</td>
<td>-</td>
<td>37°C</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of nitrates to nitrogen</td>
<td>-</td>
<td>41°C</td>
<td>±</td>
</tr>
<tr>
<td>Indol production</td>
<td>-</td>
<td>45°C</td>
<td>-</td>
</tr>
</tbody>
</table>

Characteristic are scored as follow; +, positive; ±, weakly positive; -, negative; EB-R-C, elementary branching-rod-coccus growth cycle.
Fig. 2.5: Morphology of *Rhodococcus* sp. strain A2 as seen by Phase contrast microscopy (A & B), and transmission electron microscopy (C).

Morphological differentiation in cells shape and size after cultivation for 48 h (A), and 72h (B). Cells were grown on both Basal-HC medium and nutrient broth medium. (C) Presence of fimbria-like structure (arrows) on the cell surfaces, and the V shape are typical of gram-positive bacteria. Bar = 10 μm (A & B), and bar = 0.5 μm (C).

### 2.3.3.2 Phylogenetic analysis of strain A2

To investigate the phylogenetic relationships between strain A2 and *Rhodococcus* species, the 16S rRNA gene sequence was compared with those of representative members of the genus *Rhodococcus*. The 16S rRNA data supported the results of morphological and phenological analysis. The partial 16S rRNA sequence (1374 nt) of the isolated strain A2 (AB473943) clearly demonstrated that this strain belongs to the *Rhodococcus erythropolis* 16S rRNA subclade (Fig. 2.6). The organism was most closely related to the type strains of *Rhodococcus opacus* (AY027585) with an identity of 99.1%, *Rhodococcus wratislavensis* (Z37138) with homology of 98.8% and to
Rhodococcus koreensis (AF 124342) with an identity of 98.5%. The high level of identity observed between the 16S rRNA sequence of strain A2 and several Rhodococcus species suggests that the isolate A2 is a strain of one of those species. However, it is accepted that 16S rRNA sequence comparison might indicate species level identification with a probability but are not considered to be definitive. Consequently, strain A2 should be considered as Rhodococcus sp., with a close phylogenetic relation with Rhodococcus opacus and Rhodococcus wratislaviensis.

In many studies, 16S rRNA identity values between 99.0 and 99.8 have been reported for representatives of several species of Rhodococcus (Goodfellow et al., 2002; Yoon et al., 2000) that share DNA-DNA relatedness well below the 70% cut off that is recommended for the assignment of organism to the same genomic species (Wayne et al., 1987). Despite the striking similarity of strain A2 and Rhodococcus opacus and Rhodococcus wratislaviensis based on 16S rRNA, they differ in substrate range and even shape. This demonstrates that ribosomal genetic analysis is able to provide phylogenetic relationship, indeed, but does not necessarily supply information on the specific metabolic ability of isolates.
2.3.3.3 Growth of strain A2 on homocholine and other structurally related compounds

A typical time course of *Rhodococcus* sp. strain A2 cell growth and homocholine degradation is presented in Fig. 2.7. The bacterial cells increased exponentially after 15 h of cultivation with homocholine as a substrate, although increase in cell density in the control (without substrate) was negligible throughout the experiment. About 90% of the substrate was degraded rapidly during the initial 28 h of cultivation. Thereafter, the degradation rate of the substrate slowed and complete degradation occurred after 42 h from cultivation. Complete disappearance of homocholine from the medium, together
with differential growth in media with and without the substrate, indicated that the strain used homocholine as a source of carbon and nitrogen.

Utilization of other structurally related homocholine analogues by strain A2 was examined. Results showed that strain A2 utilized trimethylamino-1-butanol, γ-butyrobetaine, L-carnitine, β-alanine betaine and glycine betaine for growth, but not choline, D-carnitine, dimethyl glyicine, dimethylamino-1-propanol or dimethylamino-1-propionic acid (Table 2.7). The inability of strain A2 to use choline, a natural structurally related compound to homocholine, in addition to its ability to grow on trimethylamino-1-butanol suggested that homocholine degrading enzymes prefer a longer chain substrate. A similar observation was reported by Hassan et al. (2007) who found that the activity of trimethylamino-1-butanol dehydrogenase was greater for increased lengths of carbon chains of the quaternary ammonium compounds. Moreover, the inability of the strain to metabolize dimethyl-containing compounds might be explained by the fact that a positively charged trimethylammonium group on the substrate is critically important for the activity of the quaternary ammonium-compound-degrading enzyme (Gadda et al., 2004; Hassan et al., 2007).
Fig. 2. Time course study of the growth and metabolism of homocholine by *Rhodococcus* sp. strain A2.

Degradation of homocholine (■) and growth of cells in basal medium supplemented with 20 mM homocholine (●). The control was the basal medium without homocholine (▲). Each value represents an average of three independent experiments.

Table 2. Utilization of homocholine and other structurally related compounds by *Rhodococcus* sp. strain A2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocholine</td>
<td>++</td>
</tr>
<tr>
<td>Choline</td>
<td>-</td>
</tr>
<tr>
<td>Trimethylamino-1-butanol</td>
<td>++</td>
</tr>
<tr>
<td>L-carnitine</td>
<td>+</td>
</tr>
<tr>
<td>D-carnitine</td>
<td>-</td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>+</td>
</tr>
<tr>
<td>γ-butyrobetaine</td>
<td>+</td>
</tr>
<tr>
<td>β-alanine betaine</td>
<td>+</td>
</tr>
<tr>
<td>Dimethyl glycine</td>
<td>-</td>
</tr>
<tr>
<td>Dimethylamino-1-propanol</td>
<td>-</td>
</tr>
<tr>
<td>Dimethylamino-1-propionic acid</td>
<td>-</td>
</tr>
<tr>
<td>Control (no substrate)</td>
<td>-</td>
</tr>
</tbody>
</table>

Growth was determined by measuring the turbidity at 660 nm (OD$_{660\text{nm}}$). Symbols: (+++) OD$_{660\text{nm}}$ ≥ 3.0; (+++) OD$_{660\text{nm}}$ ≥ 2.0; (+) OD$_{660\text{nm}}$ ≥ 1.0; -, no growth.

Each datum represents the average of three independent experiments.
2.3.4 Isolation and Characterization of Homocholine Degrading *Pseudomonas* sp.

**Strains A9 and B9b**

2.3.4.1 Morphological and physiological characteristics of the isolated strains

Quaternary ammonium compound degrading bacteria exist widely in the environment and they are usually isolated from soil. In the present study two strains (A9 and B9b) were isolated using homocholine as a substrate and identified as species of the genus *Pseudomonas*, based on the analysis of phenotypic and genotypic characteristics.

Isolated strains are aerobic, gram-negative rods and motile by means of single (B9b) or multiple (A9) polar and sub-polar flagella (Fig. 2.8). Cells of strain A9 formed short rods measuring approximately 0.5–1 × 1.5–2.0 μm in size while those of B9b formed long rods of 0.5–1 × 2.5–3.0 μm during the early growth phase on both nutrient broth and basal-HC media. The colonies of both strains are non-pigmented, convex, transparent at the edges and opaque at the center and undulate with slightly irregular margins on both basal-HC and nutrient agar medium. Colonies were about 2–3 mm in diameter after 5 days of growth on nutrient agar plates. Metabolism was respiratory not fermentative. The cytochrome oxidase test and catalase activity were positive. Nitrate reduction and denitrification were negative. Strain A9 was able to grow on basal-HC media at a wide range of temperatures (4 – 41 °C) whereas strain B9b was not able to grow at either 4 or 41 °C. Both strains showed optimum growth at temperature range of 25-30 °C and pH range of 6.0-8.0. Some other physiochemical properties of strain A9 and B9b are summarized in Table 2.8. Phenotypic and physiochemical tests suggest that both strains belong to the genus *Pseudomonas*. 
Fig. 2.8 Morphology of *Pseudomonas* sp. strain A9 and B9b as seen by phase contrast microscopy (A & C) and transmission electron microscopy (B & D), respectively.

The morphological differences in the cells size and the number of flagella.

2.3.4.2 16S rRNA gene sequence analysis of strains A9 and B9b

Phylogenetic relationships could be inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known bacteria. To investigate the phylogenetic relationships between strains A9 and B9b and *Pseudomonas* species, 16S rRNA gene sequences were compared with those of representative members of the genus *Pseudomonas*. The 16S rRNA data supported the results of morphological and phenological analysis. The phylogenetic relationship drawn from the partial 16S rRNA sequence (1408 nt) of the isolated strain A9 (FJ 605430) clearly demonstrated that this strain belongs to the *Pseudomonas putida* 16S rRNA subclade (Fig. 2.9). The organism

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was most closely clustered with the type strains of *Pseudomonas putida* (AY 973266) with a homology of 99.4 % and *Pseudomonas umsongensis* (AF 460450) with a homology of 99.3 %. Whereas, the partial (1421 nt) 16S rRNA gene sequence of strain B9b (FJ 605432) demonstrated its phylogenetic position in the *Pseudomonas fulva* subclade. The strain is closely clustered with two types of strain, *Pseudomonas plecoglossicida* (EU 594553) and *Pseudomonas fulva* (DQ 141541) having sequence similarities of 99.8 % and 99.7 %, respectively. Our 16S rRNA sequence comparison confirmed that strains A9 and B9b belong to the genus *Pseudomonas*.

The high level of similarity observed between the 16S rRNA sequence of these strains and several *Pseudomonas* species suggest that the isolated strains could be strain of one of those species. However, it is accepted that 16S rRNA sequence comparison may indicate species level identification with probability but are not considered to be definitive. In many studies, 16S rRNA similarity values more than 99.0 have been reported for representatives of several species of *Pseudomonas* (Behrendt et al., 2003; Hauser et al., 2004; Uchino et al., 2001) that share DNA-DNA relatedness well below the 70 % cut off recommended for the assignment of organism to the same genomic species (Wayne et al., 1987). In contrast, it was pointed out that strains with lower similarity in the 16S rRNA gene sequence would reassociate with rates above the species limit (Tvrzova et al., 2006). Combined genotypic and phenotypic data suggest that strains A9 and B9b merits recognition as new species within the genus *Pseudomonas*, we tentatively named them as *Pseudomonas* sp. strain A9 and *Pseudomonas* sp. strain B9b.
Fig. 2. Phylogenetic tree based on 16S rRNA sequence showing the relationship between the isolated strains A9 (FJ605430) and B9b (FJ605432), and most of the closely related *Pseudomonas* species.

Numbers at nodes indicate level of bootstrap support ≥ 50 %, based on a neighbour-joining analysis of 1000 re-sampled datasets. Bar = 0.002 nucleotide substitution per nucleotide position.

Diverse ranges of microbial taxa capable of aerobic degradation of quaternary ammonium compounds have been reported (Seim et al., 1982; Miura-Fraboni and England, 1983; Mori et al., 1988; Van Ginkel et al., 1992; Keach et al., 2005). Among them, *Pseudomonas* species are predominant, due to their versatility and capability of degrading a number of different quaternary ammonium compounds (Van Ginkel, 1996).

The genus *Pseudomonas* is present in ecologically diverse niches, playing important roles in the biogeochemical cycles. Bacteria belonging to this genus are engaged in important metabolic activities in the environment, including element cycling and the
degradation of biogenic and xenobiotic pollutants (Timmis, 2002). They play a key role in the metabolisms of quaternary ammonium compounds, e.g., choline (Nagasawa et al., 1976), γ-butyrobetaine (Miura-Fraboni and Englard, 1983), 3-N-trimethylamino-1-butanol (Hassan et al., 2007), 2,3-dihydroxy propyl-trimethyl ammonium (Keach et al., 2005) and many others long chain quaternary ammonium compounds (Van Ginkel et al., 1992; Nishihara et al., 2000).

Additionally we also reported here the isolation of two Pseudomonas strains able to degrade homocholine and to use it as a sole carbon and nitrogen source. Despite the striking similarity of strain A9 and B9b in substrate range, they differ in 16S rRNA gene sequences and even shape. This demonstrates that the metabolic ability of the isolates of the same genus might be similar although they are differing in the species level.

2.3.4.3 Utilization of several C.N. sources by strains A9 and B9b

For the determination of nutritional and biochemical properties of the isolated strains A9 and B9b, a variety of selected organic compounds were tested (Table 2.8). Both strains were able to grow on homocholine, which was used for their isolation and weakly on β-alanine betaine. Choline and its degradation pathway metabolites namely glycine betaine, dimethylglycine, and sarcosine were used as substrates for both strains. Trimethylamino-1-butanol, an analogue of homocholine in which the amino alcohol chain lengthened by one CH₂ group, and an intermediate metabolite of its degradation pathway namely γ-butyrobetaine were not utilized for growth by both strains. L-carnitine, the naturally widely occurring quaternary ammonium compound was also supported the growth of both strains, whereas D-carnitine did not. It is clear that the preference of quaternary ammonium compounds as substrates for both strains is quite
similar. The only exception is that strain B9b showed positive growth with dimethylamino-1-propanol whereas that of A9 was negative. Out of the additionally substrates tested as carbon sources D-maltose, adipate, and acrylate did not support the growth of both strains. In addition to these substrates strain B9b did not also utilized both D-mannitol and N-acetyl-glucosamine. Moreover, the cell growth of both strains under the controls (basal media without the substrate) was negligible throughout the experiment.

Table 2. Phenotypic characteristics of homocholine-degrading *Pseudomonas* sp. strains A9 and B9b isolated from soil.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>A9</th>
<th>B9b</th>
<th>Characteristic</th>
<th>A9</th>
<th>B9b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagellation</td>
<td></td>
<td></td>
<td>Assimilation of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td></td>
<td></td>
<td>D-maltose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Oxidase</td>
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<td>+</td>
<td>Caprate</td>
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<td>Adipate</td>
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<td>+</td>
<td>-</td>
<td>Malate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
<td>-</td>
<td>Citrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indol production</td>
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<td>-</td>
<td>Phenyl acetate</td>
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<td>+</td>
</tr>
<tr>
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<td>-</td>
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<tr>
<td>Arginine dehydrodase</td>
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<td>+</td>
<td>Acrylate</td>
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<td>-</td>
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<tr>
<td>β-galactosidase</td>
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<td>-</td>
<td>3-hydroxypropionate</td>
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<td>Homocholine</td>
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<td>+</td>
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<tr>
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<td>-</td>
<td>Choline</td>
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<td>+</td>
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<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td>Trimethylaminobutanol</td>
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<td>-</td>
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<tr>
<td>4 °C</td>
<td>+</td>
<td>-</td>
<td>L-carnitine</td>
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<td>+</td>
</tr>
<tr>
<td>41 °C</td>
<td>+</td>
<td>-</td>
<td>D-carnitine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH 5.0</td>
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<td>+</td>
<td>Glycine betaine</td>
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<tr>
<td>pH 8.0</td>
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<td>+</td>
<td>γ- butyrobetaine</td>
<td>-</td>
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<tr>
<td>NaCl (4 %)</td>
<td>+</td>
<td>-</td>
<td>β- alaninebetaine</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Assimilation of</td>
<td></td>
<td></td>
<td>Dimethyl glycine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-glucose</td>
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<td>+</td>
<td>Dimethylaminopropanol</td>
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<td>+</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+</td>
<td>+</td>
<td>Dimethylaminopropionic acid</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>Sarcosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>Propanol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-acetyl-glucosamine</td>
<td>+</td>
<td>-</td>
<td>Trimethylamine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Characteristics were scored as follow: +, positive; -, negative; ±, weakly positive. Each datum represents the average of three independent experiments.
2.4 CONCLUSIONS

The present study is an attempt to investigate the ability of soil microorganisms to degrade homocholine. As expected, the ability to degrade homocholine is widespread among aerobic microorganisms since representatives of the genus *Arthrobacter*, *Rhodococcus* and *Pseudomonas*, were found to grow with homocholine as a sole source of carbon and nitrogen. All these microorganisms are widely distributed in the biosphere, and particularly in the soil, they probably play an important role in the degradation of homocholine in the nature. Although there are very few reports on the presence of homocholine in mammalian brains and there is no direct evidence on the presence of such compound in plants. The widespread utilization of homocholine by soil microorganisms provides indirect evidence that the compound is widely exists in the nature and may be in the plant kingdom. In this study we reported the isolation of homocholine degrading bacteria from soil as a first study. Strains belongs to the genus *Arthrobacter*, *Rhodococcus* and *Pseudomonas* were isolated, identified and characterized for their ability to grow with homocholine and its related analogues. With few exceptions, most of the bacteria isolated and identified so far that degrade quaternary ammonium compounds were from the genus *Pseudomonas* and *Arthrobacter* (Van Ginkel et al., 1992; Van Ginkel, 1996; Hassan et al., 2007). However, *Rhodococcus* species capable of degrading quaternary ammonium compounds have not yet been reported despite their versatility and ability to degrade numerous organic compounds, including some difficult and toxic compounds. In the present study we are able to isolate *Rhodococcus* strains that metabolized homocholine as a sole source of carbon and nitrogen.
CHAPTER 3
DEGRADATION OF HOMOCHOLINE BY RESTING CELL SUSPENSIONS AND GROWING CELL CULTURES OF THE ISOLATED STRAINS

3.1 INTRODUCTION

Traditionally, organisms for bioremediation purposes have been sourced from environments exposed to the target compounds on the assumption that there is a better likelihood for these organisms to have potential to degrade these compounds. This has been undertaken in numerous studies on a variety of quaternary ammonium compounds including: cetyl trimethyl ammonium bromide (CTAB), dodecyl benzyl dimethyl ammonium chloride and didecyl dimethyl ammonium chloride (Gerike, 1982; Van Ginkel et al., 1992; Takenaka et al., 2007), choline (Shieh, 1964; Kortstee, 1970; Mori, 1981) and L-carnitine (Goulas, 1988; Mori et al., 1988, Mori et al., 1994) among others. It is thought that the soil environment exerts a selective pressure that selects for organisms with the metabolic capability to degrade the target compound as a carbon and energy source and that the likelihood of isolating an organism with degradative capabilities from these sources is greater (Margesin et al., 2003).

Quaternary ammonium compounds such as choline, glycine betaine and carnitine are widely distributed components in many organisms. These naturally occurring
quaternary ammonium compounds as well as synthetic quaternary ammonium compounds are metabolized in the nature by soil microorganisms. Many microorganisms used choline as a sole source of carbon and nitrogen has been isolated from soil and the degradation pathway of choline of these isolates was studied (Ikuta et al., 1977; Mori et al., 1980, 1992, 2002; Nagasawa et al., 1976). In microorganisms choline is oxidized by either choline oxidase (EC 1.1.3.17) or membrane bound choline dehydrogenase (EC.1.1.99.1) to betaine aldehyde, which in turn is oxidized to glycine betaine by a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (Andersen et al., 1988; Mori et al., 1980; Nagasawa et al., 1976; Tani et al., 1977).

Despite its similarity to choline in many aspects, very few microorganisms degrading homocholine as only source of carbon and nitrogen has been isolated (Chapter 2) and consequently, the catabolic pathway remains unclear. From the choline-like structure, one would expect that homocholine could be degraded in a similarly and at a rate comparable to that of choline. Thus, an osmoprotectant β-alanine betaine could be an intermediate product in the degradation pathway of homocholine. Therefore, the study of homocholine degradation pathway and the enzymes involve in is of considerable interest. From an applied standpoint, the recent findings that many marine bacteria and plant species accumulate β-alanine betaine in response to salt stress or water deficit (Hanson et al., 1994; Rathinasabapathi et al., 2000) have prompted considerable interest in research on β-alanine betaine biosynthesis, with the goal of genetically engineering water/osmotic stress resistance in beneficial bacteria and crop plants (Rathinasabapathi et al., 2000). With this in mind, an investigation was carried out to see which mechanism is followed in the degradation of homocholine by soil
microorganisms. This beside, whether β-alanine betaine is accumulate as an intermediate metabolite or not. Therefore, this study was carried out to identify and quantify the metabolites released from homocholine by the isolated strains that utilize homocholine as a carbon and nitrogen source, as well as to elucidate the degradation pathway of homocholine by these isolates. In this chapter, we have isolated and identified metabolites liberated from homocholine by the isolated strains. Furthermore, the partial pathway for homocholine degradation was also postulated.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3-N-Dimethylaminopropionic acid (DMA-Propionic acid), and p-bromophenacyl bromide were purchased from Tokyo Kasai (Tokyo, Japan). Benzoyl chloride, dehydrated pyridine and methyl iodide were from Wako Pure Chemicals Co. Ltd. (Tokyo, Japan). O-(4-Nitrobenzyl)-hydroxyamine was obtained from Fluka Chemicals (GmbH, Switzerland). Unless otherwise specified, all other reagents were of analytical grade from either Wako (Tokyo, Japan) or Sigma (St. Louis, MO, USA).

3-N-Trimethylamino-1-propanol iodide (homocholine) was prepared from 3-N-dimethylamino-1-propanol as described in Chapter 2. 3-N-Trimethylaminopropionaldehyde was also prepared from 3-aminopropion-aldehyde diethylacetal as described in Chapter 2.

3.2.2 General Methods

General weight measurements were made using IB-200H electronic balance (Shimadzu corp. Kyoto, Japan) and smaller quantities and measurements for the preparation of
standards were made using Mettler AE240 analytical balance (Mettler, Toledo, AG, Switzerland). pH was determined using Horiba F-22 pH meter (Horiba Ltd, Kyoto, Japan). Cultures and extracts were centrifuged using either a medium size centrifuge Himac CF16RX2 or big size centrifuges Himac SCR 18B and RC 20B (Hitachi Koki Co Ltd., Tokyo, Japan). Media were autoclaved at 121°C for 20 min using BS-235 High Pressure Steam Sterilizer (Tomy Seiko Co., Ltd., Tokyo, Japan). Filter sterilization of solutions was carried out using 0.22 μm disposable filters MILLEX®-GA (Millipore, Molshiem, France). Samples were vortex using a Vortex Genie 2 (Scientific Industries, Inc., USA). Benzoylation, esterification and hydroxylation of samples and standards were carried out at 90°C using Eyala MG-200 hot plate (Tokyo Rikakika Co., Ltd., Japan).

3.2.3 Chemical Synthesis

3-N-Trimethylaminopropionic acid (β-alanine betaine) was synthesized by N-methylation of dimethylaminopropionic acid (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) with methyl iodide. Methyl iodide (4 ml) was added to a suspension of dimethylaminopropionic acid (1 g, 6.5 mmol) and KHCO₃ (1.3 g, 13 mmol) in 20 ml of methanol. Then the mixture was stirred overnight at room temperature. The mixture was decanted. Subsequently, the liquid phase was concentrated and the residue was extracted using a mixed solvent (acetonitrile: methanol =10:1, v/v, 15 ml × 3). The combined extracts were dried under a nitrogen stream to give β-alanine betaine as a colourless powder (1.2 g, 63.2%). The structure and purity of β-alanine betaine were confirmed using ¹H NMR (Fig. 3.1) and capillary electrophoresis.
Fig. 3. 1 $^1$H NMR spectrum of chemically synthesized 3-$N$-trimethylamino-1-propionic acid (β-alanine betaine)

3.2.4 Growth and Homocholine Degradation by the Isolated Strains

The isolated strain was cultivated at 25°C on 75 ml of basal-HC liquid media containing 20 mM homocholine as a sole source of carbon, nitrogen and energy. At intervals of 9, 12, 15, 18 and 24h about 10 ml culture was withdrawn by pasteurized pipette and centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was collected and preserved at -20°C until used for detection of residual homocholine and the metabolites of its degradation as described below.
3.2.5 Homocholine Degradation by Resting Cells of the Isolated Strains

The isolated strain was cultivated (24 h at 25°C) on 75 ml of basal-HC liquid media containing 20 mM homocholine as a sole source of carbon, nitrogen and energy. The cells were harvested at the exponential phase by centrifugation at 10,000 × g for 20 min at 4°C, washed three times with saline solution (8.5 g/L KCl) and re-suspended in 50 mM potassium phosphate buffer (pH 7.5). The resting cell reaction was started by the addition of homocholine (20 mM) to the cell suspension. The suspension was incubated on a shaker at 120 rpm and 30°C. At appropriate time intervals (30 min, 1, 2, 3 and 6 h), aliquots of the cell suspension were withdrawn and boiled for 3–5 min to stop the reaction. After centrifugation, the supernatants were preserved at -20°C and used for metabolites detection as described below.

3.2.6 Isolation and Quantification of the Metabolites of Homocholine

3.2.6.1 Homocholine

Since quaternary ammonium compounds lack an absorbance in UV/VIS light range, derivetization is most important. The decreasing amount of homocholine was detected during the degradation of the compound by the isolated strains according to the method of Zhang et al. (2001). In this method, 0.1 ml of the supernatants of either culture filtrate or intact cell reaction mixtures and an authentic standard of homocholine (10 mM in 2-propanol containing 3% water) were mixed with 0.3 ml of benzoyl chloride (10% in pyridine). The tube was caped and heated at 80°C for 15 min. After cooling to room temperature, 1 ml of chloroform and 0.5 ml of water were added and the mixture was centrifuged at 10,000 × g for 20 min. The supernatant contained benzooylated homocholine was applied directly to CE at hydrostatic mode (25 cm height for 30 sec)
and electrophoresis was carried out at 20 kV for 20 min. Standards homocholine was treated in the same manner. Each experiment was carried out in triplicate.

3.2.6.2 Trimethylaminopropionaldehyde (TMAPaldehyde)

The supernatants of either culture filtrate or intact cell reaction mixtures (0.1 ml) were treated with 0.3 ml of O-(4-nitrobenzyl) hydroxylamine in methanol (5 mg/ml) and heated at 80°C for 15 min. After cooling to room temperature, 0.1 ml of 50 mM sodium phosphate buffer (pH 3.0) was added to the reaction mixture and the existence of the metabolite trimethylaminopropionaldehyde-oxime was detected using low pH capillary electrophoresis (Zhang et al., 1997).

3.2.6.3 β-alanine betaine and trimethylamine (TMA)

Both β-alanine betaine and TMA were detected by low pH capillary electrophoresis after esterification with p-bromophenacyl bromide following the methods of Nishimura et al. (2001) with slight modifications. In micro tube, 0.1 ml of the collected supernatants of either culture filtrate or intact cell reaction mixture and authentic standards (β-alanine betaine and TMA) were placed and mixed with 0.05 ml of a buffer solution (100 mM KH2PO4: distilled water: acetonitrile = 1:1:4). To the mixture, 0.3 ml of p-bromophenacyl bromide (20 mg/ml in acetonitrile) was added. The tube was capped and heated at 90°C for 90 min. The reaction mixture was evaporated to dryness with a centrifugal evaporator. Three hundreds microliters of 50 mM sodium phosphate buffer (pH 3.0) was added, mixed well and centrifuged at 10,000 × g for 20 min at 4°C. The supernatants, which contained esters of the metabolites β-alanine betaine and TMA, were analyzed using capillary electrophoresis.
3.2.6.4 Trimethylamine (TMA)

In glass tubes, the supernatants of either culture filtrate or intact cell reaction mixtures (1 ml) were placed and then 0.5 ml toluene and 0.5 ml of 8N NaOH were added and mixed by vortex for 15 sec. The mixtures were stand at room temperature for about 5 min, and then vortex mixed again for 15 sec, and thereafter stands at room temperature for another 5 min. The organic layers were transferred to new glass tube and tightly close. The upper layer contained the metabolite TMA, which was identified by GC–MS as described below. The authentic standard of TMA was treated in the similar way.

3.2.7 Analytical Methods

Degradation of homocholine and the production of metabolites by intact cells of the isolated strains were detected using capillary electrophoresis, GC–MS and FAB–MS.

3.2.7.1 Capillary electrophoresis (CE)

Capillary electrophoresis analysis was conducted with a device (Photal CAPI-3300; Otsuka Electronics. Co. Ltd., Osaka, Japan) equipped with a fused silica capillary of 75 μm i.d. with a total length of 80 cm (effective length of 68 cm). A new capillary was conditioned with 0.1M NaOH for 5 min followed by 3 min distilled water and 3 min electrolyte buffer (50 mM sodium phosphate buffer, pH 3.0). Samples and relative standards were injected hydrostatically (25 mm, 60 s). The applied potential was 20 kV, and the peaks of benzoyl-homocholine, trimethylaminopropionaldehyde-oxime, and TMA- and β-alanine betaine-esters were monitored, respectively, at 200, 270 and 254 nm.
3.2.7.2 Gas chromatography-mass spectrometry (GC–MS)

Gas chromatography-mass spectrometry analysis was conducted using a mass spectrometer (JEOL AX505 HA; JEOL, Tokyo, Japan) with electron-impact ionization (70 eV) coupled with a gas chromatograph (5890 series II; Hewlett-Packard Co., Wilmington, DE, USA). A fused silica capillary column (0.25 mm i.d., 30 m long) packed with DB1 (J&W Scientific Inc., Folsom, CA, USA) was used. Helium was used as the carrier gas at a flow rate of 15 ml min⁻¹. The column temperature was maintained at 50°C and samples (1 µl) were injected onto the GC at an injection port temperature of 250°C.

3.2.7.3 Fast atom bombardment – mass spectrometry (FAB–MS)

Fast atom bombardment – mass spectrometry analysis was used to detect β-alanine betaine and 3-N-trimethylaminopropaionaldehyde following the method described by Rhodes et al. (1987). The supernatants of resting-cell reaction mixtures and the culture filtrate, alongside with the authentic standards, were applied directly to the FAB–MS spectrometer (JEOL AX505 HA; JEOL, Tokyo, Japan). Aliquots of 1 µl sample and authentic standard were mixed with glycerol and applied to the FAB probe and irradiated with Xenon (3 kV, 50°C).

3.3 RESULTS AND DISCUSSION

3.3.1 Identification of Homocholine Degrading Metabolites

Degradation of quaternary ammonium compounds, such as choline, L-carnitine and trimethylamino-1-butanol, has been reported for strains of the genera Arthrobacter, Xanthomonas, Agrobacterium and Pseudomonas among others. Betaine aldehyde,
glycine betaine, dimethyl glycine, sarcosine, 3-dehydrocarnitine, trimethylamino-1-butyraldehyde and gamma-butyrobetaine were identified as intermediate metabolites (Ikuta et al., 1976; Mori et al., 1988; Hanschmann et al., 1996; Hassan, 2008). They all seem to adopt the oxidation pathway by either a dehydrogenase or oxidase enzymes.

In the previous chapter, strains belong to the genera Arthrobacter, Rhodococcus, and Pseudomonas were isolated from soil using homocholine as a sole source of carbon, energy and nitrogen.

In this chapter, the degradation of homocholine by the resting cell suspensions and growing cell cultures of isolated strains and the detection of formed metabolites were tested by capillary electrophoresis, GC-MS and FAB-MS methods. During the consumption of homocholine by the resting cell suspensions and the growing cell cultures of the isolated strains (Arthrobacter sp. strain E5, Rhodococcus sp. strain A2, and Pseudomonas sp. strain A9 and B9b), there were a concurrent formation and accumulation of some soluble metabolites as detected by capillary electrophoresis analysis (Fig. 3.2). These metabolites were found to be trimethylamine (peak 1, TMA) and β-alanine betaine (peak 2, β-AB) as compared to the authentic standards of TMA and β-AB.
Fig. 3. 2 Capillary electrophoresis chromatogram of the intermediate metabolites of homocholine biodegradation.

The chromatogram showed the generated metabolite peaks from the degraded homocholine by the isolated strains alongside with authentic standards peaks of trimethylamine (TMA) and β-alanine betaine (β-AB).

The first evidence for the accumulation of TMA was the remarkable fishy-odor of the culture filtrate of homocholine growing cells. Further analysis of the culture filtrate and the intact cell reaction mixtures of the isolated strains by GC-MS confirmed the accumulation of TMA (Fig. 3.3). The mass spectra (M$, 59$) and the retention time (1.5 min) of the observed metabolite, agreed with those of authentic standard of TMA treated and extracted in the same way. These results demonstrated that TMA is a major metabolite of the degraded homocholine by the isolated strains.
Fig. 3. GC-MS spectra of the metabolite (TMA) formed during the degradation of homocholine by the isolated strains.

Furthermore, analysis of the culture filtrate and the intact cell reaction products by FAB-MS demonstrated the accumulation of β-alanine betaine and 3-N-trimethylaminopropionaldehyde (TMAPaldehyde). The culture filtrate and the intact cell reaction products spectrum of the isolated strains (Fig. 3.4) showed signals at m/z 118 [M+H]+ of homocholine, m/z 131 [M+] of β-alanine betaine and m/z 115 [M+] of TMAPaldehyde. Those signals agreed with the signals and the theoretical masses of the authentic standards of homocholine, β-alanine betaine and TMAPaldehyde, respectively. As expected, the results demonstrated that all the isolated strains oxidized homocholine to TMAPaldehyde and β-alanine betaine.
Fig. 3. 4 FAB–MS spectra of metabolites in the degradation pathway of homocholine by the isolated strains.

Metabolites with M⁺ of 115 and M⁺ of 131 were identified respectively as TMAPaldehyde and β-alanine betaine.

3.3.2 Degradation of Homocholine by Resting Cells of the Isolated Strains

During the degradation of homocholine by resting cells of the isolated strains, the amount of homocholine decreased concomitantly with the increase of metabolites, identified as β–alanine betaine and trimethylamine. The time course degradation of homocholine by resting cells of the isolated strains is depicted in Fig. 3.5. Resting cells of the isolated strains almost completely degraded homocholine within 6 h, and the metabolites TMAPaldehyde and β-alanine betaine formed and metabolized when the reaction elevated. The transient accumulation of β-alanine betaine in the medium indicated that it was an intermediate metabolite during the degradation of homocholine and not a dead-end product. These results indicated the sequential oxidation of
homocholine to trimethylaminopropionaldehyde and β-alanine betaine. In agreement with our results, consequent oxidation of alcohol groups of the quaternary ammonium compound to aldehyde and acid was reported (Nagasawa et al., 1976; Mori et al., 1980, 1992, 2002; Hassan et al., 2007).

It is particularly notable that the detection of β-alanine betaine as an intermediate in the homocholine degradation pathway by the isolated strains is important from a biotechnological standpoint. We assume that the enzymes responsible for the formation of β-alanine betaine might be useful in biotechnology for the engineering of osmotic stress tolerant crop plants. It was proposed that genetic engineering of osmotolerance in plants could be achieved by producing betaine in non-accumulators (McCue and Hanson, 1990). This has been demonstrated in several reports where transgenic plants accumulating glycine betaine exhibit tolerance to salt, cold and heat stresses (Hayashi et al., 1997; Alia et al., 1998a, b).
Fig. 3.5 Degradation of homocholine by resting cells of the isolated strains.

Time course degradation of homocholine (■) and the generation of the metabolites, TMA (○) and beta-alanine betaine (△) by strains: (A) Arthrobacter sp. strain E5, (B) Rhodococcus sp. strain A2 and (C) Pseudomonas sp. strains A9.
Moreover, the degradation of homocholine by the isolated strain was accompanied by a stoichiometric formation of trimethylamine and degradation of the carbon skeleton. This might be a result of the cleavage of a C-N bond of β-alanine betaine by a monooxygenase enzyme. This was further confirmed by the detection of TMA in the culture filtrate of the isolated strains grown on β-alanine betaine as a sole source of carbon and nitrogen (data not shown). In addition, the intact cell reaction of these cells with β-alanine betaine again showed an accumulation of TMA as major metabolites. Similarly, utilization of choline, γ-butyrobetaine, DL-carnitine by Candida tropicalis, Acinetobacter calcoaceticus, Pseudomonas putida and Proteus vulgaris are correlated respectively with the stoichiometric formation of TMA and complete degradation of the carbon skeleton (Seim et al., 1982; Miura-Fraboni and Englard, 1983; Mori et al., 1988). Additionally, the initial microbial degradation of methylamines, including long chain quaternary ammonium compounds, tetramethylammonium chloride, and nitrilotriacetic acid, always involves the breakage of C-N linkages (Tiedje et al., 1973; Hampton and Zatman, 1983; Van Ginkel et al., 1992). Initial cleavage of the C-N bond was proposed as a general strategy of microorganisms to gain access to the alkyl chains of quaternary ammonium compounds (Van Ginkel, 1996). Furthermore, the pathways in bacteria that have been suggested for TMA formation from choline and carnitine are based exclusively on reductive cleavage of C-N bond of these compounds, and thus yielded ethylene glycol and β-hydroxybutyrate, respectively (Mori et al., 1988: Kleber et al., 1978). In accordance with the above-mentioned pathways, we proposed that cleavage of C-N bond of β-alanine betaine accompanied with the formation of TMA and 3-
hydroxypropionate. Trimethylamine is further utilized as a nitrogen source, whereas 3-hydroxypropionate is used as carbon and energy source.

Although our study showed the cleavage of C-N bond as in the aforementioned studies, but this was not the initial step in the degradation of homocholine by the isolated strains. This demonstrated the novel degradation pathway of homocholine by the isolated strain that is quite different from the above-mentioned studies. In this study, we demonstrated that the alcohol group (-OH) of homocholine was consequently oxidized to aldehyde (-CHO) and carboxyl (-COOH) group, with cleavage of C-N bond providing TMA and alkyl chain (C-3 moiety) (Fig. 3.6).

Although the possibility of the demethylation pathway, from β-alanine betaine to dimethylaminopropionic acid, particularly in Arthrobacter sp. strain E5, is still considered since the amount of the formed TMA is quite low compared to the degraded substrate (homocholine). This beside that the substrate dimethylaminopropionic acid was also utilized for growth by the isolated strain.
3.4 CONCLUSIONS

This study was attempted to characterize and identify the metabolites of homocholine degradation by the isolated strains as well as to elucidate the degradation pathway of the compound by these isolates. As expected, the metabolites TMAPaldehyde, β-alanine betaine and trimethylamine were detected in the culture filtrate and intact cell reaction mixtures of the isolated strains.

The degradation pathway of homocholine was found to be through consequent oxidation of alcohol group (\(-\text{OH}\)) to aldehyde (\(-\text{CHO}\)) and acid (\(-\text{COOH}\)), and thereafter cleavage of C-N bond of β-alanine betaine to give trimethylamine and alkyl chain (C3-moiety).
It is particularly notable that the detection of β-alanine betaine as an intermediate during homocholine degradation pathway by the isolated strains is important from a biotechnological standpoint. We assume that the enzymes responsible for the formation of β-alanine betaine might be useful in biotechnology for the engineering of osmotic stress tolerant crop plants. Therefore, our further research will be focused on isolation, characterization and possible application of homocholine degrading enzymes. This information will be important to understand the mechanisms involved in the degradation pathway of this compound.
CHAPTER 4
ENZYMATIC ACTIVITY IN THE DEGRADATION PATHWAY OF HOMOCHOLINE BY THE ISOLATED STRAINS

4.1 INTRODUCTION

Enzymes are remarkable catalysts capable of accepting a wide array of complex molecules as substrates and catalyzing reactions with unparalleled chiral (enantio-) and positional (regio-) selectivities (Schmid et al., 2001). Such high selectivity also affords efficient reactions with few by-products, thereby making enzymes an environmentally friendly alternative to conventional chemical catalysts (Schmid et al., 2001). Among various classes of enzymes, oxidoreductases represent a highly versatile class of biocatalysts for specific reduction and oxidation reactions, and currently used for the production of a wide variety of chemical and pharmaceutical products (Johannes et al., 2006). To this decade, more than 1,000 oxidoreductases are known from which 80% use NADH as cofactor, 10% use the corresponding phosphates. Flavins and pyrroloquinoline quinone are involved more rarely (Peters, 2000). The glucose-methanol-choline (GMC) oxidoreductase enzyme superfamily is composed of a group of enzymes that are able to catalyze the conversion of alcohols to the corresponding aldehydes or ketones. This family includes alcohol dehydrogenase, glucose oxidase,
cholesterol oxidase, cellobiose dehydrogenase, pyranose 2-oxidase, choline dehydrogenase, choline oxidase and betaine aldehyde dehydrogenase (Cavener, 1992). The later three enzymes play an important role in the metabolism of choline in both mammalian and microorganisms.

In choline degradation pathway, choline is oxidized to glycine betaine by two reactions: (i) choline to betaine aldehyde by either choline oxidase or choline dehydrogenase (Ikuta et al., 1977; Landfald et al., 1986; Nagasawa et al., 1976; Rathinasabapathi et al., 1997; Rozwadowski et al., 1991; Russell et al., 1994; Russell et al., 1998; Tsuge et al., 1980; Yamada et al., 1977) and (ii) betaine aldehyde to glycine betaine by a NAD⁺-dependent betaine aldehyde dehydrogenase (Arakawa et al., 1987; Falkenberg et al., 1990; Nagasawa et al., 1976; Mori et al., 1992; Mori et al., 1980; Mori et al., 2001; Valenzuela-Soto et al., 1994).

TMA-Butanol, a choline analogue, had a similar pathway as choline. In oxidative reaction, TMA-Butanol is converted to TMABaldehyde by a NAD⁺-dependent TMA-balcohol dehydrogenase. Consequently, TMABaldehyde is oxidized to γ-butyrobetaine by a NAD⁺-dependent TMABaldehyde dehydrogenase. Both enzyme have been isolated and purified from *Pseudomonas* sp. 13CM (Hassan, 2008). On the other hand, enzymatic studies on the degradation pathway of homocholine, an analogue of choline and TMA-Butanol, by soil microorganisms were not yet carried out.

In the previous chapters, the author postulated the degradation pathway of homocholine by strains belongs to the genera *Arthrobacter*, *Rhodococcus* and *Pseudomonas*. Similar to choline and TMA-Butanol, homocholine was consequently oxidized to TMAPaldehyde and β-alanine betaine. Thereafter, cleavages of β-alanine...
betaine C-N bond yielded trimethylamine and alkyl chain. In this chapter, screening and preliminary studies on the oxidative enzymes in the degradation pathway of homocholine by *Pseudomonas* sp. strain A9 will be described.

**4.2 MATERIALS AND METHODS**

**4.2.1 Materials**

3-Dimethylamino-1-propanol (DMA-Propanol), 4-dimethylamino-1-butanol (DMA-Butanol), and 4-aminobutanol were purchased from Tokyo Kasei (Tokyo, Japan). Homocholine iodide was prepared from 3-dimethylamino-1-propanol according to the method described in Chapter 1. TMAPaldehyde iodide was prepared from 3-aminopropionaldehyde dimethylacetal according to the method described previously in Chapter 1. Protease inhibitor cocktail Set II was from Calbiochem (Darmstadt, Germany; Table 4.1). TSK-gel G3000SW was from Tosoh (Tokyo, Japan). Standard proteins for gel filtration were from BIO-RAD (Hercules, USA). All other reagents were commercial products of analytical grade from Wako Pure Chemicals Co. Ltd. (Tokyo, Japan).

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>Target protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF hydrochloride</td>
<td>Serine protease</td>
</tr>
<tr>
<td>Bestatin</td>
<td>Aminopeptidase B, leucine aminopeptidase</td>
</tr>
<tr>
<td>E-64, protease inhibitor</td>
<td>Cysteine protease</td>
</tr>
<tr>
<td>EDTA, disodium</td>
<td>Metalloprotease</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Aspartic protease</td>
</tr>
</tbody>
</table>

Table 4.1 Protease inhibitor cocktail
4.2.2 General Methods

Novaspec II from Amersham Pharmachia Biotech was used to check the turbidity. HITACHI HIMAC SCR 18B (rotor: RPR9-2) was used for centrifugation. For measurement of enzyme activity, Shimadzu UV-2100S was used. For HPLC system, Gilson HPLC System was used. Unipoint TM HPLC system control software was used for data analysis. AE-6400 Rapidus-NirenMinisurabu Electrophoresis Apparatus from ATTO Corporation was used for polyacrylamide gel electrophoresis.

4.2.3 Screening for Homocholine degrading Enzymatic Activities

4.2.3.1 Plate replica staining method

An additional screening step was setup in order to narrow the selection range and to see the metabolic ability of the isolated strains. In this step, the isolated strains (30 highly growth strains) were screened for homocholine oxidation activities using plate replica activity staining methods. In this method, the isolated strains were transfer individually using sterilized tooth stick to a single basal-HC agar plate medium. The plate was incubated at 30°C for either 24 or 48 h. The grown colonies were replicated onto a filter paper, treated with 1 ml of lysing solution (0.5% lysozyme and 10 mM EDTA in 100 mM potassium phosphate buffer, pH 8.0) and incubated at 37°C for 30 min. After lysis of the cells, the filter paper was sunk in a solution containing inhibitors of energy-generating system (10 mM of NaN₃, 10 mM of NaF, 1 mM of Na₂AsO₄) for 5 min. Then, the filter paper was frozen and thawed three times to inactivate the energy-generating system. After drying, the dried filter paper was sunk in 2 ml of activity staining solution containing 100 mM of glycine-NaOH buffer (pH 9.5), 64 µM of 1-methoxy phenazine methosulfate, 0.24 mM of nitroblue tetrazolium (NTB), 1 mM of
NAD⁺ and either 2 mM homocholine or 1 mM of TMAPal iodide. The reduction of the NTB lead to the formation of the purple color is an indictor of the enzymatic activity. Strains showed positive result with replica staining were selected and used for preparation of cell free extract to confirm the enzyme activity by the spectrophotometric methods.

4.2.3.2 Cell free extract preparation

To check the enzyme activities of the isolated strains, the strains were inoculated into 75 ml of basal-HC liquid medium and incubated at 25°C for 2 days in reciprocal shaker. At the end of cultivation period, bacterial cells were harvested by centrifugation at 10,000 x g for 20 min and washed with 0.85 % KCl. The harvested bacterial cells were suspended in 30 ml of 0.2 M potassium phosphate buffer pH 7.5 containing 1mM dithiothreitol (DTT) and disrupted by either sonication at 4~11°C for 15 min with 1.5 min run intervals or by glass bead (0.1 mm) on dry ice or in a cold room for 10 cycles with 30 sec for each cycle. Cell free extract was obtained by centrifugation at 10,000 x g for 20 min as supernatant and assayed for dehydrogenase and oxidase activities.

4.2.3.3 NAD⁺-dependent homocholine dehydrogenase

Homocholine dehydrogenase activity was measured as an increase in the absorbance at 340 nm at 30°C. The standard reaction mixture (1.5 ml) contained 225 µmol of Tris-HCl buffer (pH 7.5), 33.3 µmol of homocholine iodide, 3 µmol of NAD⁺ and an appropriate amount of the enzyme. The reaction was started by the addition of NAD⁺ and the enzyme activity was calculated using an extinction coefficient of 6,200 M⁻¹·cm⁻¹ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of
enzyme that catalyzes the formation of 1 μmol of NADH per min under the assay conditions. Specific activity was defined as units of enzyme activity per mg protein.

4.2.3.4 NAD⁺-dependent TMAPaldehyde dehydrogenase

The standard reaction mixture (1.5 ml) contained 225 μmol of potassium phosphate buffer (pH 8.0), 15 μmol of TMAPaldehyde iodide, 3 μmol of NAD⁺, and an appropriate amount of the enzyme. The reaction was started by the addition of NAD⁺ and the enzyme activity was calculated using an extinction coefficient of 6,200 M⁻¹·cm⁻¹ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min under the assay conditions. Specific activity was defined as units of enzyme activity per mg protein.

4.2.3.5 Membrane-bound homocholine dehydrogenase

Membrane-bound homocholine dehydrogenase activity was measured as a decrease in the absorbance at 600 nm at 30°C using phenazine methosulfate (PMS)-2,6-dichlorophenol-indophenol (DCIP) assay system, according to the method of Nagasawa et al. (1976). The assay mixture (1.5 ml) contained 67.5 μmol of potassium phosphate buffer (pH 8.0), 1.5 μmol of potassium cyanide, 0.375 μmol of 1-methoxy PMS, 0.15 μmol of DCIP, 22.5 μmol of homocholine iodide and an appropriate amount of enzyme. The assay was started by the addition of homocholine iodide. The activity was calculated using an extinction coefficient of 21,500 M⁻¹·cm⁻¹ for oxidized DCIP at 600 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmole of DCIP per min under the standard assay conditions.
4.2.3.6 Homocholine oxidase

Homocholine oxidase activity was measured by the modification of the procedure described by Tani et al. (1977). The reaction mixture contained 135 µmol of potassium phosphate buffer (pH 8.0), 45 µmol of homocholine iodide and a suitable amount of enzyme in a total volume of 3.0 ml. The reaction was carried out with shaking at 30°C for 10 min. One ml of the reaction mixture was mixed with 1 ml of 0.1% solution of 2,4-dinitrophenyhydrazine in 1 M HCl and heated in a boiling water bath for 7 min. The mixture was cooled to room temperature and then added to 6.4 ml of distilled water. After the addition of 1.6 ml of 2 M NaOH, the absorbance at 440 nm was measured. TMAPaldehyde was used to create the standard curve to calculate the enzyme activity. One unit of the enzyme activity was defined as the amount of the enzyme that catalyses the formation of 1 µmol of TMAPaldehyde per min under the above assay conditions.

4.2.3.7 NAD⁺-dependent 3-hydroxypropionate dehydrogenase

3-Hydroxypropionate dehydrogenase activity was measured as an increase in the absorbance at 340 nm and at 30°C. The standard reaction mixture (1.5 ml) contained 225 µmol of Tris-HCl buffer (pH 7.5), 33.3 µmol of 3-hydroxypropionate, 3 µmol of NAD⁺ and an appropriate amount of the enzyme. The reaction was started by the addition of NAD⁺ and the enzyme activity was calculated using an extinction coefficient of 6,200 M⁻¹·cm⁻¹ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of NADH per min under the assay conditions. Specific activity was defined as units of enzyme activity per mg protein.
4.2.4 Time Course of the Growth and Enzyme Formation of *Pseudomonas* sp. strains A9

In order to determine the cultivation time needed for high enzyme activity formation, time course experiment was performed. Seed culture was prepared by cultivation of *Pseudomonas* sp. strain A9 in 5 ml basal-HC medium at 25°C for 24h with reciprocal shaking at 122 strokes/min. Then the turbidity of the seed culture was determined by measuring the optical density at 660 nm. At the growth of 2.0 (\( \text{T}_{660} = 2.0 \)), 200 μl of the seed culture broth was inoculated in 300 ml of basal-HC medium. Cultivation was carried out at 25°C on a reciprocal shaker. At intervals of 12, 15, 18, 22, and 26h the growth was estimated and the bacterial cells were collected by centrifugation at 6,000 \( \times \) g (HITACHI HIMAC SCR 18B rotor: RPR9-2) for 30 min at 4°C and washed three times with 0.85% KCl. The harvested bacterial cells were suspended in 10 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 1mM dithiothreitol (DTT) and 1 mM EDTA, and disrupted by glass bead (0.1 mm) on dry ice for 5 min. Cell free extract was obtained by centrifugation at 10,000 \( \times \) g for 30 min as a supernatant and assayed for homocholine dehydrogenase activity.

4.2.5 Stability of Homocholine Dehydrogenase Activity of Strain A9

In order to select homocholine degrading strain, stability of the homocholine dehydrogenase was evaluated. Cell free extract of each selected strain was dialyzed against potassium phosphate buffer, tris-HCl buffer and triethanolamine buffer (50 mM, pH 7.5) containing 1 mM of DTT and 1mM EDTA for overnight at 4°C. The dialyzed extracts were stored at 4°C for 0 to 10 days and the remaining enzyme activity was estimated. The stability of the enzyme against storage was also evaluated. The cell free
extracts were stored at 4°C for 0 to 10 days and the remaining activity was measured. The stability of homocholine dehydrogenase was also examined in the presence of SH protecting reagents such as DTT, 2-mercaptethanol and glutathione, as well as in the presence of stabilizing reagents such as ethanol, glycerol and ethylene glycol.

4.2.6 Formation of Homocholine Dehydrogenase on Various Media

_Pseudomonas_ sp. strain A9 was grown on basal medium supplemented with 1% of quaternary ammonium compounds such as homocholine, choline, TMA-butanol and L-carnitine as a sole source of carbon and nitrogen. The strain was also cultivated on basal-homocholine medium supplemented with either glucose (1%) or glycerol (1%) as a carbon source or ammonium sulfate (0.5%) as a nitrogen source. Cultivation was carried out in 300 ml medium/1L cultural flask on a reciprocal shaker (120 strokes/min) at 25°C for 24h.

4.2.7 Measurement of Protein Content

Protein was measured by Lowry method (Lowry _et al._, 1951) using bovine serum albumin as a standard protein (Fig. 4.1), or by the absorbance at 280 nm.
4.2.8 Measurement of Molecular Mass

The molecular mass of the enzyme was estimated by gel filtration on TSK-gel G3000SW column (0.8 x 30 cm) equilibrated with 0.1 M of potassium phosphate buffer (pH 7.5) containing 1 mM DTT, 1 mM EDTA and 5% ethanol. The standard proteins used were thyroglobulin, γ-globulin, ovalbumin, myoglobin and vitamin B₁₂.

4.2.9 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (native-PAGE) was done using either 10% or 7.5% gels according to the methods of Laemmli (1970). Protein was stained by commassie brilliant blue (CBB) R-250 or was checked for enzyme activity. The reaction mixture contained 150 mM of tris-HCl buffer (pH 7.5), 64 μM of 1-methoxy PMS, 0.24 mM of nitro-tetrazolium blue, 3 mM of homocholine iodide and 1 mM of NAD⁺ (Table 4.2).
Table 4.2 Reaction mixture for activity staining on native-PAGE gels

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl buffer (pH 7.5, 450 mM)</td>
<td>6.70 ml</td>
</tr>
<tr>
<td>Homocholine chloride (500 mM)</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>β-NAD⁺ (60 mM)</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>1-Methoxy PMS (6.4 mM)</td>
<td>0.20 ml</td>
</tr>
<tr>
<td>Nitro tetrazolium blue (2.4 mM)</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.00 ml</td>
</tr>
</tbody>
</table>

4.2.10 Intact and Dry Cell Reaction of *Pseudomonas* sp. Strain A9

To further confirm the metabolic route of homocholine in *Pseudomonas* sp. strain A9, both intact and dry cell reaction experiments were carried out. Intact cell reaction was carried out as described in chapter 3. The only exception is that at intervals of 0, 30, 60, 90, 120, 150 and 180 min, aliquots of the cell suspension were withdrawn and boiled to stop the reaction.

To prepare dried cells, cells suspension was spread on glass Petri dish and dried at room temperature with electric fan. Then the collected cells were further dried in a vacuum discator and finely grounded using mortar. The dried cells (75 mg) were suspended in 18 ml of 50 mM potassium phosphate buffer (pH 7.5). The dried cell reaction was started by the addition of homocholine (20 mM) with or without NAD⁺ (5 mM) to the cell suspension. The suspension was incubated on a shaker at 120 rpm and 30°C. At appropriate time intervals (0, 30, 90, 150, 210, and 300 min), aliquots of the cell suspension were withdrawn and boiled for 3–5 min to stop the reaction. After centrifugation, the supernatant was preserved at -20°C and used for metabolites detection as described in chapter 3. An exception is that TMAPaldehyde concentration in the reaction mixture was quantified by DNPH methods as described elsewhere (Tani 87).
et al., 1977). The amount of TMAPaldehyde in the reaction mixture was calculated from the standard curve that generated using different concentration of TMAPaldehyde.

4.2.11 Detection of 3-Hydroxypropionate by LC/MS/MS

Analyses of the intact cell reaction mixtures of *Pseudomonas* sp. strain A9 and the authentic standard of 3-hydroxypropionate were carried out using a Waters LC/MS instrument consisting of a Waters 2695 liquid chromatograph coupled with a Waters Quatromicro API mass spectrometer. LC separations were made using a 4.6 × 150 mm Waters Symmetry C18 column at room temperature and at flow rate of 0.2 ml/min. Solvent A was 0.1% formic acid in acetonitrile, and solvent B was 0.1% formic acid in distilled water. The following gradient of B was applied: 0 min, 100%; 5 min, 100%; 15 min, 0%; 25 min, 0%; 27 min, 100%. MS analysis was done in the negative ESI-MS/MS mode.
4.3 RESULTS AND DISCUSSION

4.3.1 Screening for Homocholine degrading Activity

Screening of the homocholine oxidation activity in the isolated strains by replica staining and spectrophotometer assays demonstrated that NAD⁺-dependent dehydrogenase enzymes are predominant in homocholine degrader. Another oxidation activities such as oxidase and membrane-bound dehydrogenase were not detected in all isolates. In replica staining screening test, about 10 strains showed positive results (formation of purple color), which indicated the presence of NAD⁺-dehydrogenase activities in these isolates (Fig. 4.2a). Cell free extracts of these isolates again confirmed the presence of both NAD⁺-dependent alcohol and aldehyde dehydrogenase activities (Fig. 4.2b). Those isolates were chosen as the most superior homocholine degraders and were identified to the specific level (as described in chapter 2) according to general principles of microbial classification. From those isolates, *Pseudomonas* sp. strain A9 was selected as a preferred homocholine degrader, since it showed quite higher activity of both NAD⁺-dependent homocholine and TMAPaldehyde dehydrogenases. In the preliminary experiment, these enzymes were found to be unstable during the cell free preparation and against dialysis. Although *Rhodococcus* and *Arthrobacter* strains were also showed both activities but these strains were excluded because cell free extract preparation is quite difficult and need more extraction time that significantly affect the enzyme stability. Moreover, *Rhodococcus* and *Arthrobacter* strains require more cultivation time (48h) compare to *Pseudomonas* strains (24h).
Fig. 4.2 Screening of NAD⁺-dependent dehydrogenase activity in the isolated strains by (A) replica staining and (B) spectrophotometric assays

4.3.2 Time Course of the Growth and Enzyme Formation of *Pseudomonas* sp.

**Strains A9**

The time course of the formation of homocholine dehydrogenase and cell growth of *Pseudomonas* sp. strain A9 was examined in basal-HC medium (Fig. 4.3). The
production of homocholine dehydrogenase activity was significantly increased with the increase in the cell growth (T 660 nm). The maximum enzyme activity formation was observed in late exponential phase at about 24h. Thereafter, the activity was rapidly decreased after 24h cultivation. Hassan (2008) also found that the formation of TMA-Butanol dehydrogenase activity by *Pseudomonas* sp. 13CM was rapidly decreased after 6 h cultivation. This phenomenon looks similar in quaternary ammonium alcohol degrader. To this point we do not know the actual reasons for the rapid decrease in the enzyme activity after maximum formation. It might be resulted from the inhibition of the cellular enzymes by low molecular weight metabolites that accumulated in high concentration in the culture medium during the degradation of the substrate and then penetrate into the cellular components. These metabolites might be homocholine analogue such as trimethylamine. During the growth of strain A9 on homocholine basal medium, the pH of the medium was decreased gradually from 7.0 to 6.3.
Fig. 4.3 Time course of the growth and homocholine dehydrogenase activity formation of *Pseudomonas* sp. strain A9.

Cell-free extract was prepared from cells that grown on 300 ml of culture broth.
4.3.3 Formation of Homocholine Dehydrogenase on Various Media

To assess the expression of homocholine dehydrogenase by *Pseudomonas* sp. strain A9, the bacterium was cultivated on basal media of various homocholine analogues as C and N source, as well as on basal-homocholine medium supplemented with additional C or N sources. The cell free extracts of the above media was prepared and assayed for NAD⁺-dependent homocholine dehydrogenase activity. The results (Table 4.3) showed that homocholine dehydrogenase activity could only be observed in the cell-free extract of cultures grown on homocholine. In assays with cell extracts from cultures grown on choline, 4-N-trimethylamino-1-butanol (TMA-Butanol) and glucose, homocholine dehydrogenase activity could not be observed. It is also notable that glucose exerted a total repression of homocholine dehydrogenase activity induction, since no activity was detected on homocholine-glucose growing cells. Similar observation was reported for betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa* (Nagasawa *et al.*, 1976; Velasco-Garcia *et al.*, 2006). Moreover, the intact cell reaction of strain A9 grown on homocholine and glucose was preformed. The metabolites such as TMAPaldehyde, β-alanine betaine and TMA were only detected in the reaction mixture of homocholine growing cells, whereas they were not detected in the reaction mixture of glucose growing cells. These observations demonstrated that the enzymes responsible for degradation of homocholine were induced during the growth on homocholine. The induction of quaternary ammonium compounds degrading activities was also observed in many reports (Nagasawa *et al.*, 1976; Velasco-Garcia *et al.*, 2006; Setyahadi, 1998) among others.
### Table 4.3 Formation of Homocholine dehydrogenase on various media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth (1% substrate)</th>
<th>Enzyme activity (mU/ml broth)</th>
<th>Specific activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocholine</td>
<td>2.57</td>
<td>6.21</td>
<td>30.0</td>
</tr>
<tr>
<td>Choline</td>
<td>2.65</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>TMA-Butanol</td>
<td>0.58</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Glucose &amp; 0.5% (NH₄)₂SO₄</td>
<td>3.09</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Supplemented with carbon source (0.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>1.63</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.85</td>
<td>3.98</td>
<td>22.2</td>
</tr>
<tr>
<td>Supplemented with nitrogen source (0.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>3.05</td>
<td>9.34</td>
<td>21.5</td>
</tr>
</tbody>
</table>

Basal medium: 1% substrate, 0.2% K₂HPO₄, 0.2% KH₂PO₄, 0.05% MgSO₄, 7H₂O, 0.05% yeast extract, and 0.1% polypeptone.

#### 4.3.4 Stability of Homocholine Dehydrogenase of Pseudomonas sp. Strain A9

In a preliminary experiment, the stability of homocholine dehydrogenase was evaluated under several conditions of storage, pH, stabilizers and SH-group protecting reagents in order to optimize the cell free extract preparation and enzyme assay conditions. The overall observation is that the enzyme is unstable and liable to degradation by other enzymes. Addition of protease inhibitor cocktail besides the dithiothreitol (1mM) stabilized the enzyme to some extent, and after dialysis the activity was significantly decreased. Replacement of protease inhibitor cocktail by EDTA (1 mM) in the buffer gave similar results (data not shown), which indicated that metallo-protease might degrade homocholine dehydrogenase. Moreover, the effect of some stabilizers such as dithiothreitol, glutathione (1mM), ethanol (5%) and ethylene glycol (10%) were also tested. The results (Fig. 4.4) showed that ethanol and ethylene glycol are effective stabilizers of homocholine dehydrogenase activity. About 70% of the enzyme activity was retained after 7 days at 4°C in 50 mM potassium phosphate buffer, pH 7.5,
containing either 5% ethanol or 10% ethylene glycol. Furthermore, different dialysis buffers were examined, and 50 mM potassium phosphate buffer gave better results compared to others buffers. On the other hand, the cell free extract was preserved at 4°C without dialysis. The results (Fig. 4.5) showed that homocholine dehydrogenase retained about 98% of its activity after 3 days. However, after 7 days only 40% of the enzyme activity was retained. This instability of homocholine dehydrogenase makes the purification and characterization of this enzyme is a challenge. In choline degradation pathway, choline dehydrogenase (E.C. 1.1.99.1) is an inner mitochondrial membrane protein that catalyzes the four-electron oxidation of choline to glycine betaine via a betaine aldehyde intermediate and requires an electron acceptor other than oxygen. To date, no in depth biochemical or kinetic characterization has been performed on choline dehydrogenase, mainly due to the difficulty in its purification because of the instability of the enzyme in vitro (Ghanem, 2006). Recently, Gadda and McAllister-Wilkens (2003) reported the first recombinant choline dehydrogenase. This recombinant form choline dehydrogenase was highly unstable in vitro, which hindered any further biochemical and mechanistic investigations of the enzyme (Gadda and McAllister-Wilkens, 2003).
**Fig. 4.4** Effect of stabilizers on the stability of homocholine dehydrogenase of *Pseudomonas* sp. strain A9.

Cell free extract from homocholine-growing cells was prepared by glass bead in 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT and 1 mM EDTA. About 1 ml of this extract was dialyzed overnight against 50 mM potassium phosphate buffer containing either 1 mM DTT (●), 1 mM glutathione (●), 5% ethanol (▲), or 10% ethylene glycol (▲) and stored at 4°C for 1 week.

**Fig. 4.5** Evaluation of the stability of homocholine dehydrogenase during storage at 4°C.
4.3.5 Intact and Dry Cell Reaction of *Pseudomonas* sp. Strain A9

During the degradation of homocholine by resting cells of *Pseudomonas* sp. strain A9, the amount of homocholine decreased concomitantly with the increase of metabolites, identified as TMAPaldehyde, β-alanine betaine and TMA (Fig. 4.6). The results confirmed the sequential oxidation of homocholine to TMAPaldehyde and β-alanine betaine. Thereafter, cleavage of C-N bond of β-alanine betaine provided TMA and alkyl chain.

![Graph showing degradation of homocholine](image)

**Fig. 4.6** Degradation of homocholine by resting cells of *Pseudomonas* sp. strain A9.

Time course degradation of homocholine (■) and the generation of the metabolites, TMAPaldehyde (△) beta-alanine betaine (▲) and TMA (●) by intact cells of *Pseudomonas* sp. strains A9.

Dry cell reaction was carried out using homocholine (20 mM) as a substrate with and without NAD⁺. The results (Fig. 4.7a) showed that addition of NAD⁺ to the reaction mixture significantly increased the degradation rate of homocholine, as well as the production rate of the metabolites TMA and β-alanine betaine. Whereas in the
reaction mixture without NAD⁺, the degradation rate of homocholine, as well as the metabolites formation was very slow (Fig. 4.7b). The slight degradation of homocholine in the absence of added NAD⁺ might be resulted from the remained NAD⁺ with the dried cells. The results demonstrated that the enzymes responsible for the metabolism of homocholine are alcohol and aldehyde dehydrogenases that require NAD⁺ as electron acceptor.

![Graph A](image)

**Fig. 4. 7** Degradation of homocholine by dried cells of *Pseudomonas* sp. strain A9.

Time course degradation of homocholine (■) and the generation of the metabolites beta-alanine betaine (▲) and TMA (●) by dried cells of *Pseudomonas* sp. strains A9. Dry cell reaction was preformed with (A) and without (B) NAD⁺.
4.3.6 Substrate Specificity of Homocholine Dehydrogenase

An attempt was made to determine the substrate spectrum of homocholine dehydrogenase in the crude preparation, because the enzyme is unstable and lost its activity during purification processes. The substrate specificity of homocholine dehydrogenase was determined at 30°C and pH 7.5 using various alcohols and quaternary ammonium compounds in the presence of NAD\(^+\) as a cofactor. Among alcohols tested, DMA-butanol appears to be the most favorable substrate for homocholine dehydrogenase followed by TMA-Butanol, homocholine (TMA-Propanol), 1-butanol, 4-amino-1-butanol and dimethylamino-1-propanol (DMA-Propanol) (Table 4.4). It is particularly interesting that no detectable activity was found for choline, an analogue of homocholine that is found ubiquitously in nature and the ability to degrade choline are widespread amongst microorganisms. Thus, the ability to degrade homocholine does not go alongside with the ability to catabolize choline. Furthermore, the inability of homocholine dehydrogenase to oxidize choline, in accordance with the induction of the enzyme activity with homocholine, ruled out the enzyme to be a choline-oxidizing enzyme. It was also interesting that homocholine dehydrogenase of strain A9 showed high preference to DMA-Butanol and TMA-Butanol, although this bacterium was unable to grow on these substrates. The inability of the bacterium to grow on these substrates as well as the induction of the enzyme only by homocholine also excluded the enzyme to be a TMA-Butanol-oxidizing enzyme. Recently, a NAD\(^+\)-dependent TMA-Butanol dehydrogenase was purified and characterized from *Pseudomonas* sp. 13CM. This enzyme did not react with TMA-Propanol, DMA-Propanol and choline (Hassan, 2008). Thus, it could be assumed that
homocholine dehydrogenase is a new enzyme and is not a choline or TMA-Butanol oxidizing enzyme.

Table 4.4 Substrate specificity of homocholine dehydrogenase from strain A9

<table>
<thead>
<tr>
<th>Substrate (33.3 mM)</th>
<th>Activity (mU/ml)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocoline (CH₃)₃N⁺(CH₂)₃CH₂OH</td>
<td>649</td>
<td>100</td>
</tr>
<tr>
<td>TMA-Butanol (CH₃)₃N⁺(CH₂)₂CH₂OH</td>
<td>902</td>
<td>139</td>
</tr>
<tr>
<td>TMA-Pentanol (CH₃)₃N⁺(CH₂)₄CH₂OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TMA-Hexanol (CH₃)₃N⁺(CH₂)₅CH₂OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Choline (CH₃)₃N⁺CH₂CH₂OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-methyl choline (CH₃)₂N⁺CH(CH₂)OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMA-Propanol (CH₃)₂N(CH₂)₂CH₂OH</td>
<td>141</td>
<td>22</td>
</tr>
<tr>
<td>DMA-Butanol (CH₃)₂N(CH₂)₃CH₂OH</td>
<td>1020</td>
<td>157</td>
</tr>
<tr>
<td>DMA-Hexanol (CH₃)₂N(CH₂)₅CH₂OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-Amino-1-butanol H₂N⁺(CH₂)₃CH₂OH</td>
<td>215</td>
<td>33</td>
</tr>
<tr>
<td>1-Butanol CH₃(CH₂)₂CH₂OH</td>
<td>236</td>
<td>36</td>
</tr>
<tr>
<td>1-Propanol CH₃CH₂CH₂OH</td>
<td>43</td>
<td>7</td>
</tr>
<tr>
<td>2-Propanol CH₃CH(OH)CH₃</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol CH₃CH₂OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methanol CH₃OH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Carnitine (CH₃)₂N⁺CH₂CH(OH)CH₂COOH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Carnitine (CH₃)₂N⁺CH₂CH(OH)CH₂COOH</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

To ascertain if the activity detected in the cell free extract of homocholine growing cells with different substrate was catalyzed by the same enzyme, crude cell free extract was analyzed by native-PAGE, and then, NAD⁺ dependent activity was located in gels using different substrates. The result showed that only single band of activity was detected (Fig. 4.8). The activity band with DMA-buanol as a substrate was much more intense than that with homocholine as a substrate. This result is in good agreement with the activity measured \textit{in vitro} for both substrate (Table 4.4). The results obtained demonstrated that homocholine dehydrogenase has a broad substrate range. Broad
substrate specificities were also reported for many primary and secondary alcohol and aldehyde dehydrogenases (Vangnai and Arp, 2001; Schenkels and Duine, 2000; Tani et al., 2000; Jaureguibeitia et al., 2007; Jo et al., 2008).

Fig. 4. 8 Native-PAGE analysis of crude homocholine dehydrogenase.

Native gels (10%), after PAGE, were stained for NAD$^+$ dependent dehydrogenase activity for 5 min without substrate (lane 1) and with the substrates; homocholine (lane 2), trimethylamino-1-butanol (lane 3) dimethylamino-1-propanol (lane 4), dimethylamino-1-butanol (lane 5), 4-amino-1-butanol (lane 6) and 1-buanol (lane 7)

4.3.7 Measurement of the Native Molecular Mass of Homocholine Dehydrogenase

The native molecular mass of the enzyme was estimated by size exclusion chromatography on TSK-gel. The result showed that the enzyme has a molecular mass of 160 kDa (Fig. 4.9). Such high molecular masses (150~170 kDa) were also reported for medium-chain alcohol dehydrogenase from Acinetobacter sp. strain M-1 (Tani et al., 2000), a nicotinoprotein alcohol dehydrogenase from Rhodococcus erythropolis DSM 1069 (Schenkels and Duine, 2000) and an aldehyde dehydrogenase from Rhodococcus erythropolis UPV-1 (Jaureguibeitia et al., 2007).
4.3.8 Detection of 3-hydroxypropionate Dehydrogenase Activity

As described in chapter 3, β-alanine betaine degraded to TMA and C-3 moiety such as 3-hydroxypropionate. A NAD⁺-dependent 3-hydroxypropionate-dehydrogenase activity was also detected in the cell free extract of *Pseudomonas* sp. strain A9. This activity was only detected in the cell free extract of homocholine-growing cells and no activity was detected on both choline- and glucose-growing cells (Table 4.5). Furthermore, this enzyme activity was also detected on native-PAGE only in the cell free extract of homocholine growing cells (Fig. 4.10). These results demonstrated the induction of 3-hydroxypropionate dehydrogenase activity by homocholine and indicated the presence of 3-hydroxypropionate as an intermediate metabolite in the degradation pathway of homocholine by *Pseudomonas* sp. strain A9. The upper band was assumed to be for homocholine dehydrogenase activity because it appeared in the same position of
homocholine dehydrogenase activity band.

**Table 4.5** NAD⁺-dependent 3-hydroxypropionate dehydrogenase activity in the cell extracts of homocholine-, choline- and glucose-growing cells

<table>
<thead>
<tr>
<th>Medium Substrate</th>
<th>Activity (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocholine</td>
<td>125</td>
</tr>
<tr>
<td>Choline</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 4.10** Native-PAGE analysis of crude 3-hydroxypropionate dehydrogenase.

Non-denaturing gel (7.5% gel) stained for NAD⁺-dependent 3-hydroxypropionate dehydrogenase activity. Lane 1; homocholine-growing cells, lane 2; choline-growing cells, and lane 3; glucose-growing cells.

Moreover, to confirm the presence of 3-hydroxypropionate as an intermediate metabolite, the intact cell reaction mixtures of strain A9 were analyzed by LC-MS. The results demonstrated the accumulation of 3-hydroxypropionate as an intermediate
metabolite (Fig. 4.11). The mass spectrum ($M^+ 90.08$) and the retention time (6.7 min) of the observed metabolite, agreed with those of authentic standard of 3-hydroxypropionate.

![LC/MS/MS analysis of 3-hydroxypropionate](image)

**Fig. 4.11** LC/MS/MS analysis of 3-hydroxypropionate in the degradation pathway of homocholine by the intacted cells of *Pseudomonas* sp. strain A9.

(A) Intact cell reaction mixture of strain A9 and (B) authentic standard of 3-hydroxypropionate.

In a similar study, cleavage of C-N bond of choline by *Candida tropicalis* was
accompanied by formation of trimethylamine and ethylene glycol (Mori et al., 1988). 3-Hydroxypropionate is of special interest in view of that the biodegradable polymers of it can potentially replace lot kinds of traditional petrochemistry-based polymers and be used in some new fields such as surgical biocomposite materials and drug release material (Jiang et al., 2009).

4.4 CONCLUSIONS

This study was carried out to investigate the enzymatic activities in the degradation pathway of homocholine by the isolated strains. Screening of homocholine oxidation activity in the isolated strains by replica staining and spectrophotometer assays showed that NAD⁺-dependent dehydrogenase enzymes are predominant in the isolates. Furthermore, dried cell reaction of Pseudomonas sp. strain A9 cells with homocholine in the presence and absence of NAD⁺ demonstrated that enzymes responsible for the metabolism of homocholine are alcohol and aldehyde dehydrogenases that require NAD⁺ as electron acceptor.

Moreover, in the cell free extract of Pseudomonas sp. strain A9 an inducible NAD⁺-dependent homocholine dehydrogenase was detected. The crude enzyme has broad substrate specificity and was unstable in vitro which makes the purification of the enzyme is a challenge.

Furthermore, an inducible NAD⁺-dependent 3-hydroxypropionate dehydrogenase activity was also detected in the cell free extract of Pseudomonas sp. strain A9. This result indicated the presence of 3-hydroxypropionate as an intermediate metabolite in the degradation pathway of homocholine by Pseudomonas sp. strain A9.
Overall, it could be concluded that in *Pseudomonas* sp. strain A9, homocholine is oxidized to TMAPaldehyde by a NAD$^+$-dependent homocholine dehydrogenase, and consequently, TMAPaldehyde oxidized to β-alanine betaine by a NAD$^+$-dependent aldehyde dehydrogenase. Thereafter, cleavage of β-alanine betaine C-N bond yielded trimethylamine and 3-hydroxypropionate (C-3 moiety). 3-Hydroxypropionate was further oxidized to malonate semi-aldehyde by a NAD$^+$-dependent 3-hydroxypropionate dehydrogenase (Fig. 4.12).

**Fig. 4.12** Proposed degradation pathway of homocholine by *Pseudomonas* sp. strain A9

\[
(CH_3)_3N\text{-}CH_2CH_2CH_2OH \xrightarrow{\text{Homocholine dehydrogenase}} (CH_3)_3N\text{-}CH_2CH_2CHO \xrightarrow{\text{TMAPaldehyde dehydrogenase}} (CH_3)_3N\text{-}CH_2CH_2COOH \xrightarrow{\text{β-alanine betaine}} (CH_3)_3N \xrightarrow{\text{Trimethylamine}} \xrightarrow{3\text{-Hydroxypropionate dehydrogenase}} \text{HOCHCH}_2\text{COOH} \xrightarrow{\text{3-Hydroxypropionate}} \text{OHCCH}_2\text{COOH} \xrightarrow{\text{Malonate semi-aldehyde}} \text{Biomass}
\]
CHAPTER 5
GENERAL CONCLUSIONS

Quaternary ammonium compounds (QACs) are either naturally distributed in the biosphere with more than 100 reported examples including well-known representatives such as choline, glycine betaine and β-alanine betaine or widely used in commercial and consumer applications as disinfectant, fabric softeners, hair conditioners and emulsifying agents. The massive production and utilization of QACs has led to their extensive discharge into the environment, raising concern globally. Biological treatment has been found to be an effective way to degrade QACs and especially aerobic treatment processes can provide rapid biodegradation via bacteria. Although extensive research has been conducted on the microbial degradation of choline and its structurally related compounds, no research has been done on homocholine, a compound that resemble choline in many aspects of cholinergic metabolisms. Furthermore, the reaction catalyzes the oxidation of choline to glycine betaine is of considerable medical and biotechnological applications, because the accumulation of glycine betaine in the cytoplasm of many plants and human pathogens enables them to counteract hyperosmotic environments. Similarly, the study of homocholine degradation enzymes may has potential for the improvement of the stress resistance of plant by introducing an efficient biosynthetic pathway for β-alanine betaine in genetically engineered economically relevant crop plant. Therefore, this research was conducted to investigate the degradation of homocholine by soil microorganisms and to elucidate its degradation pathway.
Pure cultures are indispensable resources to develop an understanding of the degradation pathway of homocholine and the enzymes involved in. In order to explore the degradation ability of homocholine by soil microorganisms, enrichment cultures were prepared from soil samples taken from various locations in Tottori University and around Tottori City. The findings of this study demonstrate that the ability to degrade homocholine is widespread among aerobic microorganisms since representatives of the genus *Arthrobacter*, *Rhodococcus* and *Pseudomonas* were found to grow with homocholine as a sole source of carbon and nitrogen. All these microorganisms are widely distributed in the biosphere, and particularly in soil, they probably play an important role in the degradation of homocholine in the nature. Although there are very few reports on the presence of homocholine in mammalian brains and there is no direct evidence on the presence of such compound in plants. The widespread utilization of homocholine by soil microorganisms provides indirect evidence that the compound is widely exists in the nature and may be in the plant kingdom. In this study, we reported the isolation of four new homocholine degrading microbial species as a first study. Strains belongs to the genus *Arthrobacter*, *Rhodococcus* and *Pseudomonas* were isolated, identified and characterized for their ability to grow with homocholine and its related analogues. With few exceptions, most of the bacteria isolated and identified so far that degrade quaternary ammonium compounds were from the genus *Pseudomonas* and *Arthrobacter*. However, *Rhodococcus* species capable of degrading quaternary ammonium compounds have not yet been reported despite their versatility and ability to degrade numerous organic compounds, including some difficult and toxic compounds. In the present study we are able to isolate *Rhodococcus* strains that metabolized homocholine as a sole source of carbon and nitrogen.
The study in chapter 3 was attempted to characterize and identify the metabolites of homocholine degradation by the isolated strains as well as to elucidate the degradation pathway of the compound by these isolates. During the degradation of homocholine by growing and resting cells of the isolated strains, the amount of homocholine decreased concomitantly with the increase of metabolites, identified as trimethylaminopropionaldehyde, β-alanine betaine and trimethylamine. These findings demonstrate the sequential oxidation of homocholine by these isolates. Thus, the degradation pathway of homocholine was revealed to be through consequent oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), and thereafter cleavage of C-N bond of β-alanine betaine to give trimethylamine and alkyl chain (C3-moiety).

It is particularly notable that the detection of β-alanine betaine as an intermediate metabolite during homocholine degradation by the isolated strains is important from a biotechnological standpoint. We assume that the enzymes responsible for the formation of β-alanine betaine might be useful in biotechnology for the engineering of osmotic stress tolerant crop plants.

This study in chapter 4 was carried out to investigate the enzymatic activities in the degradation pathway of homocholine by the isolated strains. Screening of homocholine oxidation activity in the isolated strains by replica staining and spectrophotometer assays showed that NAD⁺-dependent dehydrogenase enzymes are predominant in all isolates. Furthermore, dried cell reaction of *Pseudomonas* sp. strain A9 cells with homocholine in the presence and absence of NAD⁺ demonstrated that the enzymes responsible for the metabolism of homocholine are alcohol and aldehyde dehydrogenases that require NAD⁺ as electron acceptor. Moreover, in the cell free extract of *Pseudomonas* sp. strain A9 an inducible NAD⁺-dependent homocholine
dehydrogenase was detected. The crude preparation of this enzyme has broad substrate specificity. Although various buffering conditions and stabilizing reagent were applied to stabilize the enzyme activity, the enzyme was found to be unstable in vitro and lose its activity soon after and during the purification processes. This phenomena makes the purification of the enzyme is a challenge. Furthermore, an inducible NAD\(^+\)-dependent 3-hydroxypropionate dehydrogenase activity was also detected in the cell free extract of \textit{Pseudomonas} sp. strain A9. This result indicated the presence of 3-hydroxypropionate as an intermediate metabolite in the degradation pathway of homocholine by this strain. Thus, in \textit{Pseudomonas} sp. strain A9, homocholine is oxidized to trimethylaminopropionaldehyde by a NAD\(^+\)-dependent homocholine dehydrogenase, and consequently, trimethylaminopropionaldehyde oxidized to \(\beta\)-alanine betaine by a NAD\(^+\)-dependent aldehyde dehydrogenase. Thereafter, cleavage of \(\beta\)-alanine betaine C-N bond yielded trimethylamine and 3-hydroxypropionate (C-3 moiety). Thereafter, 3-hydroxypropionate was further oxidized to malonate semi-aldehyde by a NAD\(^+\)-dependent 3-hydroxypropionate dehydrogenase.

Overall, this study provides basic information on the microbial degradation pathway of homocholine and illustrates its degradation metabolites and the enzymes involved in. This information is important in order to explore these metabolites and enzymes in biotechnology to overcome hyperosmotic environmental stresses. Further research will be focused on isolation, characterization and possible application of homocholine-degrading enzymes.
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Isam Ali Mohamed Ahmed
March, 2010
ABSTRACT

Microbial degradation and metabolism of quaternary ammonium compounds such as choline, TMA-butanol, and L- and D-carnitine have been well investigated. Furthermore, the degradation pathways of these compounds in microorganisms have been studied. Although extensive research has been conducted on the microbial degradation of choline and its structurally related compounds, no research has been done on homocholine, a compound that resemble choline in many aspects of cholinergic metabolisms. Therefore, this research was conducted to investigate the degradation of homocholine by soil microorganisms and to elucidate its degradation pathway.

Chapter 1 defines the aim of the work, provides a literature review of the relevant studies on the distribution and function of quaternary ammonium compounds, and also presents the microbial degradation of quaternary ammonium compounds.

In chapter 2, the isolation, characterization and identification of homocholine degrading microorganism were described. Thirty strains with high growth on homocholine medium were selected from 142 strains grown on homocholine. From these strains, four strains showed the highest growth and homocholine degrading rate were selected. Strains belong to the genus *Arthrobacter*, *Rhodococcus* and *Pseudomonas* were identified and characterized by morphological, biochemical and genetical analysis. With few exceptions, most of the bacteria isolated and identified so far that degrade quaternary ammonium compounds were from the genus *Pseudomonas* and *Arthrobacter*. However, *Rhodococcus* species capable of degrading quaternary ammonium compounds have not yet been reported, in the present study we are able to
isolate *Rhodococcus* strains that metabolized homocholine as a sole source of carbon and nitrogen.

The study in chapter 3 was attempted to characterize and identify the metabolites of homocholine degradation by *Arthrobacter* sp. strain E5, *Rhodococcus* sp. strain A2 and *Pseudomonas* sp. strain A9 as well as to elucidate the degradation pathway of the compound by these isolates. The degradation of homocholine by the resting cell suspensions and growing cell cultures of these strains and the detection of formed metabolites were tested by capillary electrophoresis, GC-MS and FAB-MS methods. During the degradation of homocholine by growing and resting cells of these strains, the amount of homocholine decreased concomitantly with the increase of metabolites, identified as trimethylaminopropionaldehyde, β-alanine betaine and trimethylamine. These findings demonstrate the sequential oxidation of homocholine by these isolates. Thus, the degradation pathway of homocholine was revealed to be through consequent oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), and thereafter cleavage of C-N bond of β-alanine betaine to give trimethylamine and alkyl chain (C3-moiety).

The study in chapter 4 was carried out to investigate the enzymatic activities in the degradation pathway of homocholine by the isolated strains. Screening of the homocholine oxidation activity in the isolated strains by replica staining and spectrophotometer assays showed that NAD⁺-dependent dehydrogenase enzymes are predominant in all isolates. Furthermore, dried cell reaction of *Pseudomonas* sp. strain A9 cells with homocholine in the presence and absence of NAD⁺ demonstrated that the enzymes responsible for the metabolism of homocholine are alcohol and aldehyde dehydrogenases that require NAD⁺ as electron acceptor. Moreover, in the cell free
extract of *Pseudomonas* sp. strain A9 an inducible NAD$^+$-dependent homocholine dehydrogenase was detected. The crude preparation of this enzyme has broad substrate specificity. Although various buffering conditions and stabilizing reagent were applied to stabilize the enzyme activity, the enzyme was found to be unstable *in vitro* and lose its activity soon after and during the purification processes. Furthermore, an inducible NAD$^+$-dependent 3-hydroxypropionate dehydrogenase activity was also detected in the cell free extract of *Pseudomonas* sp. strain A9. This result indicated the presence of 3-hydroxypropionate as an intermediate metabolite in the degradation pathway of homocholine by this strain. Thus, in *Pseudomonas* sp. strain A9, homocholine is oxidized to trimethylaminopropionaldehyde by a NAD$^+$-dependent homocholine dehydrogenase, and consequently, trimethylaminopropionaldehyde oxidized to β-alanine betaine by a NAD$^+$-dependent aldehyde dehydrogenase. Thereafter, cleavage of β-alanine betaine C-N bond yielded trimethylamine and 3-hydroxypropionate (C-3 moiety). Thereafter, 3-hydroxypropionate was further oxidized to malonate semi-aldehyde by a NAD$^+$-dependent 3-hydroxypropionate dehydrogenase.

Overall, this study provides basic information on the microbial degradation pathway of homocholine and illustrates its degradation metabolites and the enzymes involved in. This information is important in order to explore these metabolites and enzymes in biotechnology to overcome hyperosmotic environmental stresses. Further research will be focused on isolation, characterization and possible application of homocholine degrading enzymes.
摘要

コリン、トリメチルアミノプタノール、L-カルニチン、D-カルニチンなどの第4級アンモニウム化合物の分解・代謝の研究は詳細になされてきた。コリンやその構造類似体の微生物分解に関する研究は数多くあるが、ホモコリンはコリン作動性代謝の面から見てコリンと類似の化合物であるにもかかわらず、その微生物分解に関する研究はなされていない。そこで、本研究ではホモコリン分解菌を土壌微生物に探索し、その分解経路を明らかにすることを目的とした。

第1章では、本研究の目的を述べ、第4級アンモニウム化合物の分布やその機能についての研究及び第4級アンモニウム化合物の微生物分解に関する研究について概説した。

第2章ではホモコリン分解微生物の単離と同定が述べられている。ホモコリンに生育できる142菌株を土壌から分離し、生育が良好であった30菌株を選抜した。さらに、ホモコリン分解速度が速い菌株として4菌株を選抜した。形態学的観察、生化学的性質の検討及び分子生物学的分析により、これら4菌株はArthrobacter属、Rhodococcus属、Pseudomonas属に属する細菌であることが明らかとなった。今までに報告されている第4級アンモニウム化合物を分解できる菌のほとんどはArthrobacter属あるいはPseudomonas属の細菌であり、本研究でも同様の結果であった。一方、これまで第4級アンモニウム化合物を分解できるRhodococcus属細菌は報告されておらず、本研究での分離が初めての例である。

第3章ではArthrobacter sp. strain E5、Rhodococcus sp. strain A2、Pseudomonas sp. strain A9によるホモコリン分解の代謝産物の同定を試みた。選抜した3菌株のホモコリン培養液や休止細胞反応液中の代謝産物をキャピラリー電気泳動、GC-MS、FAB-MSにより検出・同定を行った。培養基質であるホモコリンの減少に伴い、トリメチルアミノプタノール、オルテヒド、β-アラニペプタイシン、トリメチルアミノと同定された代謝産物が増加した。このような結果から、ホモコリンの分解経路として、ホモコリン分子中のアルコール基がアルデヒド基、カルボキシル基へと酸化され、さらに,
β-アラニンペタイン中の炭素-窒素間の結合が切断され、トリメチルアミンとアルキル鎖（C3部分）を生じるような経路が存在することを明らかにした。

第4章ではホモコリんで分解経路に関与する酵素について検討した。ホモコリン分解菌として分離した30菌株中のホモコリン酸化活性をレプリカ法と分光度法でスクリーニングし、ほとんどの菌株にはNAD依存性の脱水素酵素が存在することが明らかとなった。さらに、Pseudomonas sp. strain A9の風乾菌体を用いて酵素活性を検討したところ、ホモコリンの酸化にはNAD依存性のアルコール脱水素酵素とアルデヒド脱水素酵素が関与していることが明らかとなった。また、A9株の無細胞抽出液中にNAD依存性のホモコリン脱水素酵素活性を見出し、本酵素は誘導酵素であることを証明した。粗酵素液を用いて基質特異性を検討したところ、広い特異性を示した。酵素精製に先立ちNAD依存性ホモコリン脱水素酵素活性の安定化を検討したが、格段に安定化させる条件を見出すことはできなかった。また、NAD依存性3-ヒドロキシプロピオン酸脱水素酵素活性をPseudomonas sp. strain A9の細胞抽出液中に見出し、誘導的に生成することを明らかにした。このことは、3-ヒドロキシプロピオン酸が本菌のホモコリン分解の代謝中間体であることを示している。以上の結果から以下のような分解経路を推定した。Pseudomonas sp. strain A9では、ホモコリンはトリメチルアミンプロピオンアルデヒドを経由してβ-アラニンペタインに酸化される。これらの反応を触媒する酵素はNAD依存性ホモコリン脱水素酵素とNAD依存性アルデヒド脱水素酵素である。次に、β-アラニンペタインはそのC-N結合が開裂を受け、トリメチルアミンと3-ヒドロキシプロピオン酸が生成する。さらに、3-ヒドロキシプロピオン酸はNAD依存性3-ヒドロキシプロピオン酸脱水素酵素により酸化され、マロン酸セミアルデヒドとなる。
LIST OF ORIGINAL PUBLICATIONS

(1) Mohamed Ahmed, I.A., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N.
Isolation and characterization of 3-N-trimethylamino-1-propanol degrading
This paper partly covers Chapter 2 and 3 in the thesis.

(2) Mohamed Ahmed, I.A., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N.
Isolation and characterization of 3-N-trimethylamino-1-propanol degrading
This paper partly covers Chapter 2 and 3 in the thesis.

(3) Mohamed Ahmed, I.A., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N.
Isolation and characterization of homocholine degrading *Pseudomonas* sp. strain
A9 and B9b. *World Journal of Microbiology and Biotechnology* (In press, DOI:
10.1007/s11274-010-0320-z).
This paper partly covers Chapter 2 and 3 in the thesis.