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Isolation and Characterization of 3-*N*-Trimethylamino-1-Propanol Degrading *Arthrobacter* sp. Strain E5

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Abstract: The aim of this study was to screen for microorganism that able to utilize 3-*N*-trimethylamino-1-propanol (homocholine) as sole source of carbon and nitrogen and to see which mechanism is followed in the degradation of this compound by soil microorganisms. A gram-positive bacterium, designated, as strain E5 was isolated from soil. The strain was identified as *Arthrobacter* sp. strain E5 based on the phenotypic features, physiologic and biochemical characteristics and phylogenetic analysis. The cells of strain E5 displayed primary branching at the exponential phase and fragmented into irregular rod and coccoid elements at the stationary phase. The colonies were yellow in color, convex, round and entire with smooth and regular margins on both homocholine and nutrient agar medium. Comparative 16S rDNA sequencing studies indicated that strain E5 fall into *Arthrobacter nicotinovorans* subclade where it forms a monophyletic group with the type strains of *Arthrobacter nicotinovorans* and *Arthrobacter histidinolovorans*. Metabolites analysis by capillary electrophoresis and gas chromatography-mass spectrometry showed trimethylamine as a major metabolite beside β-alanine betaine and trimethylaminopropionaldehyde. Therefore, the possible degradation pathway of homocholine in *Arthrobacter* sp. strain E5 is through consequence oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), respectively and thereafter cleavages of C-N bond providing trimethylamine and alkyl chain.

Key words: Quaternary ammonium compounds, homocholine, 3-*N*-trimethylamino-1-propanol, 16S rDNA gene sequence, biodegradation

INTRODUCTION

3-*N*-trimethylamino-1-propanol (homocholine) an analogue of choline, in which the amino alcohol group is lengthened by one CH_2-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 Aspry, 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 the form of phosphatidylcholine. It is also a precursor of membrane and lipoprotein phospholipids and the neurotransmitter acetylcholine; it thus is important for the integrity of cell membranes, lipid metabolism and cholinergic nerve function (Zeisel and Blusztajn, 1994; Zeisel, 2000). From the simple and choline-like structure, one would expect that homocholine is degraded in

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a similar way and to rates comparable to that of choline. To date, no microorganism degrading homocholine as only 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 for its degradation.

Arthrobacter is a common genus of Gram-positive, obligate aerobic soil bacteria characterized by pleomorphism 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 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. In this ongoing study on the degradation of homocholine by soil microorganisms we isolated and identified many bacterial strains of the genus *Rhodococcus*, *Pseudomonas* and *Arthrobacter*. In this study, we reported the isolation and identification of *Arthrobacter* sp. strain E5 grown on homocholine as the only sole source of carbon and nitrogen.

MATERIALS AND METHODS

Materials

3-dimethylamino-1-propanol (DMA-Propanol), 3-dimethylaminopropionic acid (DMA-Propionic acid), 3-aminopropionaldehyde diethylacetal and *p*-bromophenacyl bromide were purchased from Tokyo kasai (Tokyo, Japan). Gram staining kit, methyl iodide and benzoyl chloride were purchased from Wako Chemicals (Tokyo, Japan). *O*-(4-nitrobenzyl) hydroxylamine was purchased from Fluka (Buchs, Switzerland). Primers used in this study were obtained from Sigma Genosys (Sigma, Japan). Unless otherwise specified, all other reagents were of analytical grade from either Wako (Tokyo, Japan) or Sigma (St. Louis, MO, USA).

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).

3-*N*-trimethylaminopropionaldehyde was prepared from 3-aminopropionaldehyde diethylacetal) by treatment with methyl iodide according to the method of Hassan *et al*. (2007).

3-*N*-trimethylaminopropionic acid (β-alanine betaine) was synthesized by *N*-methylation of dimethylaminopropionic acid (Tokyo kasai, Tokyo, Japan) with methyl iodide. Methyl iodide (4 mL) was added to a suspension of dimethylaminopropionic acid (1 g, 6.5 mmol) and $KHCO_3$ (1.3 g, 13 mmol) in 20 mL of methanol and the mixture was stirred overnight at room temperature. The mixture was decanted and the liquid phase was concentrated and the residue was extracted with a mixed solvent (acetonitrile: methanol = 10:1, v/v, 15 mL×3). The combined extracts were dried under stream of nitrogen to give β-alanine betaine as a colorless powder (1.2 g, 63.2%). The structure and purity were checked by ^{1}H NMR spectrum (JEOL-ECP 500 MHZ, NMR spectrometer) and capillary electrophoresis.

Isolation, Growth and Maintenance of Homocholine Degrading Bacteria

Enrichment cultures of soil samples from different location in Tottori University and around Tottori city were used to obtain homocholine-degrading strain. Approximately 100 mg of each soil

sample was inoculated into 5 mL basal medium (basal-homocholine media) containing 5 g L^{-1} homocholine as sole source of carbon and nitrogen; 2 g KH_2PO_4; 2 g K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g yeast extract and 1 g polypeptone (pH 7.0). Cultivation was done at 30°C for 2 to 7 days in reciprocal shaker at 144 rpm. 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 and/or meat extract and subsequently 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).

Morphological, Biochemical and Physiological Characterization

The morphological characterization of the cells was examined by using Olympus BX 41 optical microscope (Olympus corporation, Tokyo, Japan) under light and phase contrast conditions. The Gram-reaction was tested using Gram-staining kit from Wako (Tokyo, Japan) according to the manufacture instruction manual and by the KOH method (Buck, 1982). Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Motility was checked by water hanging drop method. Utilization of different carbon sources and selected metabolic activities were investigated by using API 20 NE commercial available test kit (Biomerieux, Geneva, Switzerland) following the company instruction manual. The utilization of selected carbon compounds and combined carbon and nitrogen sources was tested in liquid culture (5 mL) at pH 7.0 by following turbidity using basal media supplemented with 0.5% of the compound of choice. All tests were inoculated with cells taken from the colonies pre-grown on basal-HC media. Cultures were incubated at 25°C on shaker for 48 h and all tests were done in triplicates.

Sequencing of 16S-rDNA Gene

To identify the isolated bacteria, 16S rDNA gene fragments were amplified by colony PCR with the following primers sets: 314F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'). The resultant PCR products were then used for sequencing. The 16S rDNA sequences were compared with those available in the DDBJ database using both FASTA and BLASTN algorithms and aligned using Clustal W, ver. 1.83 with default settings. The evolutionary tree for the datasets was constructed by using the neighbour-joining method of Saitou and Nei (1987) and viewed with TreeView software. The confidence of the resultant tree topologies were evaluated by performing bootstrap analysis of the neighbour-joining method based on 1000 re-samplings.

Isolation and Quantification of the Metabolites of Homocholine

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 10000 g for 20 min at 4°C. The harvested cells were washed with saline solution (8.5 g L^{-1} KCl) before being centrifuged again at 10000 g for 20 min at 4°C. Intact cell reaction was carried out at 30°C under shaking condition and at intervals of 30 min, 1, 2, 3 and 6 h aliquots were withdrawn and the reaction was stopped by boiling for 3~5 min. After centrifugation, the supernatants of the culture were divided into four parts and processed as follows:

- The supernatant (1 mL) was treated with 0.5 mL of 8 M NaOH and the metabolite was extracted with 0.5 mL toluene. The upper layer contained the metabolite trimethylamine (TMA), which was identified by GC-MS as described below

- The supernatant (0.1 mL) was benzoylated with benzoyl chloride (100 mg mL^{-1} in pyridine) and analyzed by low pH capillary electrophoresis (Zhang *et al*., 2001)
- The supernatant (0.1 mL) was treated with 0.3 mL of *O*-(4-nitrobenzyl) hydroxylamine (5 mg mL^{-1} in methanol) and the metabolite trimethylaminopropionaldehyde-oxime was detected by low pH capillary electrophoresis (Zhang *et al*., 1997)
- The supernatant (0.1 mL) was esterified with *p*-bromophenacyl bromide following the methods of Nishimura *et al*. (2001) and the metabolites β-alanine betaine (β-AB) and TMA were detected by low pH capillary electrophoresis

Analytical Methods

Degradation of homocholine and the production of metabolites by intact cells of *Arthobacter* sp. strain E5 were detected by capillary electrophoresis and GC-MS methods.

Capillary electrophoresis (CE) 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. and 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, 270, 254 nm.

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out using JEOL AX505HA mass spectrometer (JEOL, Tokyo, Japan) with electron-impact ionization (70 eV) coupled with Hewlett Packard 5890 series II gas chromatography (Wilmington, DE, USA). A fused silica capillary column (0.25 mm i.d., 30 m long) packed with DB1 (J and W scientific, Folsom, CA, USA) was used. Helium was used as carrier gas at a flow rate of 15 mL min^{-1}. Column temperature was maintained at 50°C and samples (1 µL) were injected to the GC at an injection port temperature of 250°C.

RESULTS AND DISCUSSION

Enrichment, Isolation and Growth of Strain E5

By the enrichment for homocholine degrading microorganisms under conditions described above, 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.

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. 1). The colonies are yellow in color, convex, round and entire with smooth and regular margins on both homocholine and nutrient agar medium. 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 grown at either 4 or 41°C. Some other physiochemical properties of strain E5 are shown in Table 1. Phenotypic and physiochemical tests suggest that the isolated strain E5 is belongs to the genus *Arthrobacter*.

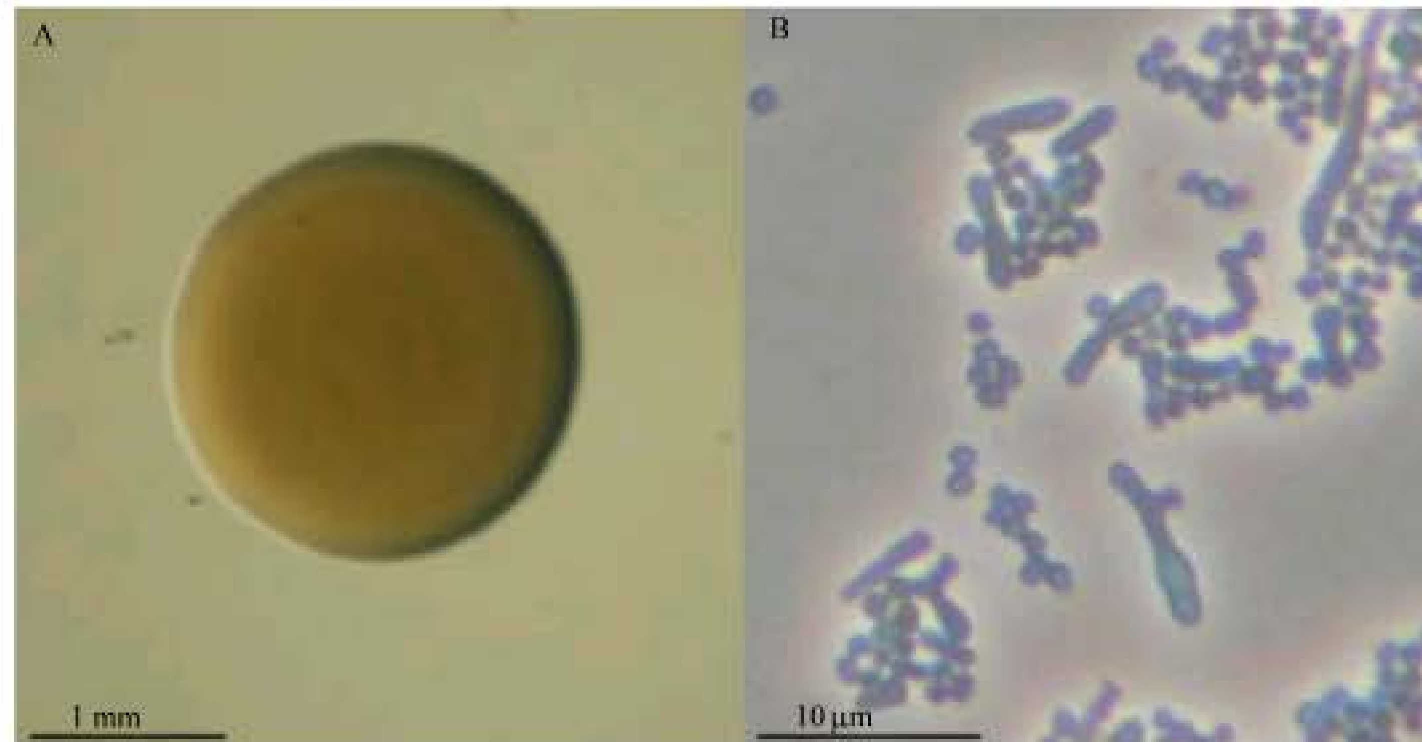

Fig. 1: Morphology of isolated strain *Arthrobacter* sp. E5 as seen by light microscopy under (A) optical (B) phase contrast conditions. The colony morphology in panel (A) and the morphological differentiation in cells shape and size after cultivation for 48 h (B) are typical of gram-positive bacteria

Table 1: Morphological and physiological characteristics of *Arthrobacter* sp. strain E5

Parameters	Characteristics	Parameters	Characteristics
Morphogenic sequence	R-C	Assimilation of	
Colony morphology	Entire and convex	D-glucose	+
Colony color	Yellow	L-arabinose	+
Gram reaction	+	D-mannose	+
Motility	Non	D-mannitol	+
Oxidase	-	N-acetyl-glucosamine	+
Catalase	+	D-maltose	+
Urease	-	Gluconate	+
Reduction of nitrates to nitrite	-	Caprate	-
Reduction of nitrates to nitrogen	-	Adipate	-
Indol production	-	Malate	+
Glucose fermentation	-	Citrate	+
Arginine dehydrolase	-	Phenyl acetate	+
β-galactosidase	+	Growth with (0.5%)	
β-glucosidase	+	Propionate	+
Hydrolysis of		Acrylate	+
Esculin	-	3-hydroxypropinate	-
Gelatin	+	Malonate	-
Growth at		Homocholine	+
4°C	-	β-alaninebetaine	+
37°C	+	Dimethylaminopropanol	+
45°C	-	Dimethylaminopropionic acid	w
pH 5.0	-	Trimethylamine	w
pH 8.0	+	Dimethylamine	+
		Monomethylamine	w

+: Positive utilized; w: Weakly positive; -: Negative; R-C: Rod-coccus growth cycle

16S rDNA Gene Sequence Analysis

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 rDNA gene sequence was compared with those of representative members of the genus *Arthrobacter*. The 16S rDNA data supported the results of morphological and phenological analysis. The phylogenic tree drawn from the partial 16S rDNA

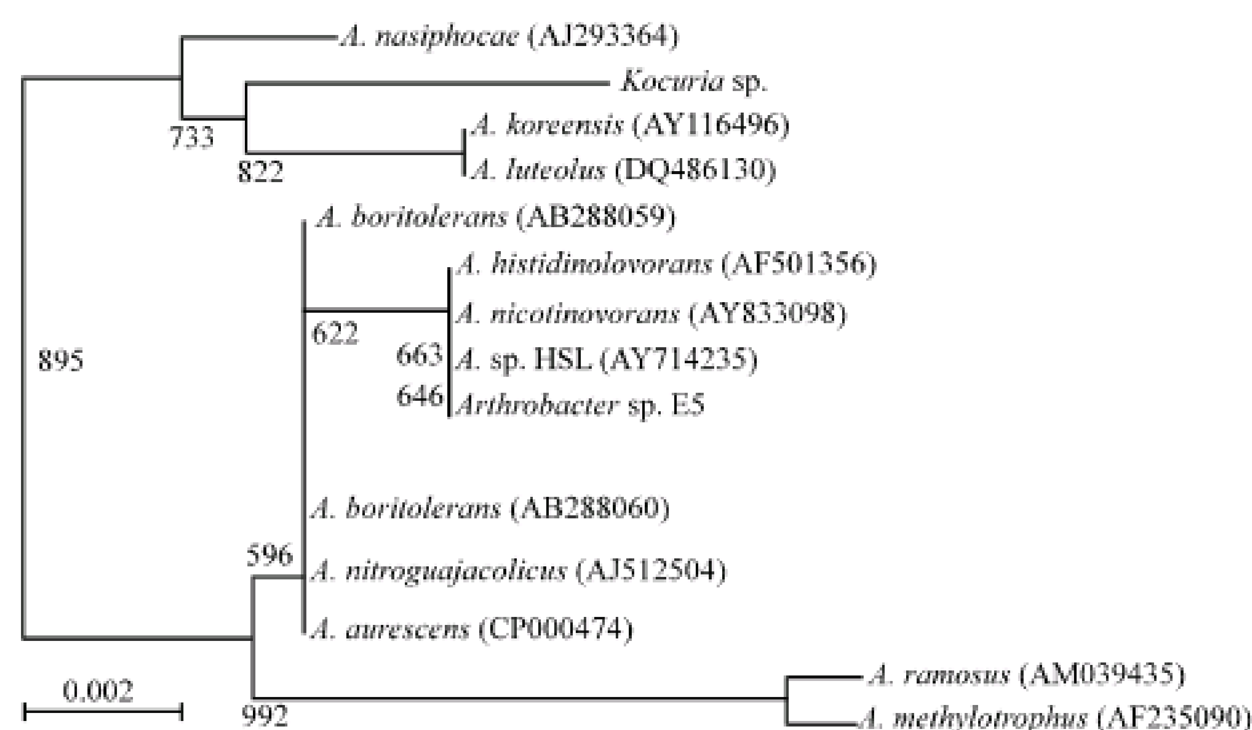

Fig. 2: Phylogenetic tree based on 16S rDNA sequence showing the relationship between strain E5 and most closely related species of the genus *Arthrobacter*. Numbers at nodes indicate level of bootstrap support ≥50%, based on a neighbour-joining analysis of 1000 re-sampled datasets. Bar = 0.005 nucleotide substitution per nucleotide position

sequence (544 nt) of the isolated strain E5 (accession number FJ595954) clearly demonstrated that this strain belongs to the *Arthrobacter nicotinovorans* 16S rDNA subclade (Fig. 2). The organism was most closely related to the type strains of *Arthrobacter nicotinovorans* (accession number AY833098) with a homology of 99.8%, to *Arthrobactes* sp. HSL strain (accession number AY714235) with homology of 99.8% and to *Arthrobacter histidinolovorans* (accession number AF501356) with homology of 99.8%. The high level of similarity observed between the 16S rDNA sequence of the isolated strain E5 and several *Arthrobacter* species suggest that strain E5 could be a strain of one of those species. However, it is accepted that 16S rDNA sequence comparison may indicate species level identification with 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*.

Utilization of Several C N Sources 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 1). 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 were utilized for growth by the isolated strain E5. Out of the tested substrates, C_1-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 N-containing compounds that have dimethyl group instead of tri- and mono methyl group. Most of the substrates tested as carbon sources were utilized for growth except caprate, adipate, malonate and 3-hydroxypropionate.

Degradation of Homocholine by the Resting Cells of Strain E5

The degradation of homocholine by the resting cells of strain E5 and the detection of formed metabolites were tested by capillary electrophoresis and GC-MS methods. During the consumption of homocholine by the resting cells of strain E5, there were a concurrent formation and accumulation of some soluble metabolites as detected by capillary electrophoresis analysis (Fig. 3). These metabolites were found to be trimethylamine (peak 3, TMA) and β-alanine betaine (peak 4, β-AB) as

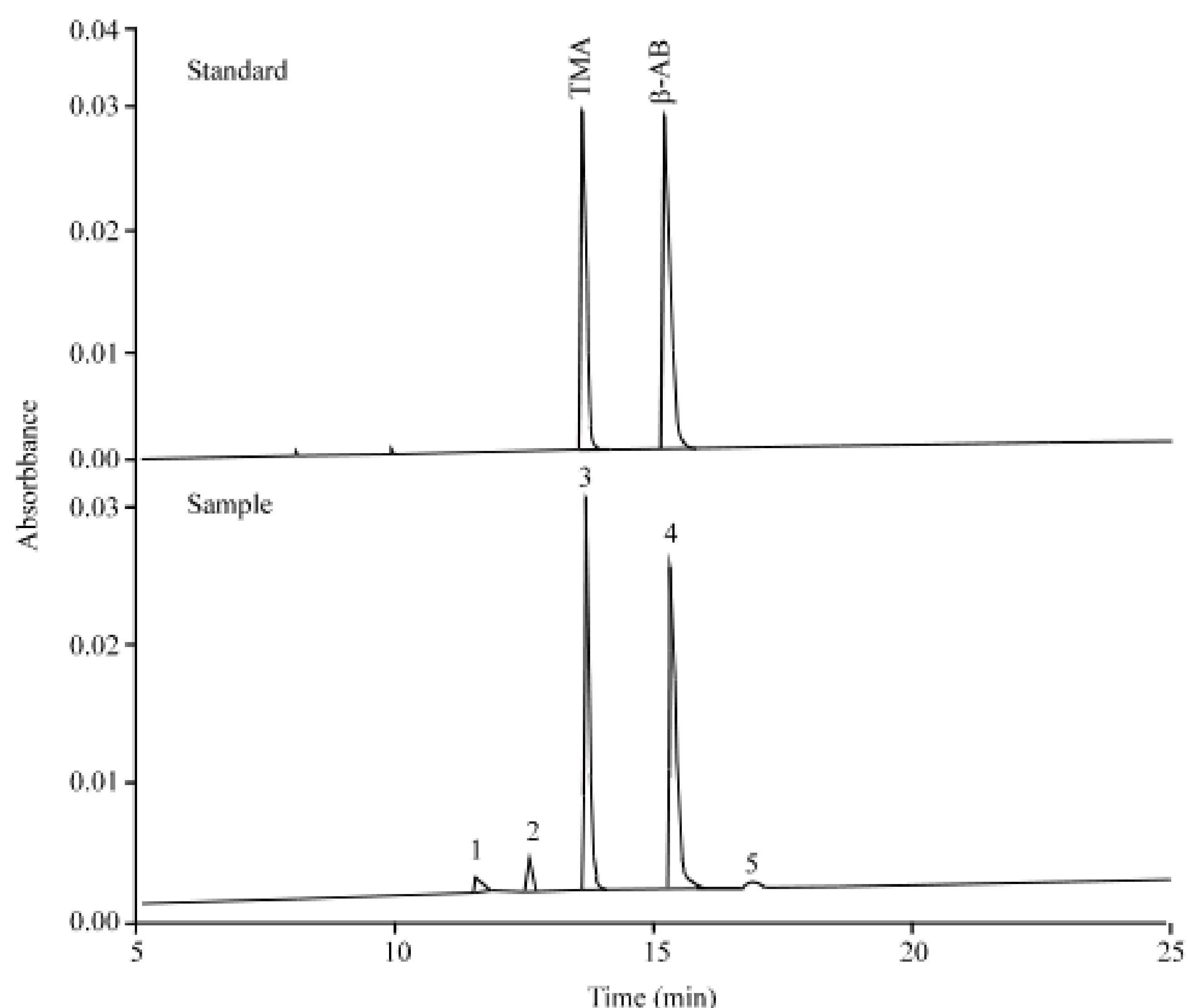

Fig. 3: Capillary electrophoresis chromatogram of the culture filtrate of strain E5 and authentic standards of trimethylamine (TMA) and β-alanine betaine (β-AB). Peaks 3 and 4 were coincided with the authentic standards of TMA and β-AB, respectively

compared with the authentic standards of TMA and β-AB. The first evidence for the accumulation of TMA was the remarkable fishy-odor of the culture filtrate. Further analysis of the culture filtrate and the intact cell reaction products by GC-MS confirmed the accumulation of TMA (data not shown). 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. The time course degradation of homocholine by resting cells of strain E5 is shown in Fig. 4. Resting cells of strain E5 degraded homocholine to near completion in 6 h and the metabolites trimethylaminopropionaldehyde and β-AB formed and metabolized when the reaction elevated. There is also accumulation of TMA with the degradation of homocholine. It is clear that the formation of TMA was resulted from cleavage of C-N bond of the β-AB. This was further confirmed by the detection of TMA in the culture filtrate of strain E5 grown on β-AB as sole source of carbon and nitrogen. Moreover, the intact cell reaction of the cells of strain E5 with β-AB showed again the accumulation of TMA as major metabolite. Similarly, the formation of TMA and complete degradation of the carbon skeleton has been reported in the utilization of many quaternary ammonium compounds (QACs) such as; choline, γ-butyrobetaine, DL-carnitine by *Candida tropicalis*, *Acinetobacter calcoaceticus*, *Pseudomanas putida* and *Proteus vulgaris*, respectively (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, always involves the breakage of C-N linkages (Tiedje *et al*., 1973; Van Ginkel *et al*., 1992). Initial cleavage of the C-N bond, was proposed to be a general strategy of microorganisms to gain access to the alkyl chains of quaternary ammonium compounds (Van Ginkel, 1996). Although present study showed the cleavage of C-N bond as in the aforementioned studies, but this was not the initial step in the degradation of

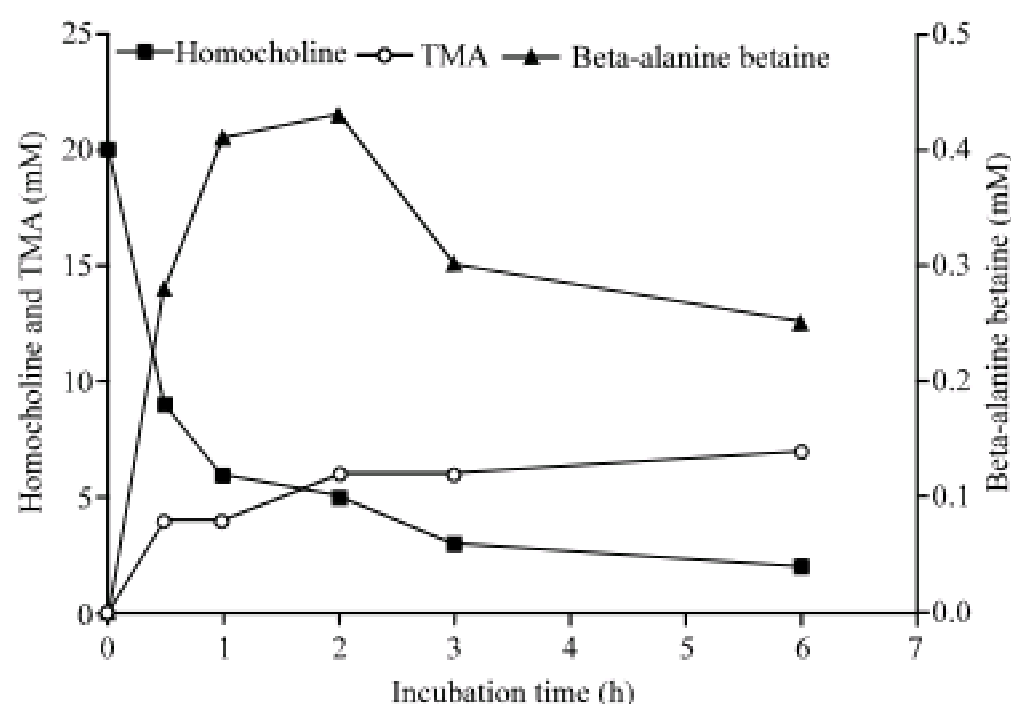

Fig. 4: Degradation of homocholine (20 mM homocholine) and accumulation of trimetylamine (TMA) and β-alanine betaine by a washed cell suspension of *Arthrobacter* sp. strain E5 pre-grown on homocholine as sole of carbon and nitrogen

homocholine by strain E5. This demonstrated the novel degradation pathway of homocholine by strain E5 that is quite different than the above-mentioned studies. In the present study, we found that the alcohol group of homocholine was consequently oxidized to aldehyde and carboxyl group and thereafter cleavage of C-N bond to give TMA and alkyl chain. Although the possibility of the demethylation pathway, from β-AB to dimethylaminopropionic acid, 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.

Interestingly, the detection of β-alanine betaine as an intermediate in the degradation pathway of homocholine by the isolated strains is of much importance from the biotechnological standpoint. Thus, we assumed that the enzymes responsible for the formation of β-alanine betaine might be useful in biotechnology for the engineering of osmotic stress tolerant crop plant. It was proposed that genetic engineering of osmotolerance in plants could be achieved by producing betaine in nonaccumulators (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 (Alia *et al*., 1998; Sakamoto *et al*., 1998).

CONCLUSION

We report here the isolation of *Arthrobacter* sp. strain E5 as the first strain that metabolized homocholine as the only source of carbon and nitrogen. The general strategy of this strain to metabolize homocholine was assumed to be through consequence oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), respectively and thereafter cleavages of C-N bond providing trimethylamine and alkyl chain. Our further research is focused on the enzymatic degradation pathway of homocholine to elucidate the enzymatic mechanisms. This information is important for better understanding the metabolic pathway of short chain quaternary ammonium compounds.

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