Utilization of 3-*N*-trimethylamino-1-propanol by *Rhodococcus* sp. strain A4 isolated from Natural Soil

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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 a sole source of carbon and nitrogen. The aerobic degradation of homocholine has been found by a gram-positive *Rhodococcus* sp. bacterium isolated from soil. The isolate was identified as *Rhodococcus* sp. strain A4 based on the phenotypic features, physiologic and biochemical characteristics, and phylogenetic analysis. The cells of the isolated strain grown on both basal-TMAP and nutrient agar medium displayed elementary branching mycelia fragmented into irregular rod and coccoid elements. Comparative 16S rDNA sequencing studies indicated that the strain A4 falls into the *Rhodococcus erythropolis* subclade and forms a monophyletic group with the type-strains of *R. opacus*, and *R. wratislaviensis*. Metabolites analysis by capillary electrophoresis, fast atom bombardment-mass spectrometry, and gas chromatography- mass spectrometry, showed trimethylamine (TMA) as the major metabolite beside β-alanine betaine and trimethylaminopropionaldehyde. Therefore, the possible degradation pathway of trimethylamino propanol in the isolated strain is through consequence oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), and thereafter the cleavage of β-alanine betaine C-N bonds yielded trimethylamine and alkyl chain.

Keywords—Homocholine, 3-*N*-trimethylamino-1-propanol, Quaternary ammonium compounds, 16S rDNA gene sequence.

I. INTRODUCTION

QUATERNARY ammonium compounds (QACs) are widely distributed in the biosphere with more than 100 reported examples, including such well-known representatives as choline, glycine betaine, and L-carnitine [1]. They have different biological functions, such as adaptation of the organisms toward the environmental stresses and transportation of chemical groups in a number of metabolic processes among others [1]. It is well known that choline is constituent of cell membrane as phospholipids, glycine betaine is an osmoprotectant in plants and microorganisms, and L-carnitine plays an important role during beta-oxidation of activated fatty acids in mitochondria. Homocholine is similar to choline in many aspects of cholinergic metabolisms. 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 ([3], [5]). It is also effective in preventing fat infiltration both in fat and cholesterol fatty livers [4]. From the choline-like structure, one would expect that homocholine could be degraded in a similar way and rate 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.

The genus *Rhodococcus* is comprised of genetically and physiologically diverse bacteria, which have been isolated from various habitats such as soil and seawater plants [9]. The large genome of rhodococci, the redundant and versatile catabolic pathways, the ability to uptake and metabolize various organic compounds such as aliphatic and aromatic hydrocarbons, halogenated compounds, nitriles and various herbicides, make them suitable industrial microorganisms for biotransformation and the biodegradation of many environmentally important organic compounds [10]. In this report, we have isolated and identified *Rhodococcus* sp. strain A4 grown on homocholine as the sole source of carbon and nitrogen.

II. MATERIALS AND METHODS

2.1. Chemical synthesis: 3-*N*-trimethylamino-1-propanol iodide (Homocholine) was prepared by *N*-methylation of DMA-Propanol (Tokyo kasai, Tokyo, Japan) according to the method of Hassan [8]

3-*N*-trimethylaminopropionaldehyde was prepared from 3-aminopropionaldehyde diethylacetal (Tokyo kasai, Tokyo, Japan) by treatment with methyl iodide according to the method of [8].

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 KHCO3 (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 x 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

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World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:3, No:3, 2009

were checked by ^{1}H NMR spectrum (JEOL-ECP 500 MHz, NMR spectrometer) and capillary electrophoresis.

*2.2.Isolation, growth and maintenance of homocholine-degrading bacteria:*Enrichment cultures of soil samples from different location at Tottori University and around Tottori city, Japan were used to obtain homocholine-degrading strain. Approximately 100 mg of each soil sample was inoculated into 5 ml basal medium (basal-HC media) containing per liter 5 g homocholine (HC) as the sole source of carbon and nitrogen; 2 g KH_2PO_4; 2 g K_2HPO_4; 0.5 g $MgSO_4$ $7H_2O$; 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 r.p.m. Subsequently, 200 μl of the culture was transferred to fresh basal-HC media for another 2 days. 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).

2.3.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, and by transmission electron microscope (JEOL 100 CX II Tokyo, 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 1% uranyl acetate. After air-drying the grids were examined by the electron microscope. The gram-reaction was tested using Gram-staining kit from (Wako pure chemicals, Tokyo, Japan) according to the company instruction manual, and by the KOH method [2]. 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 commercially available test kit (Biomerieux, Geneva, Switzerland) following the manufacture instruction manual. The utilization of selected carbon compounds, and combined carbon and nitrogen sources was tested by measuring turbidity in the liquid culture (5 ml) at pH 7.0 using basal media supplemented with 0.5% of the compound of choice. All tests were inoculated with cells taken from the colonies pregrown on basal-HC media. Cultures were incubated at 25°C on shaker for 48 h, and all tests were done in triplicates.

2.4.Sequencing of 16S rDNA gene: To identify the isolated bacteria, 16S-rDNA gene fragments were amplified by colony PCR with the following primer sets: 6F (5′-GGAGAGTTAGATCCTGGCTCAG-3′) and 1510R (5′-GTGCTGCAGGGTTACCTTGTTACGACT-3′), and primers 16f27 (5′- AGAGTTTGATCCTGGCTCAG-3′) and 16r1488 (5′-CGGTTACCTTGTTAGGACTTCACC-3′) those were obtained from Sigma Genosys (Sigma Aldrish, Japan). 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 using the neighbour-joining method of Saito and Nei [16] and viewed with Tree View software. The confidence of the resultant tree topologies were evaluated by performing bootstrap analyses of the neighbour-joining method based on 1000 resamplings.

2.5. 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) and centrifuged again at 10000 g for 20 min 4°C. Intact cell reaction was carried out at 30°C under shaking condition, and at intervals of 30 min, 1h, 2h, 3h, and 6h 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 described previously [12].

2.6. Analytical methods: Degradation of homocholine and the production of metabolites by intact cells of *Rhodococcus* sp. strain A4 were detected by capillary electrophoresis, GC-MS, and FAB-MS.

2.6.1. 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, and 254 nm ([14], [23], [24]).

2.6.2. 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&W scientific, Folsom, CA, USA) was used. Helium was used as carrier gas at a flow rate of 15 ml/min. Column temperature was maintained at 50°C, and samples (1μl) were injected to the GC at an injection port temperature of 250°C.

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:3, No:3, 2009

2.6.3. Fast Atom Bombardment Mass Spectrometry (FAB-MS) analysis was used to detect β-alanine betaine and 3-*N*-trimethylaminopropionaldehyde following the method described by Rhodes et al [15]. Glycerol was used as matrix.

III. RESULTS AND DISCUSSION

3.1. Enrichment, isolation, and growth of strain A4: By the enrichment for homocholine degrading microorganisms under conditions described in the "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 A4 showed the highest growth. This strain also degraded homocholine at a higher rate than others, therefore was selected for further study.

3.2. Morphological and physiological characteristics of strain A4: Strain A4 is an aerobic, non-motile, and Gram-positive bacterium that forms mycelium and long rods of variable length during the early growth phase on basal-HC agar media.
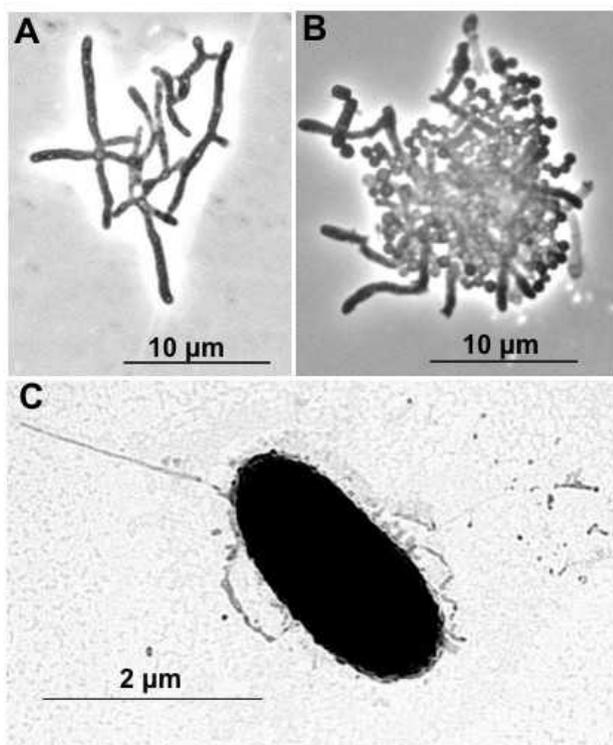

Fig. 1. Morphology of isolated strain Rhodococcus sp. strain A4 as seen by Phase contrast microscopy (A & B), and transmission electron microscopy (C). Morphological differentiation in cells shape and size after cultivation for 48 h (A), and 72h (B). Arrows in (C) showed the presence of fimbria-like structure on the cell surfaces is typical of gram-positive bacteria. Bar = 10 μm (A & B), and bar = 2 μm (C). 16S rDNA sequence analysis

Most of the cells formed filaments or showed elementary branching at early growth phase (Fig. 1), and latter fragmented into irregular short rods and/or cocci towards the end of 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 medium. Colonies were about 1.5-3.0 mm in diameter after 6 days of growth on nutrient agar plates. Strain A4 showed catalase activity, but no oxidase activity. The bacterium was able to grow on basal-HC media 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. Phenotypic and physiochemical tests suggest that strain A4 belongs to the genus *Rhodococcus*.

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 A4 and *Rhodococcus* species, a 16S rDNA gene sequence was compared with those of representative members of the genus *Rhodococcus*. The 16S rDNA data supported the results of morphological and phenological analysis. The phylogenetic relationship drowns from the partial 16S rDNA sequence (1376 nt) of the isolated strain A4 clearly demonstrated that this strain belongs to the *Rhodococcus erythropolis* 16S rDNA subclade (Fig. 2). The organism was most closely related to the type strains of *Rhodococcus opacus* with a homology of 99.1%, *Rhodococcus wratislaviensis* with homology of 98.8 %, and to *Rhodococcus koreensis* with homology of 98.5 %. The high level of similarity observed between the 16S rDNA sequence of A4 and several *Rhodococcus* species suggest that the isolated strain A4 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 A4 should be considered as *Rhodococcus* sp., with a close phylogenetic relationship with *Rhodococcus opacus* and *Rhodococcus wratislaviensis*.
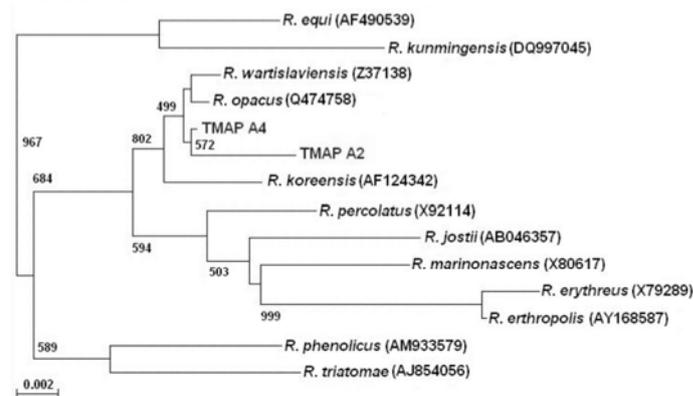

Fig. 2. Phylogenetic tree based on 16S rDNA sequence showing the relationship between strain A4 and most of the closely related Rhodococcus species. Numbers at nodes indicate level of bootstrap support ≥ 50%, based on a neighbour-joining analysis of 1000 re-sampled datasets. Bar = 0.005 nucleotide substitution per nucleotide position.

In many studies, 16S rDNA similarity values between 99.0 and 99.8 have been reported for representatives of several species of *Rhodococcus* ([6], [22]) that share DNA-DNA relatedness well below the 70% cut off recommended for the assignment of organism to the same genomic species [21]. Despite the striking similarity of strain A4 and *Rhodococcus opacus*, and *Rhodococcus wartislaviences* based on 16S

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:3, No:3, 2009

rDNA, they differ in substrate range and even shape. This demonstrates that ribosomal and genetic analysis was provided phylogenetic relationship, indeed, but did not necessarily supply information on the specific metabolic ability of isolates.

3.3. Utilization of several C.N. sources by strain A4: For the determination of nutritional and biochemical properties of the isolated strain, *Rhodococcus* sp. strain A4, a variety of selected organic compounds were tested (Table 1). The strain was found to grow on homocholine, which was used for its isolation, whereas choline, and D-carnitine did not support the growth. Trimethylamino-1-butanol, an analogue of homocholine in which the amino alcohol chain lengthened by one CH_2 group, and an intermediate metabolite of its degradation pathway namely γ-butyrobetaine were utilized for growth by *Rhodococcus* sp. strain A4. Out of the substrates tested C.N.-containing compounds only L-carnitine, β-alanine betaine (β-AB), glycinebetaine, sarcosine and glycine supported the growth, whereas dimethyl glycine, dimethylamino-1-propanol, dimethylaminopropionic acid, and C_1-compounds such as trimethylamine (TMA), dimethylamine, and monomethylamine did not.

TABLE I UTILIZATION OF HOMOCHOLINE AND STRUCTURALLY RELATED
COMPOUNDS BY *RHODOCOCCUS* SP. STRAIN A4

Compound	Growth
Homocholine	+ +
Choline	−
Trimethylamino-1-butanol	+ + +
L-carnitine	+ +
D-carnitine	−
Glycine betaine	+ +
γ- butyrobetaine	+ +
β- alanine betaine	+
Dimethyl glycine	−
Dimethylamino-1-propanol	−
Dimethylamino-1-propionic acid	−
Control (no substrate)	−

Growth was determined by measuring the turbidity at 660 nm (OD660 nm).
Symbols: (+ + +) OD660 nm ≥ 3.0; (+ +) OD660 nm ≥ 2.0; (+) OD660 nm ≥ 1.0; −, no growth. Each data represent the average of three independent experiments

3.4. Degradation of homocholine by the resting cells of strain A4: The degradation of homocholine by the resting cells of strain A4, 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 cells of strain A4, there were a concurrent formation and accumulation of some soluble metabolites as detected by capillary electrophoresis analysis. These metabolites were found to be trimethylamine and β-alanine betaine as 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 product 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. Furthermore, analysis of the culture filtrate and the intact cell reaction products by FAB-MS demonstrated the accumulation of β-AB and trimethylaminopropionaldehyde (TMAPAL). The culture filtrate spectrum of strain A4 (fig. 3A) showed signals at *m/z* 118 $[M+H]^+$ of homocholine, *m/z* 131 $[M^+]$ of β-AB, and *m/z* 115 $[M^+]$ of trimethylaminopropionaldehyde. The disappearance of the signal at *m/z* 118 after 48h cultivation indicated the complete degradation of the provided substrate (homocholine).
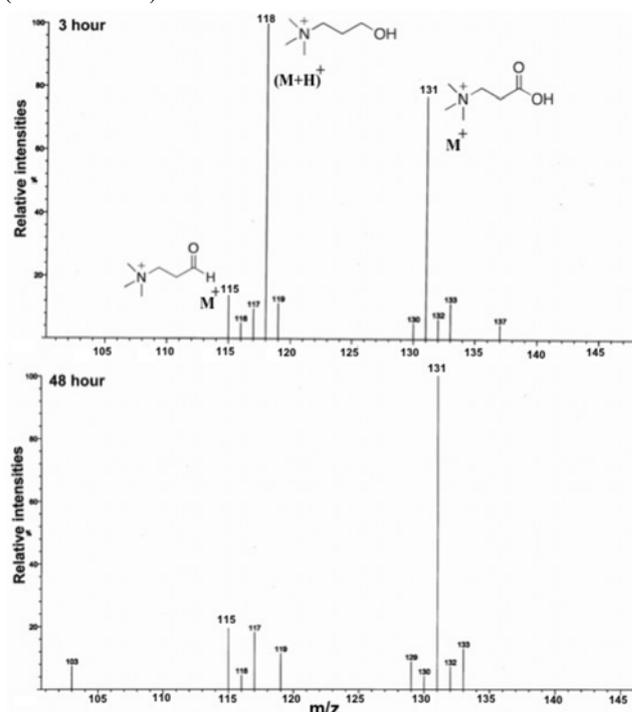

Fig. 3. FAB-MS spectra of metabolites in the degradation pathway of homocholine by strain A4. The metabolites with M+ of 115 and M+ of 131 were identified as trimethylaminopropionaldehyde and β-alanine betaine, respectively.

The time course degradation of homocholine by resting cells of strain A4 is shown in figure 4. Resting cells of strain A4 almost completely degraded homocholine in 6 h, and the metabolites TMAPAL and β-AB formed and metabolized when the reaction elevated. In contrast, TMA was accumulated with the degradation of homocholine. It is clear that the stoicheiometric formation of TMA 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 A4 grown on β-AB as sole source of carbon and nitrogen. In addition intact cell reaction of these cells with β-AB showed again the accumulation of TMA as major metabolites. Similarly, utilization of homocholine, choline, γ-butyrobetaine, and DL-carnitine by *Arthrobacter* sp. strain E5, *Candida tropicalis*, *Acinetobacter calcoaceticus*, *Pseudomanas putida*, and *Proteus vulgaris*, respectively, are

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:3, No:3, 2009

correlated with the stoichiometric formation of TMA and complete degradation of carbon skeleton ([11], [12], [13], [17]). 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 ([7], [18], [19]). 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 [20]. 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 strain A4. This demonstrated the novel degradation pathway of homocholine by strain A4 that is quite different than 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 and thereafter cleavage of C-N bond providing TMA and alkyl chain.
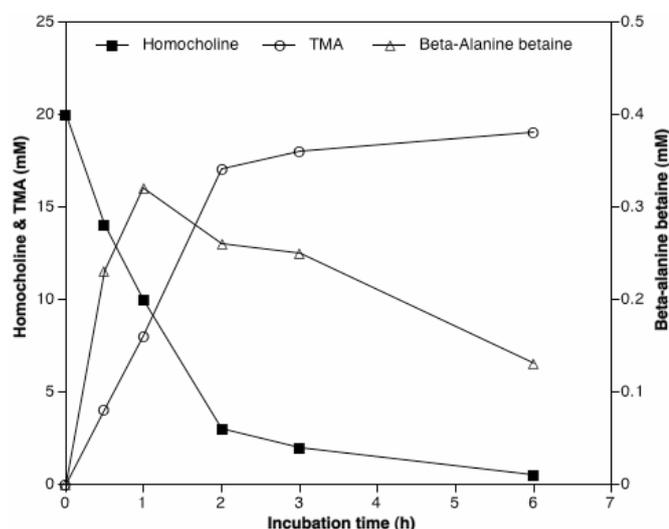

Fig. 4. Degradation of homocholine (20 mM homocholine), and accumulation of trimetylamine (TMA) and beta-alanine betaine by a washed cell suspension of Rhodococcus sp. A4 pre-grown on homocholine as sole source of carbon and nitrogen.

IV. CONCLUSION

With view exceptions, most of the bacteria isolated and identified so far for their capability to degrade quaternary ammonium compounds were from the genus *Pseudomonas* ([8], [19], [20]). To the best of our knowledge, *Rhodococcus* species capable of degrading quaternary ammonium compounds were not yet reported, despite their versatility and ability to degrade a large number of organic compounds, including some of the most difficult and toxic compounds. Interestingly, we report here the isolation of *Rhodococcus* sp. strain A4 as the first strain that metabolized homocholine as the only source of carbon and nitrogen. The degradation pathway of homocholine was found to be through consequence oxidation of alcohol group to aldehyde and acid, respectively. Our further research is focused on the enzymatic

degradation pathway of homocholine to elucidate the enzymatic mechanisms, metabolites and specificity of the degradation of homocholine. This information will be important to understand the mechanisms involved in the degradation pathway of this compound.

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World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:3, No:3, 2009

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