Enzymatic Study on the Microbial Degradation of Homocholine

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ABSTRACT

This research was conducted to investigate the enzymatic activities in the degradation pathway of homocholine by bacterial strains isolated from the soil. Screening of the homocholine oxidation activity in the isolated strains, by replica staining method and spectrophotometric assay, showed that NAD^+ - dependent dehydrogenase enzymes were 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 were 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 unstable *in vitro* and lost 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.

Key words: Homocholine; microbial degradation; β-alanine betaine; trimethylaminopropionaldehyde

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. The glucose-methanol-choline (GMC) oxidoreductase 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 (Nagasawa *et al*., 1976; 1997; Russell *et al*., 1998) and (ii) betaine aldehyde to glycine betaine by a NAD^+-dependent betaine aldehyde dehydrogenase (Nagasawa *et al*., 1976; Mori *et al*., 1980; Mori *et al*., 1992; Mori *et al*., 2001). Moreover, 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-butanol dehydrogenase. Consequently, TMABaldehyde is oxidized to γ-butyrobetaine by a NAD^+-dependent TMABaldehyde dehydrogenase (Hassan, 2008).

Homocholine (3-*N*-trimethylamino-1-propanol), an analogue of choline in which the amino alcohol group is lengthened by one CH2-group, resembles choline in many aspects of cholinergic metabolism (Boksa and Collier, 1980). After transportation into rat brain synaptosome, it is acetylated and released as acetylhomocholine from a superior cervical ganglion and the 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 in both fat and cholesterol affected fatty livers (Channon *et al*., 1937). Despite its similarity to choline in many aspects, reports on microorganisms degrading homocholine are scarce (Mohamed Ahmed *et al*., 2009a, b; Mohamed Ahmed *et al*., 2010) and consequently, its catabolic pathway remains unclear. Based on its structural similarity to choline, one would expect that homocholine could be degraded in a similar way and at a rate comparable to that of choline. It was found that, an osmoprotectant β-alanine betaine was an intermediate product in the degradation pathway of homocholine (Mohamed Ahmed *et al*., 2009a, b; Mohamed Ahmed *et al*., 2010). Therefore, the study of homocholine degradation pathways and the enzymes involved in them is of considerable interest. Using metabolomics, we previously postulated the degradation pathway of homocholine by strains belonging to the genera *Arthrobacter*, *Rhodococcus* and *Pseudomonas* (Mohamed Ahmed *et al*., 2009a; b; Mohamed Ahmed *et*

al., 2010). 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. However, enzymatic activities in the degradation pathway of homocholine by isolated strains were not yet carried out. Thus, the aim of the present study was to initially investigate the enzymatic activities in the degradation pathway of homocholine by the isolated strains and particularly *Pseudomonas* sp. strain A9.

MATERIALS AND METHODS

Materials

3-Dimethylamino-1-propanol (DMA-Propanol), 4-dimethylamino-1-butanol (DMA-Butanol), and 4-aminobutanol were purchased from Tokyo Kassei (Tokyo, Japan). Homocholine iodide was prepared from 3-dimethylamino-1-propanol, according to the method described previously (Mohamed Ahmed *et al*., 2009a). TMAPaldehyde iodide was prepared from 3-aminopropoinaldehyde dimethylacetal, according to the method described previously (Mohamed Ahmed *et al*., 2009a). Protease inhibitor cocktail Set II was from Calbiochem (Darmstadt, Germany; Table 1). 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).

Methods

Screening for enzymatic activities by plate replica staining method: In this method, 30 rapid growing strains isolated from the soil were screened for homocholine oxidation activities using plate replica activity staining methods. The isolated strains were transfered individually using sterilized tooth sticks to basal-homocholine agar plates containing, 5 g homocholine (HC), 2 g KH_2PO_4, 2 gK_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 1 g polypeptone, and 15 g agar (pH 7.0) per liter. The plates were incubated at 30°C for either 24 or 48 h. The grown colonies were replicated onto filter papers, 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 papers were sunk in a solution containing inhibitors of energy-generating system (10 mM of NaN_3, 10 mM of NaF, 1 mM of Na_2AsO_4) for 5 min. Then, the filter papers were frozen and thawed three times to inactivate the energy-generating system. After drying, the dried filter papers were sunk in 2 ml each 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 led to the formation of a purple color as an indicator of enzymatic activity. Strains showing positive results

with replica staining were selected and used for preparation of cell free extract to confirm the enzyme activity by the spectrophotometric methods.

Cell free extract preparation: To check enzyme activities of the isolated strains, the strains were inoculated into 75 ml of basal-homocholine 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 × *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 beads (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 × *g* for 20 min as supernatant and assayed for dehydrogenase and oxidase activities.

Homocholine dehydrogenase activities: NAD^+-dependent homocholine dehydrogenase activity was measured as an increase in the absorbance at 340 nm at 30ºC. 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 $M^{-1}\cdot cm^{-1}$ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed 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.

Membrane-bound homocholine dehydrogenase activity was measured as a decrease in the absorbance at 600 nm at 30ºC 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 67.5µmol of potassium phosphate buffer (pH 8.0), 1.5 µmol of potassium cyanide, 0.375µmol of 1-methoxy PMS, 0.15µmol 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 $M^{-1}\cdot cm^{-1}$ for oxidized DCIP at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 µmole of DCIP per min under the standard assay conditions.

TMAPaldehyde dehydrogenase activity: The standard reaction mixture (1.5 ml) contained 225µmol of potassium phosphate buffer (pH 8.0), 15 µmol of TMAPaldehyde iodide, 3µmol 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 $M^{-1}\cdot cm^{-1}$ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed 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.

3-hydroxypropionate dehydrogenase activity: 3-Hydroxypropionate dehydrogenase activity was measured as an increase in the absorbance at 340 nm and at 30ºC. The standard reaction mixture (1.5 ml) contained 225 µmol of Tris-HCl buffer (pH 7.5), 33.3 µmol of 3-hydroxypropionate, 3µmol 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 $M^{-1} \cdot cm^{-1}$ of NADH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed 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.

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-homocholine 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 (Novaspec II, Amersham Pharmachia Biotech, Sweden). At the growth of 2.0 (T660 =2.0), 200 µl of the seed culture broth was inoculated in 300 ml of basal-homocholine medium. Inoculation was at 25°C on a reciprocal shaker. At intervals of 12,15, 18, 22, and 26 h, the growth was estimated and the bacterial cells were collected by centrifugation at 6,000 × 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 beads (0.1 mm) on dry ice for 5 min. The cell free extract was obtained by centrifugation at 10,000 × *g* for 30 min as a supernatant and assayed for homocholine dehydrogenase activity.

Stability of the homocholine dehydrogenase activity of *Pseudomonas* sp. strain A9: In this method, cell free extracts were dialyzed against potassium phosphate buffer, tris-HCl buffer and triethanolamine buffer (50mM, pH7.5) containing 1mM of DTT and 1mM EDTA overnight at 4ºC. The dialyzed extracts were stored at 4ºC for 0 to10 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 4ºC 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-mertcaptoethanol and glutathione, as well as in the presence of stabilizing reagents such as ethanol, glycerol and ethylene glycol.

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 in 300 ml medium in one litre culture flask shaken on a reciprocal shaker (120 strokes/min) at 25ºC for 24h.

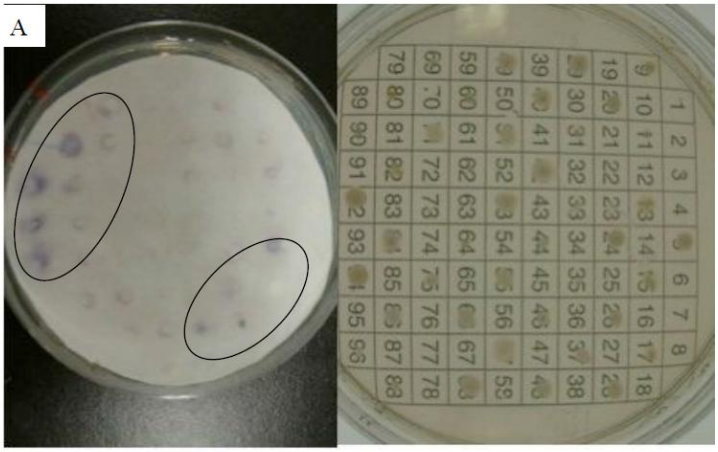
Intact and dry cell reaction of *Pseudomonas* sp. strain A9: To further confirm the metabolic route of homocholine in *Pseudomonas* sp. strain A9 both intact and dry cell reaction experiments were carried out. Intact cell reaction was carried out as described previously (Mohamed Ahmed *et al*., 2009a). 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, cell suspension was spread on glass a Petri dish and dried at room temperature with an electric fan. Then the collected cells were further dried in a vacuum desiccator and finely ground using mortar. The dried cells (75 mg) were suspended in18 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 previously (Mohamed Ahmed *et al*., 2010). An exception is that TMAPaldehyde concentration in the reaction mixture was quantified by DNPH methods as described elsewhere (Tani *et al*., 1977). The amount of TMAPaldehyde in the reaction mixture was calculated from the standard curve generated using different concentrations of authentic standard of TMAPaldehyde.

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.

RESULTS AND DISCUSSION

Screening for homocholine degrading activities

Screening of the homocholine oxidation activities in the isolated strains by replica staining and spectrophotometer assays demonstrated that NAD^+-dependent dehydrogenase enzymes were predominant in homocholine degrader. 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. 1a). Cell free extracts of these isolates again confirmed the presence of both NAD^+-dependent alcohol and aldehyde dehydrogenase activities (Fig. 1b). Those isolates were chosen as the most superior homocholine degraders and were identified to the specific level (Mohamed Ahmed *et al*., 2009a, b; Mohamed Ahmed *et al*., 2010) 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 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 also showed both activities, these strains were excluded because cell free extract preparation was quite difficult and needed more extraction time that significantly affected the enzyme stability. Moreover, *Rhodococcus* and *Arthrobacter* strains required more inoculation time (48h) compared to *Pseudomonas* strains (24h).



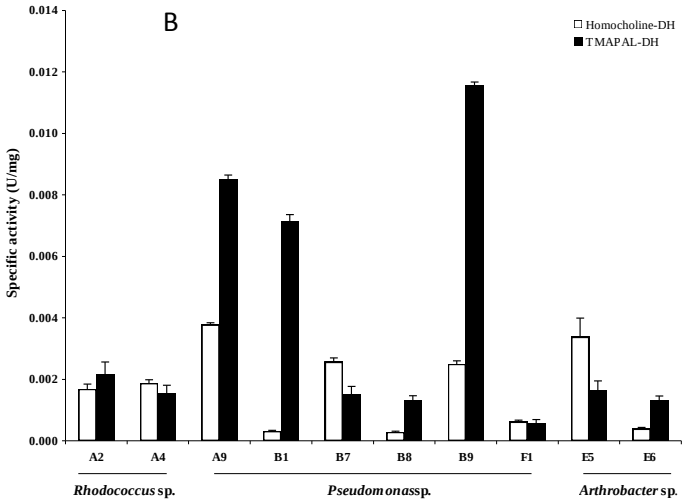


Fig. 1. Screening for dehydrogenases activity in the isolated strains by (A) replica staining and (B) spectrophotometric assays.

Time course of 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. 2). 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 24 h. Thereafter, the activity rapidly decreased after 24h inoculation. Hassan (2008) found that the formation of TMA-Butanol dehydrogenase activity by *Pseudomonas* sp. 13CM rapidly decreased after 6 h inoculation. This phenomenon looks similar in quaternary ammonium alcohol degrader. At the moment we do not know the actual reasons for the rapid decrease in the enzyme activity after maximum formation. However, it might have 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 penetrated into the cellular components. These metabolites might be homocholine analogues such as trimethylamine.

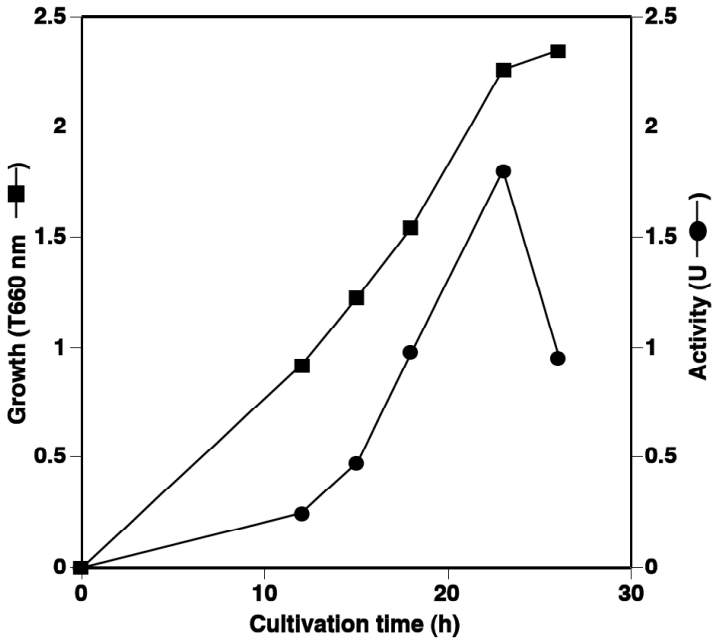


Fig 2. Time course of the growth and homocholine dehydrogenase activity formation of *Pseudomonas* sp. strain A9. Cell-free extract was prepared from cells grown on 300 ml of culture broth.

Formation of homocholine dehydrogenase on various media

To assess the induction of homocholine dehydrogenase by *Pseudomonas* sp. strain A9, the bacterium was cultivated on basal media of various homocholine analogues as C and N sources, 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 homocholine dehydrogenase activity. The results (Table 1) 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 was also notable that glucose exerted a total repression of homocholine dehydrogenase activity induction, since no activity was detected on homocholine-glucose growing cells. A 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 carried out. 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).

Table 1. Formation of Homocholine dehydrogenase on various media

Medium (1.0%)	Growth ($_{T660\ nm}$)	Enzyme activity (mU/ml broth)	Specific activity (mU/mg)
Homocholine	2.57	6.21	30.0
Choline	2.65	0.00	0.0
TMA-Butanol	0.58	0.00	0.0
Glucose & 0.5% $(NH_4)_2\ SO_4$	3.09	0.00	0.0
Supplemented with carbon source (0.5%)			
Glucose	1.63	0.00	0.0
Glycerol	2.85	3.98	22.2
Supplemented with nitrogen source (0.1%)			
$(NH_4)_2\ SO_4$	3.05	9.34	21.5

Basal medium: 1% substrate, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4$. $7H_2O$, 0.05% yeast extract, and 0.1% polypeptone.

Stability of homocholine dehydrogenase of *Pseudomonas* sp. strain A9

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 was that the enzyme was 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. 3) showed that ethanol and ethylene glycol were 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 other buffers. On the other hand, the cell free extract was preserved at 4°C without dialysis. The results (Fig. 4) 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 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).

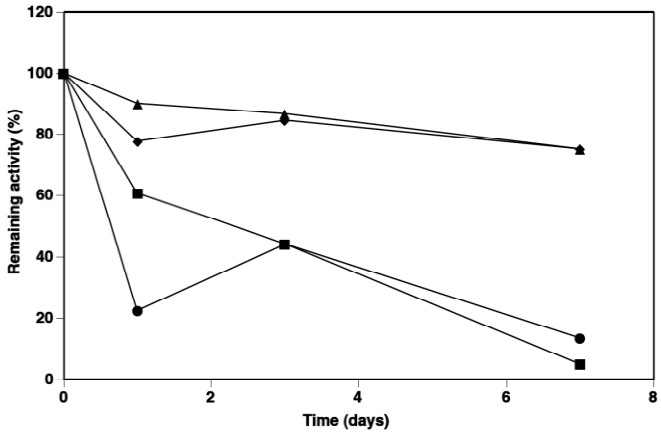


Fig 3. Effect of stabilizers on the stability of homocholine dehydrogenase of *Pseudomonas* sp. strain A9. Cell free extract (1 ml) was dialyzed overnight against 50 mM potassium phosphate buffer containing either 1mM DTT (■), 1mM glutathione (●), 5% ethanol (▲), or 10% ethylene glycol (♦) and stored at 4°C for 1 week

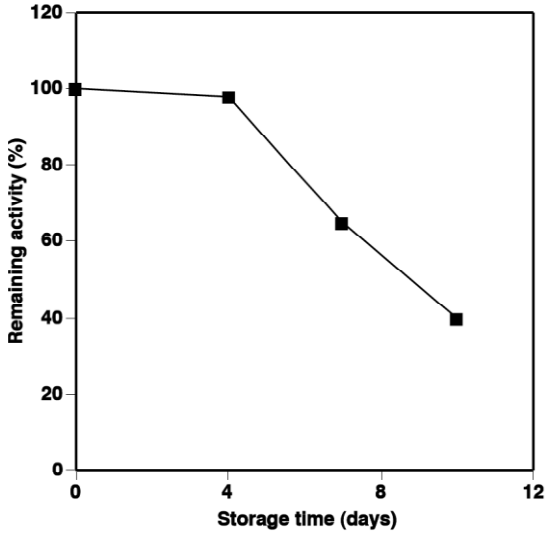


Fig 4. Evaluation of the stability of homocholine dehydrogenase during storage at 4°C.

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. 5). 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.

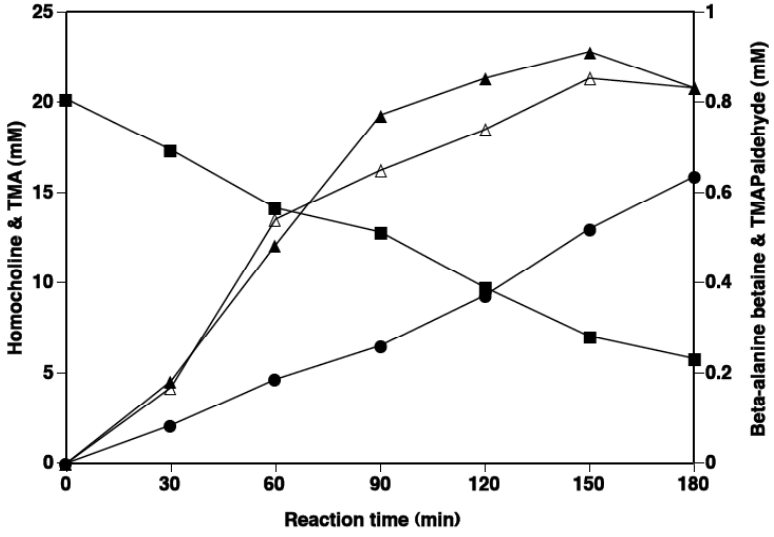
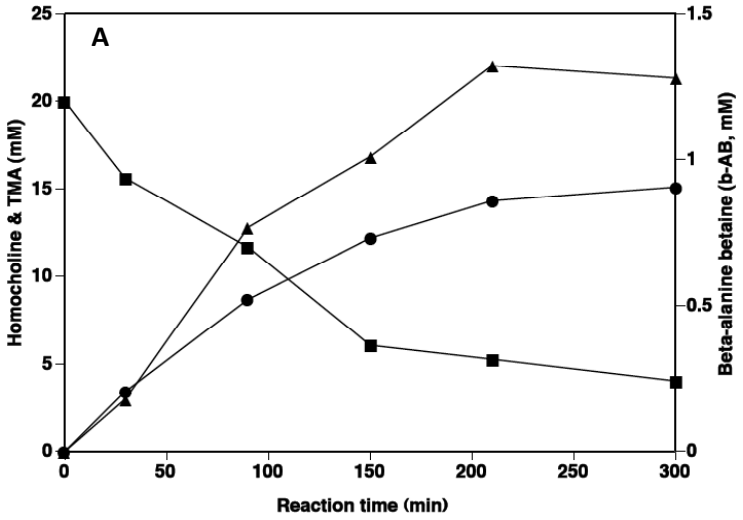


Fig 5. Degradation of homocholine by resting cells of *Pseudomonas* sp. strain A9. Time course degradation of homocholine (■) and the generation of the metabolites, TMAPaldehyde (△) beta-alanine betaine (▲) and TMA (●) by intact cells of *Pseudomonas* sp. strains A9.

Dry cell reaction was carried out using homocholine (20 mM) as a substrate with and without NAD^+. The results (Fig. 6a) 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. 6b). The slight degradation of homocholine in the absence of added NAD^+ might have resulted from the remained NAD^+ with the dried cells. The results demonstrated that the enzymes responsible for the metabolism of homocholine were alcohol and aldehyde dehydrogenases that required NAD^+ as electron acceptor.



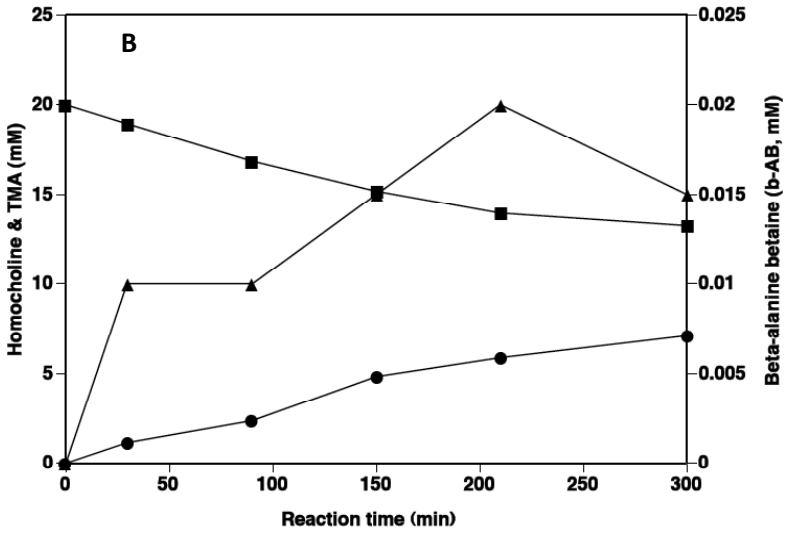


Fig. 6. Degradation of homocholine by dried cells of *Pseudomonas* sp. strain A9. Time course degradation of homocholine (■) and the generation of the metabolites beta-alanine betaine (▲) and TMA (●) by dried cells of *Pseudomonas* sp. strains A9. Dry cell reaction was performed with (A) and without (B) NAD^+.

Substrate specificity of homocholine dehydrogenase

An attempt was made to determine the substrate spectrum of homocholine dehydrogenase in the crude preparation, because the enzyme was 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 appeared 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 2). 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 is 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 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 homocholine, Demethyl amino-1- propanol (DMAP), 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. 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 a single band of activity was detected (data not shown). 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 *in vitro* for both substrates (Table 2). The results obtained demonstrated that homocholine dehydrogenase had 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).

Table 2. Substrate specificity of homocholine dehydrogenase from strain A9

Substrate (33.3 mM)		Activity (mU/ml)	Relative activity (%)
Homocoline	$(CH_3)_3N^+(CH_2)_2\ CH_2OH$	649	100
TMA-Butanol	$(CH_3)_3N^+(CH_2)_3\ CH_2OH$	902	139
TMA-Pentanol	$(CH_3)_3N^+(CH_2)_4\ CH_2OH$	0	0
TMA-Hexanol	$(CH_3)_3N^+(CH_2)_5\ CH_2OH$	0	0
Choline	$(CH_3)_3N^+CH_2CH_2OH$	0	0
β-methyl choline	$(CH_3)_3N^+CH(CH_2)OH$	0	0
DMA-Propanol	$(CH_3)_2N(CH_2)_2\ CH_2OH$	141	22
DMA-Butanol	$(CH_3)_2N(CH_2)_3\ CH_2OH$	1020	157
DMA-Hexanol	$(CH_3)_2N(CH_2)_5\ CH_2OH$	0	0
4-Amino-1-butanol	$H_2N^+(CH_2)_3\ CH_2OH$	215	33
1-Butanol	$CH_3\ (CH_2)_2\ CH_2OH$	236	36
1-Propanol	$CH_3\ CH_2\ CH_2OH$	43	7
2-Propanol	CH_3 CH (OH) CH_3	0	0
Ethanol	CH_3CH_2OH	0	0
Methanol	CH_3OH	0	0
L-Carnitine	$(CH_3)_3N^+CH_2CH(OH)CH_2COOH$	0	0
D-Carnitine	$(CH_3)_3N^+CH_2CH(OH)CH_2COOH$	0	0

Detection of 3-hydroxypropionate dehydrogenase activity

A NAD^+-dependent 3-hydroxypropionate dehydrogenase activity was 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/or glucose-growing cells. Furthermore, this enzyme activity was also detected on native-PAGE only in the cell free extract of homocholine growing cells (Fig. 7a). 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. Moreover, to confirm the presence of 3-hydrpxypropionate as an intermediate metabolite, the intact cell reaction mixtures of strain A9 were analyzed by LC/LC-MS. The results demonstrated the accumulation of 3-hydroxypropionate as an intermediate metabolite (Fig. 7b). 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. 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 as it is a biodegradable polymer and can potentially replace several 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).

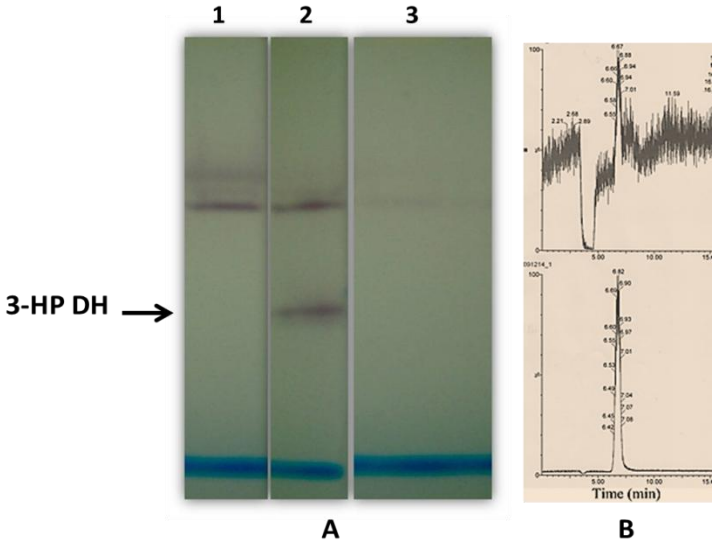


Fig 7. (A) Non-denaturing gel (7.5% gel) stained for NAD^+-dependent 3-hydroxypropionate dehydrogenase activity. Lane 1; homocholine-growing cells, lane 2; choline-growing cells, and lane 3; glucose-growing cells. (B) LC/MS/MS analysis of 3-hydroxypropionate in the degradation pathway of homocholine by the intact cells of *Pseudomonas* sp. strain A9. Intact cell reaction mixture of strain A9 (upper) and authentic standard of 3-hydroxypropionate (lower).

CONCLUSION

Hmocholine degrading enzymes are widespread in aerobic microorganisms such as *Arthrobacter*, *Rhodococcus* and *Pseudomonas*. In *Pseudomonas* sp. strain A9, homocholine was oxidized to TMAPaldehyde by homocholine dehydrogenase, and consequently, TMAPaldehyde was oxidized to β-alanine betaine by a NAD^+-dependent aldehyde dehydrogenase. 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. 8). Further studies should specifically focus on the purification and characterization of homocholine degrading enzymes.

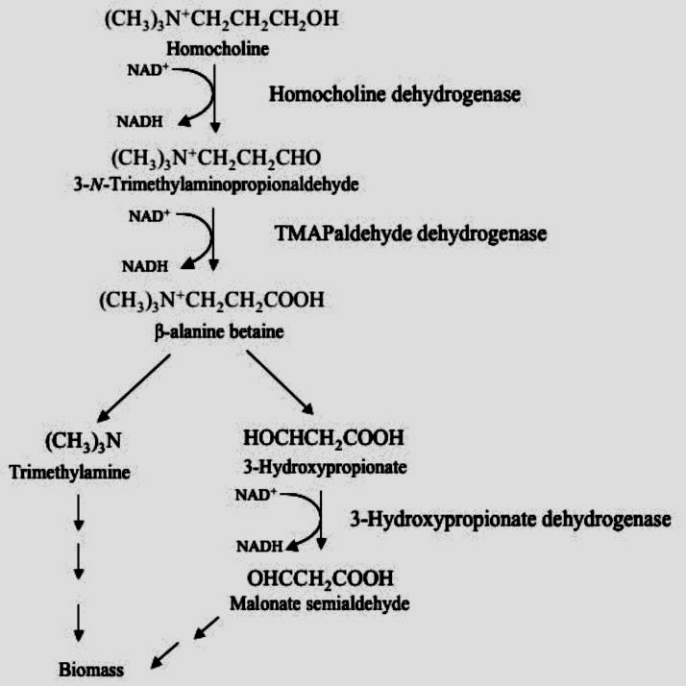


Fig 8. Proposed degradation pathway of homocholine by *Pseudomonas* sp. strain A9

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