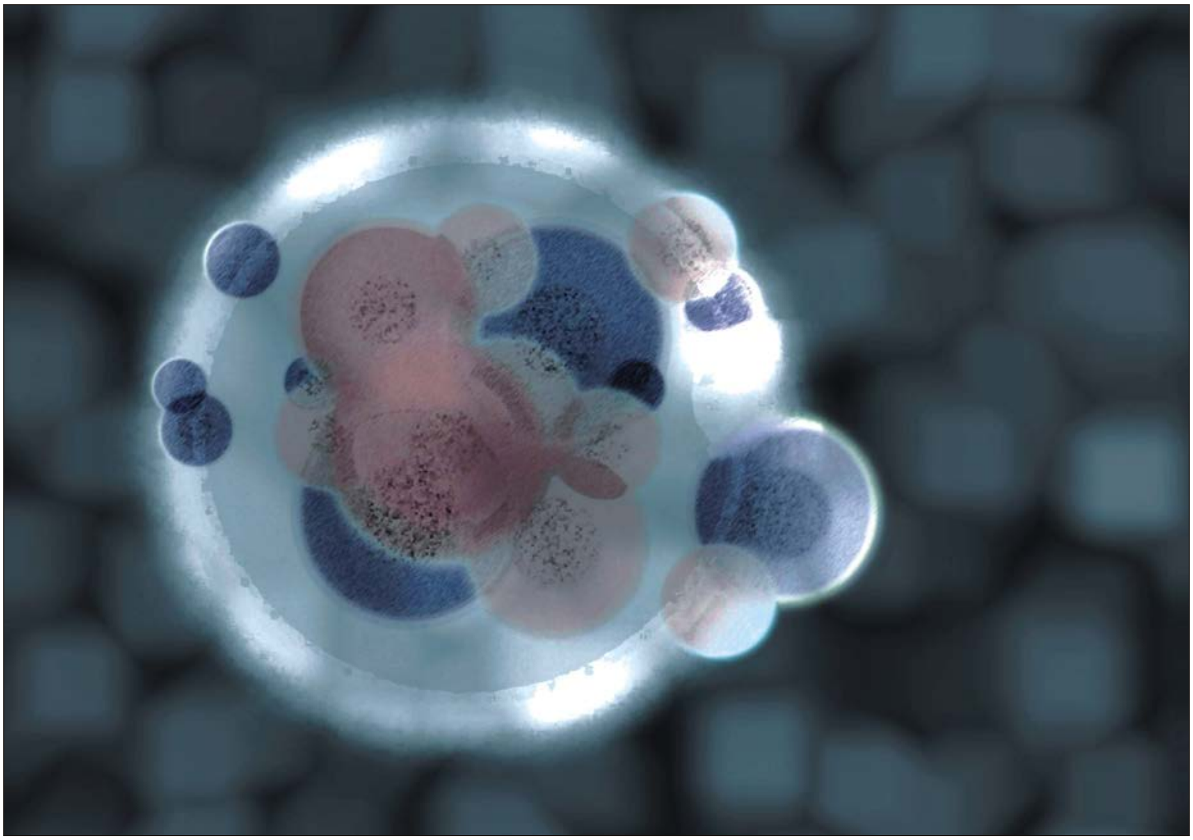

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 7 (2): 315-320, 2007
ISSN 1727-3048

Purification and Characterization of Membrane-Bound Quinoprotein Alcohol Dehydrogenase from a Native Strain of *Acetbacter*

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Abstract: A Pyrroloquinoline quinone alcohol dehydrogenase of a native strain of *Acetobacter* sp. 15 has been purified and characterized in order to its biotechnological and industrial application. Enzyme assay method is done with the potassium ferricyanide (as an electron acceptor), McIlvaine buffer 0.1 M (pH 4), Triton X-100 10%, ethyl alcohol 1 M and enzyme solution. In the presence of SDS the Enzyme was dissociated into submits with four molecular weight: 13.7, 14, 23.3 and 40 kD. At pH 6 of phosphate buffer 0.01 M, the enzyme has K_m 1.75 mM for ethanol as a substrate. In this study, substrate specificity, optimum pH and effect of Ethylenediamine tetra acetic acid (EDTA), metal ions on activity of ADH have been investigated. The data have shown that ethanol (100 mM) is the best substrate for the enzyme. The optimum pH of the enzyme activity was 4.0 and the enzyme was stable in pH 6-7.5. EDTA completely inhibited ADH activity via its binding to Ca^{2+}. Metal ions such as Ca^{2+} (1.0 mM) increased about 2-fold ADH activity whereas Fe^{2+}, Mg^{2+} and Zn^{2+} inhibited the enzyme activity. It seems that the effect of Ca^{2+} was a result of a functional role for Ca^{2+} in the enzyme, similar to what has been observed with quinoproteins glucose and methanol dehydrogenases.

Key words: Acetobacter, alcohol dehydrogenase, pyrroloquinoline quinine, membrane-bound

INTRODUCTION

Bacteria have versatile metabolic pathway that enable them to adapt to different environmental conditions. Many Gram-negative bacteria, for example, can grow on compounds as different as methylamine, ethanol and glucose as their sole source of carbon and energy (Groen *et al*., 1986), (Schie *et al*., 1989). Acetic acid bacteria, *Acetobacter* and *Gluconobacter*, as well known as vinegar producers. These bacteria are able to oxidize ethanol to acetic acid by two sequential catalytic reactions of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH), which are localized on the periplasmic side of the cytoplasmic membrane, that function by transferring electrons from ethanol to ubiquinone embedded in the membranous phospholipids (Matsushita *et al*., 1992, 1994; Ameyama *et al*., 1981, 1992). Quinoproteins are oxidoreductases that posses one of the four different quinone compounds instead of nicotinamide or flavine cofactors (Salisbury *et al*., 1979), (Ameyama *et al*., 1981; Janes *et al*., 1990; McIntire *et al*., 1991; Duine, 1991; Wang *et al*., 1996). They oxidize a broad range of substrates, such as alcohols, sugars, sugar acids and sugar alcohols and the corresponding oxidation products are accumulated in the culture medium. Based on this characteristic, several useful oxidation products are produce industrially and such processes are called oxidative fermentations. Together with acetate fermentation, L-sorbose fermentation, D-gluconate fermentation, dihydroxyacetone fermentation, keto-gluconate fermentation and so on are typical examples of oxidative fermentations by these strains (Matsushita *et al*., 1994, 2002). These proteins contain Pyrroloquinoline quinone (PQQ, 2, 4, 7-tricarboxy-1 H-pyrrolo [2, 3-f] quinoline-4, 5-dione) cofactor form the best characterized and largest quinoprotein subclass (Anthony, 1992; Duine *et al*., 1986, 1987; Duine and Jungian, 1989; Duine, 1991). Two different types of PQQ-containing alcohol dehydrogenases (ADHs) have been characterized: quinoprotein ADH and quinohemoprotein ADH. These latter enzymes contain a covalently bound heme c. (Groen *et al*., 1986) Coupled with ethanol oxidation; ADH reduces phenazine methosulfate, dichlorophenolindophenol, or ferricyanide as an artificial electron acceptor. (Ameyama and Adachi, 1982) since ferricyanide reacts with the heme components having a high redox potential, the heme c sites in ADH complex should reduce ferricyanide. PQQ-dependent enzymes, quinoproteins, have certain properties which make them superior to other dehydrogenases in analytical applications, especially in biosensor applications (Matsushita *et al*., 1992).

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Therefore quinoproteins provide an ideal alternative to traditional dehydrogenases. When NAD (P)-dependent enzymes are utilized in analytical applications a soluble coenzyme must be either continuously added to the system or immobilized in it. However, retaining the small molecule in the system can be problematical. In FAD-dependent dehydrogenases, on the other hand, the coenzyme is tightly bound to the enzyme but dissolved oxygen influences the catalytic activity.

In this study, a successful example of a complete purification of a membrane-bound ethanol dehydrogenase oxidizing ethanol is described by this method for *Acetobacter* sp. 15. After many disappointing failures in enzyme solublization and subsequence enzyme purification, special case for purifying the quinoprotein has finally lead us a successful enzyme purification. In addition, in this study, several properties of the purified membrane-bound ethanol dehydrogenase are presented.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were commercial products. DEAE-Sephadex and hydroxyapatite were purchased from Sigma Chemical Company. Potassium ferricyamide was also purchased from Sigma Chemical Company, Agar and yeast extract were kind from Microbiology Lab. of Alzahra University. Total protein assay kit was also purchased from Chem. Enzyme Company.

Microorganisms: The *Acetobactere* sp. Strain SSM15 used in this study was kind donation from the Microbiology Lab. in Alzahra University. The strain was previously isolated from home-made vinegar in Takestan-Iran.

Medium and cultivation: Basal medium employed in this study contained 50 g D-glucose, 10 g yeast extract, 30 g $CaCO_3$, 25 g Agar, 20 mL ethyl alcohol in 1 L of tap water. The type culture of acetic acid bacteria grown on the yeast extract slant was inoculated to 100 mL of the medium in 500 mL shaking flask and the cultivation was carried out at 37°C for 24 h with reciprocal shaking.

Enzyme assay: In this study, assay method is done with potassium ferricyamide as an electron acceptor. The rate of reduction of ferricyamide to ferrocyamide gives a quantitative amount of ethanol oxidation. The reaction mixture contained 0.1 mL potassium ferricyamide 0.1 M, 0.5 mL McIlvaine buffer 0.1 M (pH 4.0), 0.1 mL Triton X-100 10%, 0.1 mL ethyl alcohol 1 M, enzyme solution in total volume of 1 mL. The reaction was carried out at 37°C by the addition of ethanol solution and stopped by adding 0.5 mL of the ferric-dopanol reagent. Then, 3.5 mL of water was further added to the reaction mixture and well mixed. The resulting stabilized Prussian blue color formed was measured by spectrophotometer at 660 nm after standing for 20 min at 37°C. One unit of enzyme activity was defined as amount of enzyme catalyzing the oxidation of 1 μmol of ethanol per min under these assay conditions (Adachi *et al*., 1978).

Protein determination: The protein concentration was estimated by measuring by Total Protein Chem. Enzyme Assay Kit.

Protein concentration in sample (g dL^{-1}) = (Sample observation/standard observation) standard concentration.

Electrophoresis: For estimation of purity of enzyme preparations, slab gel electrophoresis was performed under essentially the same conditions as described by Laemmli using 12.5% of polyacrylamide gel and TrisHCL buffer, pH 8.3, Sodium Dodecyl Sulfate (SDS)-acrylamide gel electrophoresis was performed to determine purity and subunit composition of the enzyme (Laemmli, 1970).

RESULTS

Preparation of cell homogenate: Cells were harvested by centrifugation at 12,000 x g for 20 min and washed with saline 0.9%. The cell paste was suspended in 0.01 M potassium phosphate buffer, pH 6.0, (1 g of wet cell/10 mL buffer) and sonicated this suspension with sonicator at 100 W for 5 steps (5 min) with intervolves (2 min). Intact cells were removed by centrifugation at 5000 x g for 5 min. The resulting supernatant was disintegrated as cell homogenate. Appropriate concentrations of phosphate buffer were used for all purification steps.

Solubilization of enzyme: The membrane fraction is suspended in 0.01 M buffer, pH 6.0 and the protein concentration is adjusted to 30 mg mL^{-1} 10% Triton X-100 and 2-mercaptoethanol are added to a final concentration of 1.0% and 1 mM, respectively. The suspension is gently stirred for 3 h at 0°C and centrifuged at 68,000 x g for 60 min. A rose-red supernatant is obtained as the solubilized enzyme.

DEAE-Sephadex column chromatography I: To the solubilized enzyme solution, polyethylene glycol 6000 is added to 20% to precipitate the enzyme. After 30 min of stirring in an ice bath, the enzyme solution is centrifuged

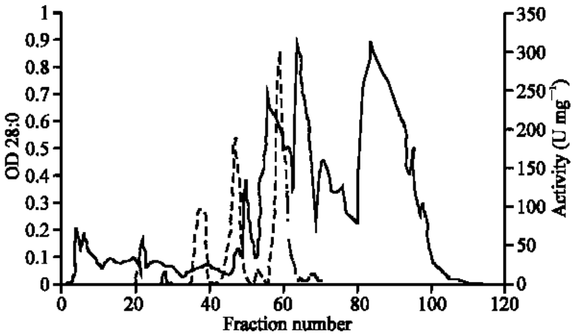

Fig. 1: Chromatography on DEAE-Sephadex A50 (1). AD solution from proceeding step was adsorbed onto column of DEAE-Sephadex A50 (I). Yield and Purification fold of this step was 65.58 and 7.67, respectively. (—) was protein content and (- - -) was enzyme activity

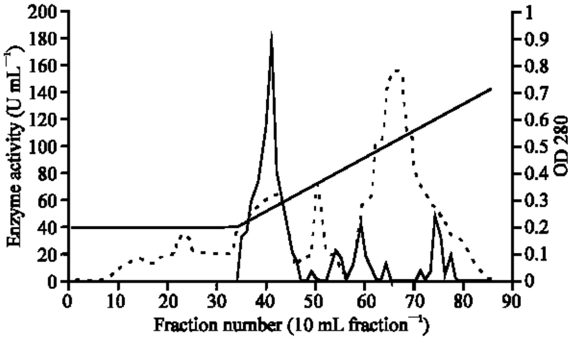

Fig. 2: Chromatography on DEAE-Sephadex A50 (II). Elution is made by a linear gradient elution formed by 500 mL of 0.015 M and 500 mL of 0.06 M buffer. Yield and purification fold of this step was 44.38 and 20.89, respectively. (—) was enzyme activity and (- - -) was protein content

at 12,000 x g for 20 min. The precipitate is suspended in small volume of 0.01 M buffer and the thick suspension is dialyzed overnight against 0.002 M buffer containing 0.1% Triton X-100. The dialyzed solution is applied to a DEAE-Sephadex A50 column (4.5×30) that has been equilibrated with 0.002 M buffer containing 0.1% Triton X-100. The column is washed with 1.5 L of the same buffer to remove nonadsorbed materials. The enzyme is eluted with 0.05 M buffer containing 0.1% Triton X-100 and polyethylene glycol 6000 is added to 20% to the pooled fractions to precipitate the enzyme. The precipitate collected by centrifugation at 12,000 x g for 20 min is dissolved in a minimum volume of 0.01 M buffer, containing 0.1% Triton X-100 and dialyzed thoroughly against the same buffer (Fig. 1).

DEAE-Sephadex column chromatography II: The dialyzed enzyme is applied to the second column DEAE-Sephadex A50 (3.5×20), which has been equilibrated with the buffer used for dialysis. After washing with 0.015 M buffer, elution is made by a linear gradient elution formed by 500 mL of 0.015 M buffer and 500 mL of 0.06 M buffer. Triton X-100 is supplemented to 0.1% to both buffer solutions. Each 15 mL of fractions collected and the enzyme was eluted at 300 to 600 mL of eluate. Judging from the elution pattern, peak fraction was corresponded to the buffer concentration of 0.035 M. pooled enzyme solution was precipitated by polyethylene glycol 6000 to 20%, after 30 min of stirring in an ice bath; the enzyme solution is centrifuged at 12,000 x g for 20 min. The precipitate is suspended in small volume of 0.01 M buffer and it is used for the next step (Fig. 2).

Hydroxyapatite fractionation I: The dialyzed enzyme from preceding step was applied to a fractionation using Hydroxyapatite, which had been equilibrated with 0.002 M buffer. After gently mixing the enzyme solution and hydroxy apatite, the mixture was stayed for 5 h for adsorption the enzyme to the beads of Hydroxyapatite. Elution of the enzyme was made by a 0.14 M buffer. Pooled enzyme solution was dialyzed 0.002 M buffer thoroughly (2 days).

Hydroxyapatite fractionation II: The dialyzed enzyme from preceding step was applied to a fractionation with Hydroxyapatite, which had been equilibrated with 0.002 M buffer. This step was done like previous step but the enzyme elution was 0.12 M buffer.

Summary of typical purification of the enzyme is shown in Table 1.

Kinetic analysis of enzyme activity: A steady-state kinetic analysis of the ADH reaction was performed in 100 mM KPB (pH 6.0). To determine the apparent K_m value for

Table 1: A summary of the purification steps of the enzyme is given in result. The overall purification was 53.19 fold with a yield of 21.77%. The purified enzyme has a specific activity of 150 U mg^{-1}

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} Pr)	Yield (%)	Purification fold
Cell homogenate	24385.2	68890.4	2.82	100.00	1.00
Solublizied enzyme	6085.8	52866.4	8.68	76.74	3.08
DEAE-Sephadex I	2088.6	45182.0	21.63	65.58	7.67
DEAE-Sephadex II	519	30571.2	58.90	44.38	20.89
Hydroxya-phatite I	255	21352.8	83.73	30.99	29.69
Hydroxya-phatite II	100	15000.4	150.00	21.77	53.19

ethyl alcohol, its concentration was varied from 10 to 100 µM. The K_m values of ADH toward ethanol were calculated from the Line weaver-Burk plot to be 1.75 mM. In all cases, regular saturation curves of the activity versus the substrate concentration were observed. (Fig. 3).

Substrate specificity: The substrate specificity of the enzyme was determined using the same enzyme assay method, except that various substrate solutions (100 mM) were used instead of ethyl alcohol. These substrates include methanol, ethanol, isopropanol, n-butanol, formaldehyde, benzaldehyde, glycerol, D-glucose, D-fructose, lactate (Table 2).

Effects of metal ions and EDTA: The effects of metal ions and inhibitors on the activity of the enzyme were method. Each compound solution was stirred into the basal reaction mixture and the reaction was started with the addition of the enzyme. Each compound was added to examined by measuring the activity using the assay the reaction mixture at a concentration of 1.0 mM, except that the concentration of EDTA was 5.0 mM (Table 3).

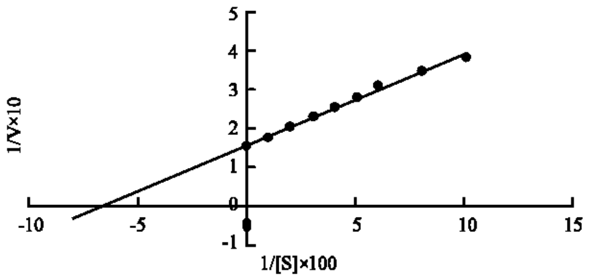

Fig. 3: Line weaver-Burk graph of oxidation of ethanol by ADH. Enzyme activity was measured at various concentration of ethanol as indicated

Table 2: Substrate specificity of the purified enzyme from *Acetobacter* sp. 15. The reaction rate with ethanol is expressed as 100

Substrate	Relative activity (%)
Methanol	1
Ethanol	100
Iso propanol	0
n-Butanol	85
Formaldehyde	0
Benzaldehyde	0
Glycerol	0
D-Glucose	0
D-Fructose	0
Lactate	0

Table 3: Effect of EDTA and metals on the activity of the purified enzyme. The reaction rate without any additive is expressed as 100

Compound	Relative activity (%)
None	100.0
EDTA	10.5
$CaCl_2$	212.0
Fe_2SO_4	39.9
$ZnCl_2$	24.3
$MgCl_2$	88.9

Optimal pH and pH stability: The correlation between the reaction rate of the ADH and pH values of the reaction mixture was determined by the same assay method as described above, except that various pHs and buffer McIlvaine buffer pH 4.0-8.0, were used for this step (Fig. 4 and 5).

Electrophoretic analysis: Dissociation into subunits was observed by SDS gel electrophoresis in the determination of molecular weight of the enzyme. In the present of SDS the enzyme was dissociated into subunits with four

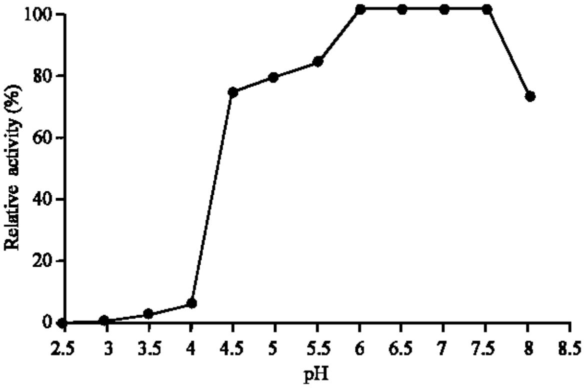

Fig. 4: The pH stability graph of ADH. Enzyme solution was diluted with various pH of Macllvaine buffer from 2.5 to 8 as indicated and stored for 24 h at 4°C. Thereafter, an aliquot of stored enzyme solution was picked up for the standard assay of enzyme activity

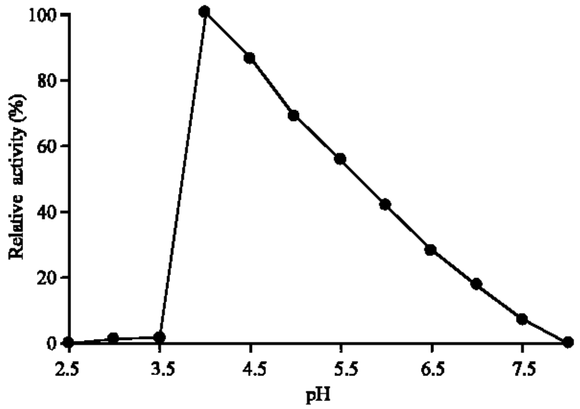

Fig. 5: The pH dependency of ADH activity was assayed under standard conditions except that pH of the buffer (Macllvaine buffer) was varied as indicated above

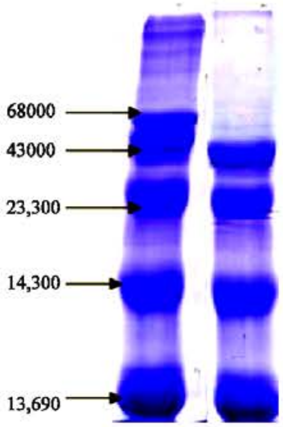

Fig. 6: SDS-Gelelectrphoresis of ADH. Twenty microliter of the last step purified enzyme was loaded on the top of the (right gel), standard marker proteins (left) were ribonuclease A, lysozyme, trypsine, ovalbumin and bovine serum albumine from top to bottom, respectively

molecular weight 13.7, 14, 23.3 and 40 kD from the top to bottom of the gel column as shown in Fig. 6.

DISCUSSION

PQQ ADH was purified to homogeneity on SDS-PAGE from the cell extract of Acetobacter sp15 by sequential column chromatographies. The purified enzyme consisted of four subunits. As shown in Table 2, ADH catalyzed the oxidation of ethanol not methanol so it is concluded that the PQQ ADH belongs to the hemoquinoprotein family of ADHs which is called ethanol dehydrogenase (Groen *et al*., 1986). Purified enzyme has been shown to possess substrate specificity for primary aliphatic alcohol. Table 2 shows the relative activity for the reductive reaction of ADH for ethanol is 100%. The enzyme act on linear chain alcohols including ethanol and n-butanol; however, it showed no activity toward secondary alcohols like isopropanol and polyoles like glycerol. The highest activity was observed with n-butanol (85% compared to the activity of ethanol). Benzaldehyde and formaldehyde were not suitable substrates for the enzyme, so aldehyde did not serve as a good substrate. The substrate spectra observed were not similar alcohol dehydrogenase like glucose dehydrogenase. The results revealed that the ADH has not a broad substrate spectrum. Primary aliphatic alcohol was rapidly oxidized but not methanol. Ethanol is best substrate for ADH; with concern the tertiary structure of alcohol dehydrogenase, it might relate with the shape of active site. In addition above, the substrate would probably enter through the hydrophobic mouth of a channel leading to the active site cavity and located between PQQ and heme-domains in the case of ADH. (Matsushita *et al*., 2004). So, the enzyme is suitable for acetic acid industrial production. Secondary and tertiary alcohols and cyclic alcohol could not be oxidized.

The enzyme is stable in neutral pH but its catalytic activity is high in pH 4 (Fig. 4 and 5), this shows that the enzyme can be regulated by pH. Alcohol-oxidizing ability and ADH activity are the most important characteristics of acetic acid bacteria. Therefore, although the occurrence the inactive ADH seems to be strange with respect to alcohol oxidation, some suggestive evidence has been reported for emergence of some kinds of inactive forms of ADH in acetic acid bacteria. In *Acetobacter*, ethanol oxidation ability was greatly decreased concomitantly with decreasing pH in culture medium (Duine *et al*., 1989).

So by shift in the pH of the culture medium, inactive ADH can presumably be converted to active ADH in the growing *Acetobacter* When *Acetobacter* grew in alkaline pH ADH activity enhanced gently, in the other word the enzyme did not face to pH shock.

As shown in Table 3, ADH Activity has been shown to increase in the presence of 1 mM Ca^{+2} and inhibited in presence the same concentration of ethylenediamine tetra acetic acid (EDTA). In the mechanism of binding Ca^{+2} to the quinoprotein alcohol dehydrogenase that catalyzed oxidation of methanol consists of the following steps: (i) association of methanol to active site Ca^{2+}; (ii) deprotonating Ca^{2+} -bound methanol by an active site Aspartate in concert with; (iii) hydride transfer from Ca^{2+}-bound substrate to the carbonyl at C-4 position and formation of a Ca^{2+} bound formaldehyde and (iv) releasing of formaldehyde from the active site of the enzyme. The catalytic role of PQQ complexed Ca^{2+} is 3-fold: (i) modest reduction of the pK_a of CH_3-OH, (ii) polarizing the carbonyl group at the C-5 position of the PQQ moiety and (iii) placing the reaction components in position to react (Zheng *et al*. 1997). Data have shown similar mechanisms could also be operative in other quinoproteins such as ADH.

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