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RESEARCH ARTICLE

EFFECT OF DIVALENT CATIONS ON THE ACTIVITY AND CONFORMATION OF YEAST ALCOHOL DEHYDROGENASE

Rizwanul Haque, Shehwaz Anwar, Md. Fazle Alam and Hina Younus*

Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

Abstract: The divalent metal chlorides affect the activity of yeast alcohol dehydrogenase (YADH). The activity of the enzyme in the presence of 20 µM of these metal chlorides decreased in the following order: $CaCl_2$ > $MgCl_2$ > no salt > $MnCl_2$ > $CoCl_2$ > $CuCl_2$ > $CdCl_2$. The EC50 values for the incubation of YADH in the presence of $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$ and $CdCl_2$ were 14.5, 37.8, 0.25, 0.21, 0.18 and 0.005 mM, respectively. The most potent inhibitor was $CdCl_2$. Fluorescence studies revealed that the conformation of YADH undergoes huge changes in the presence of these metal chlorides. By far UV-CD spectra, some secondary structural changes were observed in YADH in the presence of $CaCl_2$, $MgCl_2$, $MnCl_2$ and $CoCl_2$, however the changes were very significant in case of $CdCl_2$ and $CuCl_2$. The removal of zinc from YADH resulted in complete loss of enzymatic activity which is regained back by addition of Zn. Mg, Ca and Mn can substitute Zn in YADH, and the enzyme exhibits comparable activity as in presence of Zn. However, Co, Cu, and Cd cannot substitute Zn in YADH. The results obtained here might be useful in the biotechnological applications of alcohol dehydrogenase.

***Keywords*:** Yeast alcohol dehydrogenase; divalent cations; activity, conformation.

Introduction

The physiological role of alcohol dehydrogenase (ADH) is to catalyze the reversible oxidation of alcohols to their corresponding carbonyl compounds (Racker, 1955). They attract major scientific interest for the evolutionary perspective, afforded by their wide occurrence in nature, and for their use in synthesis, thanks to their broad substrate specificity and stereo selectivity. ADHs are often used for synthesis of enantiomerically pure stereoisomers of chiral alcohols. Yeast ADH (YADH) belongs to the zinc containing ADH family (Leskovac *et al.*, 2002). YADH is a homotetrameric enzyme with eight zinc ions and a total molecular mass of 150 KDa (Le *et al.*, 1996). The active site at each subunit contains one zinc ion which is necessary for enzyme activity. The second zinc ion present on each subunit of the enzyme predominantly plays a conformational role, by stabilizing the tertiary structure (Magonet *et al.*, 1992). The zinc atom at the catalytic site exists as a tridentate ligand of two cysteine residues and one histidine (Zn, Cys_2, His_1) (Crow *et al.*, 1995). The fourth Zn coordinate position serves as the binding site for the hydroxyl group of ethanol, which in absence of ethanol is occupied by water.

In the present study, the effect of other divalent cations e.g. manganese chloride, magnesium chloride, calcium chloride, copper chloride, cadmium chloride on the activity and conformation of YADH was investigated. The catalytic activity of YADH was also determined after replacing the zinc bound to the enzyme by manganese, magnesium, calcium, copper and cadmium. This study was performed to know whether YADH is activated/inhibited in the presence of other divalent cations and whether any other divalent metal can replace the zinc atoms in the enzyme and confer greater/lower

Corresponding Author: **Hina Younus**
E-mail: hinayounus@rediffmail.com

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activity to the enzyme as compared to the zinc bound form. This information is expected to be useful in using the enzyme in the laboratory or industrial scale.

Materials and Methods

Tris, zinc chloride, manganese chloride and copper chloride were purchased from Qualigens Fine Chemicals, India. Cadmium chloride was from HIMEDIA chemicals, India. Yeast alcohol dehydrogenase, Nicotinamide adenine dinucleotide (NAD), magnesium chloride, calcium chloride were from Sisco Research Lab., India. Ethanol was a product of CYS, china. MilliQ water was used in all experiments. All other chemicals used were of analytical grade.

Determination of YADH concentration and activity - The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard protein. The activity of YADH was determined spectrophotometrically using ethanol as the substrate. The standard reaction mixture in a total volume of 1 ml contained an appropriate amount of YADH, 0.4 mM NAD^+ and 0.3 M ethanol in 50 mM TrisHCl buffer, pH 8.0. The reaction was initiated by the addition of ethanol, followed by incubation at 37 °C, and monitored continuously for some minutes by measuring the increase in absorbance at 340 nm due to formation of NADH.

Effects of divalent cations on the activity of YADH - The effect of divalent cations on YADH activity was determined by including 20 µM of divalent metal chlorides ($CaCl_2$, $MgCl_2$, $MnCl_2$, $CuCl_2$, $CoCl_2$ and $CdCl_2$) in the standard reaction mixture. The activity measurements were done under standard assay conditions. The activity of YADH was also determined in the presence of varying concentration of divalent metal chloride.

Effect of divalent cations on the conformation of YADH - Effect of divalent cations on the conformation of YADH was studied by fluorescence and circular dichroism (CD) spectroscopic measurements. Fluorescence measurements were performed on a Shimadzu Spectrofluorimeter (model RF-5301 PC) equipped with a water bath (Cycla NTT). The Fluorescence spectrum of YADH (0.5 mg/ml) in absence or in presence of 20 µM metal chlorides ($ZnCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, and $CdCl_2$) was measured at 25 °C with a cell of 1 cm pathlength. YADH in 50 mM TrisHCl buffer, pH 8.0 was excited at 280 nm and the emission was recorded in the wavelength of 300-400 nm. The excitation and emission slit widths were 5 nm and 10 nm, respectively.

CD measurements were performed on a Jasco Spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with d-10-camphosulphonic acid. All the measurements were performed at 25 °C using a 1 cm path length cell. Far ultraviolet (UV) (190-230 nm) CD spectra were taken at 0.5 mg/ml protein concentration in the presence of 20 µM metal chlorides ($ZnCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, and $CdCl_2$). The results are expressed as CD (mdeg) which is defined as:

$$CD\ (mdeg) = MRE\ (10 \times n \times 1 \times C_p)$$

Where MRE is the mean residual ellipticity in $deg.cm^2.dmol^{-1}$, and n is the number of amino acid residues, 1 is the path length of the cell in cm and C_p is the mole fraction.

Removal of Zinc bound to YADH (-Zn YADH) - YADH (2-4 mg/ml) was incubated with 80 mM EDTA for 4-6 h at 37 °C. It was then extensively dialyzed against 50 mM TrisHCl buffer (pH 8.0). The enzymatic activity of -Zn YADH was then determined under standard assay conditions.

Effects of divalent cations on the activity of –Zn YADH - The effect of replacing Zn by other divalent cations (Mg^{2+}, Mn^{2+}, Cu^{2+}, Co, Cd^{2+} and Ca^{2+}) on the activity of –Zn YADH was determined in the presence of 20 µM of $MnCl_2$, $CoCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, and $CdCl_2$ under standard assay conditions.

Results and Discussion

Effect of Divalent Cations on the Activity of YADH

The effect of six divalent cations, $MnCl_2$, $MgCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, and $CdCl_2$ on the activity of YADH is shown in Figure 1. The activity of YADH was higher in the presence of $CaCl_2$ and $MgCl_2$ at 20 µM concentration in the reaction mixture. The activity of YADH in presence of 20 µM $MnCl_2$ and $CoCl_2$ was comparable to the

enzyme activity in the absence of any salts. The enzyme was inhibited by $CuCl_2$ and $CdCl_2$ even at 20 µM concentration. The activity of YADH in the presence of 20 µM divalent metal chlorides decreased in the following order; $CaCl_2$ > $MgCl_2$ > no salt > $MnCl_2$ > $CoCl_2$ > $CuCl_2$ > $CdCl_2$. The effect of varying concentration of divalent metal chloride on the activity of YADH is shown in Figure 2. The enzyme was activated by $CaCl_2$, and $MgCl_2$ in the micromolar concentration range but inhibited in the millimolar range. The enzyme was inhibited by $MnCl_2$, $CoCl_2$, $CuCl_2$ and $CdCl_2$ in both micromolar and millimolar concentration range. The EC50 values for the inhibition of YADH in the presence of these metal chlorides are shown in Table 1. The most potent inhibitor was $CdCl_2$ (EC50 = 5 µM).

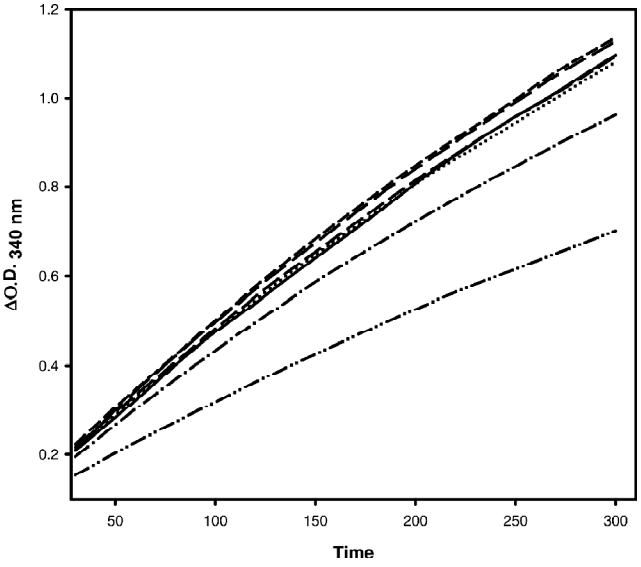


Figure 1: **Activity of YADH in the presence of 20 µM divalent metal chlorides. The activity of YADH (5 µg/ml) was determined in the presence of 20 µM concentration of each divalent metal chlorides under standard assay conditions. YADH alone (——) $CaCl_2$ (- -), $MgCl_2$ (– –), $MnCl_2$ (— —), $CoCl_2$ (....), $CuCl_2$ (– ·–), and $CdCl_2$ (– · · –).**

Table 1
EC50 Values for the Inhibition of YADH by Various Divalent Metal Chlorides

Metal chloride	*EC50 values (mM)*
$MgCl_2$	37.8
$CaCl_2$	14.5
$CuCl_2$	0.18
$MnCl_2$	0.25
$CoCl_2$	0.21
$CdCl_2$	0.005

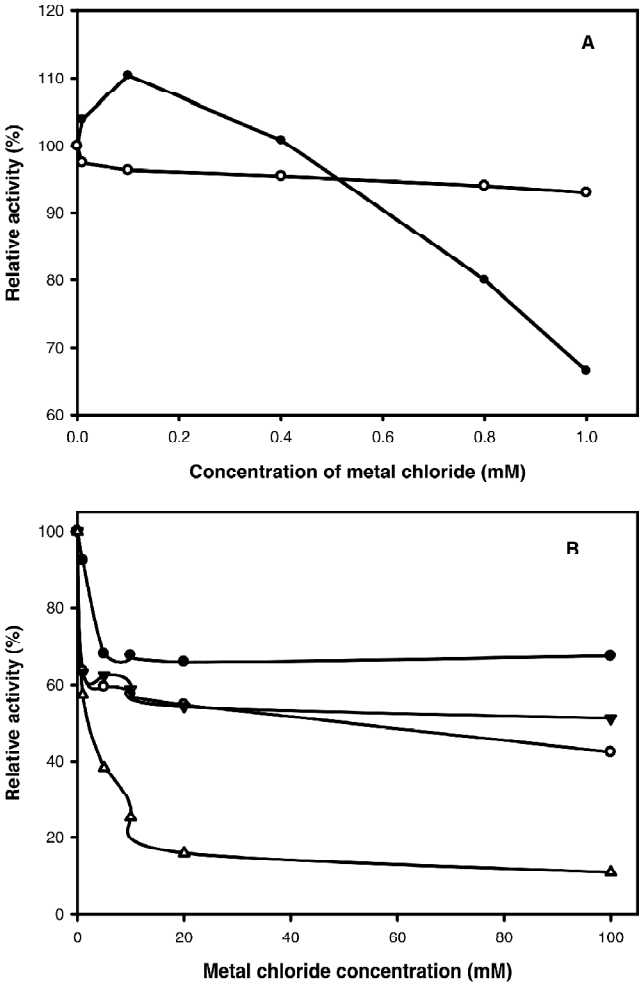


Figure 2: **Activity of YADH in the presence of divalent metal chlorides. The activity of YADH (5 µg/ml) was determined in the presence of varying concentration of divalent metal chlorides under standard assay conditions.** A: **$CaCl_2$ (●), $MgCl_2$ (○)** B: **$MnCl_2$ (●), $CuCl_2$ (○), $CoCl_2$ (▼) and $CdCl_2$ (Δ).**

Effects of Divalent Cations on the Conformation of YADH

The fluorescence spectra of YADH alone and YADH in the presence of 20 µM divalent metal chloride is shown in Figure 3. In the presence of all divalent metal chlorides used, quenching of fluorescence was observed. Therefore, the conformation of YADH changed in the presence of all these metal chlorides.

The far UV-CD spectra of YADH alone (native enzyme), in the presence of 8 M urea (denatured enzyme) and in the presence of divalent metal chloride is shown in Figure 4. It was observed that YADH undergoes some changes in the secondary structure in the presence of all divalent metal chlorides. However, since the spectra in the

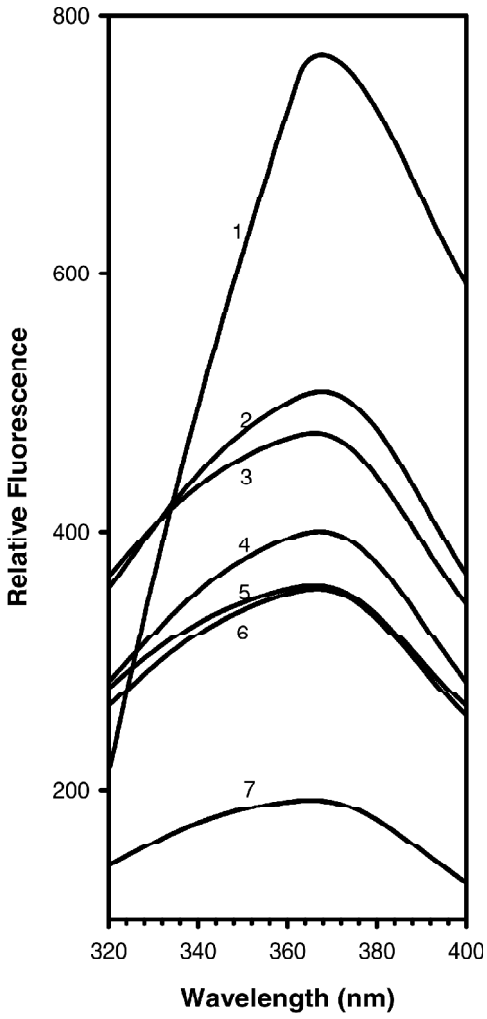
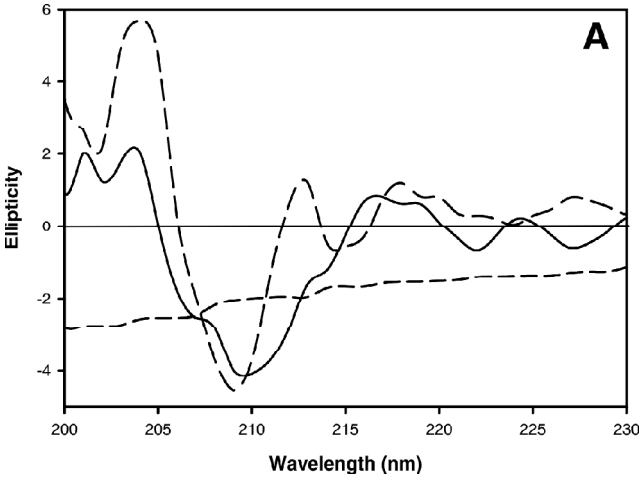
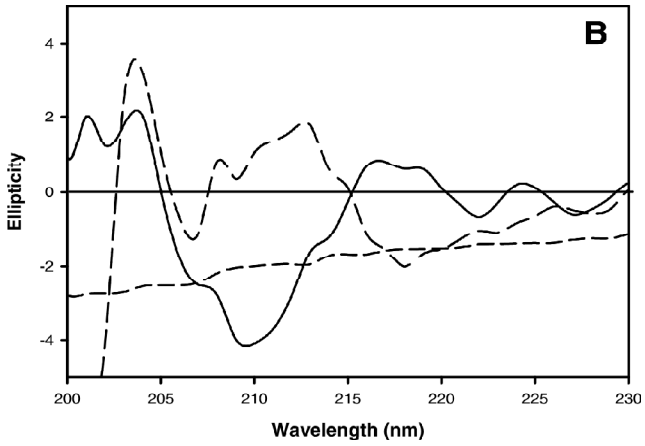
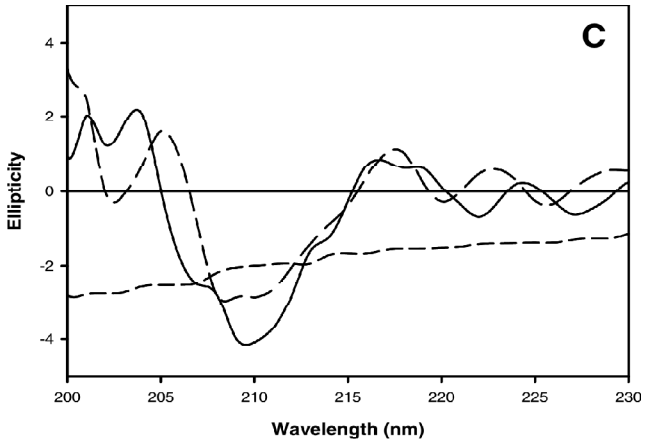
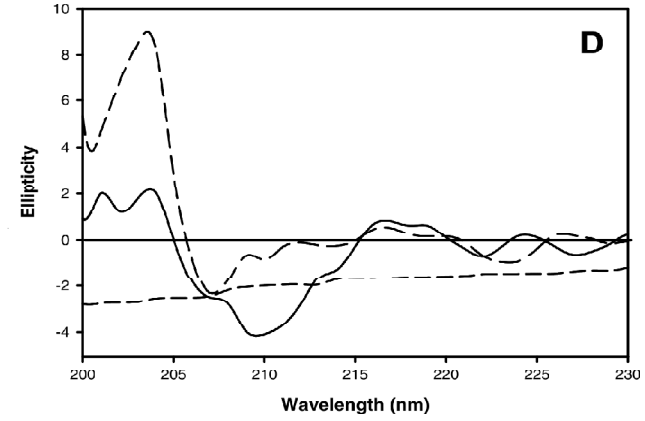


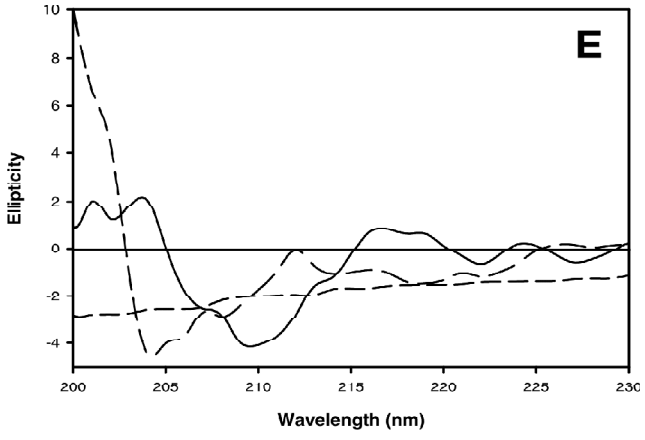
Figure 3: **Fluorescence spectra of YADH alone and in the presence of divalent metal chloride. The fluorescence spectra of YADH (0.5 mg/ml) alone and in the presence of 20 µM divalent metal chloride was measured. Samples were excited at 280 nm and the emission was recorded at 320-400 nm. YADH alone (1), YADH in presence of $MgCl_2$ (2), $CdCl_2$ (3), $MnCl_2$ (4), $CaCl_2$ (5), $CuCl_2$ (6) and $CoCl_2$ (7).**











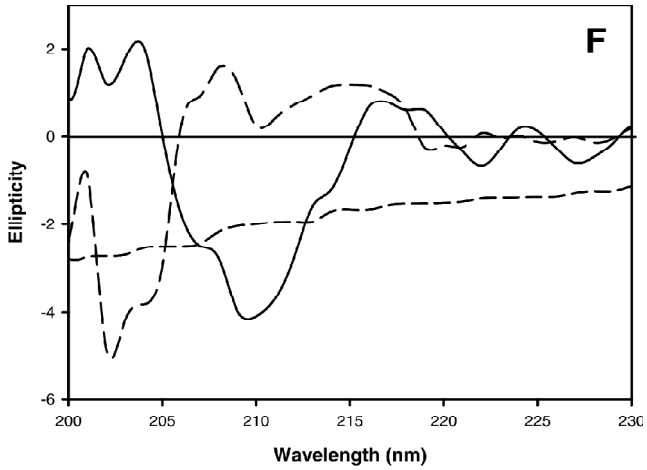


Figure 4: **Far UV-CD spectra of YADH in the absence and in the presence of divalent metal chlorides. The far UV-CD spectra was determined using 0.5 mg/ml of YADH and 20 μM divalent metal chlorides. In each plot, curves of YADH alone (——), YADH in presence of 8M urea (– –), and YADH in the presence of a specific divalent metal chloride are shown (— —). The metal chloride is $CaCl_2$ (A), $CdCl_2$ (B), $MgCl_2$ (C), $CoCl_2$ (D), $MnCl_2$ (E) and $CuCl_2$ (F).**

presence of all divalent metal chlorides were not similar to that of the denatured enzyme, hence the secondary structure was not completely lost, it is perhaps native like. Some secondary structural changes were observed in YADH in the presence of $CaCl_2$, $MgCl_2$, $CoCl_2$ and $MnCl_2$ (Figure 4 A, C, D and E). However, in the presence of $CdCl_2$ and $CuCl_2$, very significant changes in the secondary structure were observed (Figure 4 B and F).

These studies reveal that the conformation of YADH undergoes changes in the presence of all the divalent metal chlorides used in the study. Since these metal chlorides cause changes in the conformation of YADH, they alter the activity of the enzyme. $CdCl_2$ and $CuCl_2$ cause massive change in the structure of the enzyme as compared to the other metal chlorides, hence this explains why they also inhibit the enzyme to the greatest extent.

Effects of Divalent Cations on the Activity of –Zn YADH

The removal and re-insertion of metals in YADH may produce some distortion of the active site or that a different metal may be bound weakly (or strongly) to the active site residues, or may even be bound to different residues (in a different geometry) of the active site. As a consequence, a substitution of zinc just with other divalent ions may suggest novel and interesting information. In this study, we have studied the effect of replacing Zn with other divalent metals like Ca, Mg, Mn, Co, Cu and Cd on the activity of the enzyme. The removal of zinc from YADH resulted in complete loss of enzyme activity. The effects of divalent metal chlorides on the activity of –Zn YADH is shown in Figure 5. The activity of –Zn YADH in presence of 20 μM $ZnCl_2$ was regained but not completely. Therefore, the inactivation of the enzyme due to removal of Zn is reversed back by addition of Zn. The activity of –Zn YADH in presence of $MgCl_2$ was a little better than in presence of $ZnCl_2$. The activity of –Zn YADH in presence of $CaCl_2$ was comparable to that in presence $ZnCl_2$. It was a bit lower in presence of $MnCl_2$. And no activity was observed in presence of $CoCl_2$, $CuCl_2$, and $CdCl_2$. Therefore, Mg, Ca and Mn can substitute Zn in YADH, and the enzyme retains comparable activity as in presence of Zn. However, Co, Cu and Cd cannot substitute Zn in YADH.

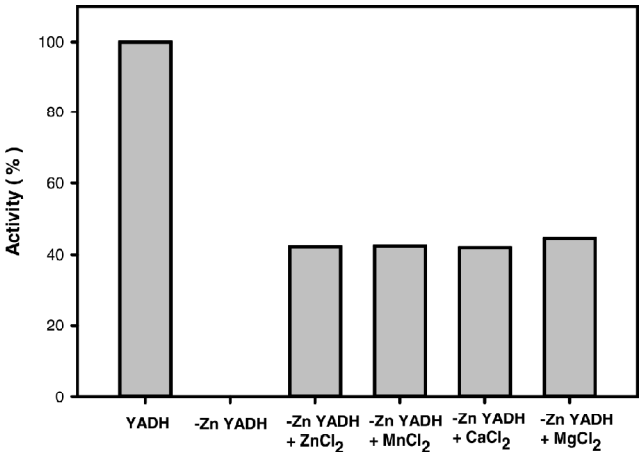


Figure 5: **Effect of divalent metal chlorides on the activity of -Zn YADH. The activity of 5 μg/ml of –Zn YADH was determined in the absence and presence of 20 μM of metal chlorides under standard assay conditions. This was compared with the activity of 5 μg/ml YADH which was taken as 100%.**

Acknowledgments

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Abbreviations

YADH, yeast alcohol dehydrogenase; NAD, nicotinamide adenine dinucleotide; CD, circular dichroism; UV, ultraviolet.

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