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Effect of AMP, ATP and 2-Mercaptoethanol on the Activity of Alcohol Dehydrogenase Isolated from Palm ((*Elaies Guineensis)*) and Raffia (*Raphia Africana*) Saps from Some Locations in Niger Delta Region of Nigeria

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Abstract
Alcohol dehydrogenases (ADHs: EC.1.1.1.1) are the critical enzymes involve in the catalysis of the last step during alcohol production. Alcohol dehydrogenase, ADH was obtained from yeast of raffia (R. hookeri) and palm (E. guineensis) saps from selected polluted and non-polluted locations in Niger Delta region: Akwa Ibom, Bayelsa, Cross River, Delta, Edo, Imo and Rivers States. The activity of the ADH was determined at the temperature of 30^{o}C and pH 8.0. Adenosine monophosphate (AMP) activated the ADH from all the locations and highest enzyme activity was seen in ADH of yeast of E. guineensis from polluted site in Delta state. Adenosine triphosphate (ATP) and 2- mercaptoethanol inhibited the ADH from all the locations. Least reduction of activity in the presence of these inhibitors was observed in E. guineensis obtained from polluted environment in Delta state. The activity of ADH of Yeast isolated from either raffia or palm saps from polluted and non-polluted environment can be regulated by these modulators in industries involve in their uses such as in brewery and bakery.

Keywords: Palm, Raffia, yeast sap(*Saccharamyces cerevissiae*), Alcohol dehydrogenase, AMP, ATP, 2-Mercaptoethanol

Introduction
Palm and raffia wines are a naturally sweet fermented beverage obtained from the saps of raphia palm (*Raphia hookeri* Mann & Wendland) and the African oil-palm (*Elaeis guineensis* Jacq.) which contains nutrient such as amino acid, proteins, vitamins and sugars as well as heavy suspension of live yeasts and bacteria (1). About 70% of the total yeast of palm wine sap is made up of *Saccharomyces cerevisiae* (2,3).
Yeasts have been isolated from many sources for industrial purposes. Such include yeasts isolated from palm wine for industrial production of ethanol (4,5,6), for single cell protein and for leavening of dough for bread-making (7), for wine production (8) and from many fermenting sources including fermenting cassava tubers (9,10).
Saccharomyces cerevisiae is the main yeast strain that is commonly reported to be responsible for alcoholic fermentation (11).
Alcohol dehydrogenases (ADH; EC 1.1.1.1) belong to the oxidoreductase family; a class of enzymes that catalyze the reversible oxidation of alcohols to corresponding aldehydes or ketones using NAD or NADP as coenzyme (12).
Alcohol dehydrogenase is made up of four subunits with each subunit containing one zinc atom (13). There are two obvious active sites per subunit: sulfhydryl groups which can be differentiated on account of differential reactivity with iodoacetate and butyl isocyanate (14).
Yeast ADH is somewhat active on the straight chain primary alcohols. It acts to a very limited extent on certain secondary and branched chain alcohols (15). *In vitro*, the enzyme is generally assayed and used in a more alkaline pH region (13).Alcohol dehydrogenase present in yeast is the most active form of the enzyme (16). Ethanol is the best substrate for Yeast alcohol dehydrogenase and its activity decreases as the size of the alcohol increases.

Materials and Method
Collection of Samples
Fresh palm wine saps obtained from raffia palm (*Raphia hookeri*) and oil palm (*Elaensis guineensis*) were collected in sterile 750ml sample containers from palm wine tapers across Niger-Delta States, Nigeria within 30-60 min of tapping. The specific towns of collection were Eboacha-Egbema and Ovuru, Mbaise (Imo state), Eleme and Borokiri (Rivers state), Ikpoba and NIFOR (Edo state), Alaja, New ogorode and Ovu (Delta state), Tobia and Imiringi (Bayelsa state), Akamkpa and Atimbo (Cross River State) and Ibeno and Metro, Uyo (Akwa Ibom State).

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Table 1: The collection locations of the *Raphia hookeri* and *Elaeis guineensis* sap samples from various Niger-Delta States in Nigeria.

Type of sap	City	State	Status
Raphia hookeri(Rafia)	Metro,Uyo	Akwa Ibom	Not Polluted
	Tombia	Bayelsa	Not Polluted
	Akamkpa	Cross River	Polluted(unicem)
	Ovu	Delta	Not Polluted
	Ikpoba slope	Edo	Polluted(Bendel Brewery)
	Eboacha-Egbema	Imo	Polluted(Gas flaring)
	Eleme	Rivers	Polluted(Petrochemical)
Elaensis guineensis(Palm)	Ibeno	Akwa Ibom	Polluted (Exxon Mobil)
	Imiringi	Bayelsa	Polluted(Shell,Nigeria)
	Atimbo	Cross River	Not polluted
	New Ogorode Alaja,	Delta	Polluted(Flour mill,ASCA,NEPA),Alaja steel company
	NIFOR	Edo	Not polluted
	Ovuru-Mbaise	Imo	Not polluted
	Borokiri	Rivers	Not polluted

Preparation of YPD Agar and Medium

YPD (yeast extract-peptone dextrose) agar was prepared by measuring 2.0 g of glucose monohydrate, 1.0 g yeast extract, 2.0 g peptone and 1.5 g agar-agar powder into a 250 ml conical flask. Little volume of distilled water was added to dissolve the flask contents and thereafter, the solution was made up to 100 ml with the distilled water. The solution was sterilized by autoclaving it for 15 min at 121°C. YPD medium was prepared following the procedure described with the exclusion of agar-agar powder in the final mixture.

Isolation of Yeast Cells from Saps

Raphia hookeri and *Elaensis guineensis* saps were allowed to sediment for a period of 10- 20 min. The sediments, which comprise dense population of yeast cells, were thereafter aseptically collected using sterile 10 ml syringes for culturing.

Growth of Isolated Yeast Cells on YPD Agar

The yeast cells isolated from raffia and palm saps were grown in a sterilized YPD agar in petri dishes and allowed to grow for 48-72 h. Five microlitre (5 µl) of a 20% antibiotic (ampicilin) was added to each culture to prevent growth of bacteria. Yeast cells (colonies) grown on culture plates were thereafter identified using microscopic approach.

Microscopic Identification of Yeast Isolates

Yeast identification was performed using microscopic observation keys and illustrated manuals 17,18). Briefly, aseptically picked yeast cell from grown colony was emulsified in a drop of saline on a slide. This was then stained with lactophenol and observed microscopically for yeast structures. This was carried out by a medical laboratory scientist in the Department of microbiology, Delta state University, Abraka, Nigeria.

Sub Culturing of Yeast Cells

Yeast cells grown on YPD agar plates were aseptically collected using sterilized tooth-picks and sub cultured into 3 ml of YPD medium. The cells were allowed to grow over duration of 48-72 h. One hundred milliliter (100ml) of the YPD medium was previously prepared with 5 µl of a 20% antibiotic (ampicilin) to prevent bacterial growth.

On microscopic examination, yeast-like cells appeared large, ellipsoidal and budding. On India ink preparation, no capsules were observed. Further identification was conducted by biochemical reactions. Isolates were able to ferment glucose, sucrose and raffinose. The yeast cells (colonies) were harvested for isolation of crude enzyme.

Isolation of Alcohol Dehydrogenase (ADH)

The isolation of alcohol dehydrogenase from the raffia and palm wine yeasts was carried out according to the method described by (19). Briefly yeast cells harvested from YPD (culture) medium were subjected to homogenization in a cold water environment using 3 ml of 0.1 M of phosphate buffer pH 8.0.This was followed by Centrifugation at 2000Xg for 10 min to separate the pellet from the supernatant. The supernatants which contained the crude enzyme extract (ADH) were then collected into labeled sample bottles and analyzed immediately for ADH activity

Alcohol Dehydrogenase Activity Assay

ADH activity was carried out according to the method of (20) with little modification. Briefly 0.5 ml of 60mM Tris-HCl (pH 8.0) buffer was added to the test tube followed by 0.25ml of 2mM ethanol and 0.5ml of 60mM NAD+. The mixture was incubated at 30°C for 10 min and the reaction was initiated by adding 0.5ml of the enzyme extract. The reaction solution was centrifuged at 4000Xg for 30 sec and the supernatant obtained. Thereafter, the increase in absorbance at a wavelength of 340 nm in the initial 10 min was recorded. Similarly, the change in absorbance at a wavelength of 340 nm within the first 10 min was also recorded for the blank (without the enzyme extract). Alcohol Dehydrogenase Activity Assay in the Presence of Effectors

ADH activity in the presence of effectors was assayed following the method described above with slight modification. Two and half millilitre (2.5 ml) of 60mM Tris-HCl (pH 8.0) buffer was added to the test tube followed by 0.4ml of 2mM ethanol, 0.1ml of 60mM NAD+ and 0.1ml of 20mM ATP . The mixture was incubated at 30°C for 10 min and the reaction was initiated by adding 0.5ml of the enzyme extract. The reaction solution was centrifuged at 4000 g for 30 sec and the supernatant obtained. Thereafter, the increase in absorbance at a wavelength of 340 nm in the initial 10 min was recorded. Similarly, the change in absorbance at a wavelength of 340 nm within the first 10 min was also recorded for the blank (without the enzyme extract).

The activity of alcohol dehydrogenase (ADH) in µmole/min was calculated using the formula:

ADH activity (µmole/min) = $[(\Delta A_{340}/\text{min Test} - \Delta A_{340}/\text{min Blank})\ (v_2)\ (df)] / \varepsilon\ (v_1)$

Where ΔA_{340} = Change in absorbance at 340 nm; df = dilution factor; $\varepsilon = 6.22 \times 10^{-3}$ µM (extinction coefficient of β-NADH at 340 nm); v_1 = volume of enzyme extract used (ml); v_2 = Total volume of reaction mixture (ml).

Statistical analysis

The assays were carried out in triplicate and analyzed with the use of one way analysis of variance (ANOVA) using SPSS version 20. Results were expressed as mean ± S.D.

Results and Discussion

The result of AMP-mediated modulations of ADH activity of yeast isolated from fresh *R. hookeri* saps from some locations in Niger Delta region of Nigeria at 30°C, pH 8.0 is shown in figure 1

The result revealed that there was increased ADH activity of yeast isolated from different locations as the concentrations of AMP increased from1.0, 2.0 and 3.0mM. Although significant increase in ADH activity was observed at 1.0 and 2.0 in some locations, highest activity was observed at 3.0mM concentration, Delta state having the highest. The order of percentage increase in activity at 3mM is Edo (15.23) > Imo (17.17) >Cross River (25.38) >Akwa Ibom (40) > Rivers (40.60) > Delta (43.14).

Increased ADH activity of yeast isolated from *E. guineensis* saps was observed in all the location as the concentration of AMP increased (figure 2). This observed increase was significant at 2.0 and 3.0mM AMP in Akwa Ibom and Delta states when compared with their respective controls ($p<0.05$). Bayelsa, Cross River, Edo and Imo demonstrated significant increase in ADH activity at 3.0mM AMP when compared with their respective controls ($p<0.05$) while Rivers state demonstrated non-significant increase(Figure 2) in ADH activity in all the three different AMP concentrations when compared with its control ($p>0.05$). The order of percentage increase in activity at 3mM is Edo (24) > Imo (30) > Cross River (32) > Bayelsa (41) > Akwa Ibom (45) > Rivers (50) >Delta (58).

Comparison of ADH activity between *R. hookeri* and *E guineensis* showed that AMP activated the ADH obtained from *E guineensis* than *R. hookeri*.

The result of the effect of ATP on the activity of ADH activity of yeast obtained from *R. hookeri* sap is shown in Figure 3. ADH activity decreased as the concentration of ATP increased in all the locations. This decrease in activity was significant at 3mM ATP in Akwa Ibom, Cross River, Delta and Rivers when compared with their respective controls ($p< 0.05$). However, the decrease in ADH activity observed in Bayelsa, Edo and Imo States was not significant when compared with their respective controls ($p>0.05$). The order of increase of percentage decrease in ADH activity at 3mM is Edo (6.0) > Delta (7.0) > (7.16) >Cross River (14.0) > Akwa Ibom (16.0) > Bayelsa (21.0). ADH of yeast isolated from *E. guineensis* (Figure 4) showed a general decrease in activity as the ATP concentrations increased and this decrease in activity was significant at 3mM ATP in Akwa Ibom,Cross River, Delta and Rivers when compared with their respective controls ($p<0.05$). The order of increasing percentage decrease in ADH activity was Delta (5.0) = Edo (5.0) > Imo (6.8) > Akwa Ibom (12.0) = Rivers (12.0) > Bayelsa (17.0).

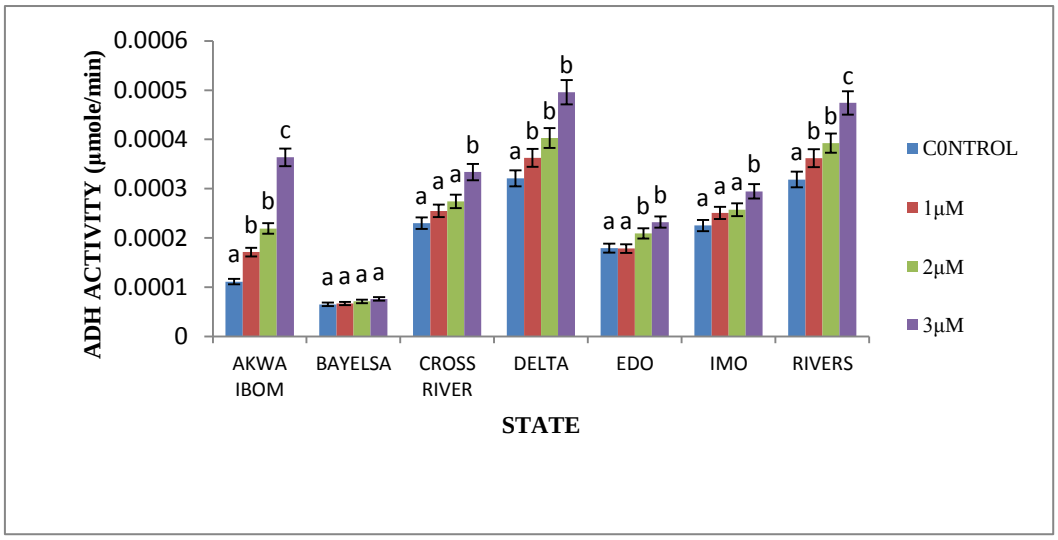

Figure 1. *AMP-mediated modulations of ADH activity of yeast isolated from fresh R. hookeri saps from some locations in Niger Delta region of Nigeria at 30°C, pH 8.0. Source of alcohol dehydrogenase: Akwa Ibom (Metro Uyo, not polluted), Bayelsa(Tobia, not polluted), Cross River (Akamkpa, Polluted), Delta (Ovu, not polluted), Edo (Ikpoba Slope, polluted) Imo (Eboacha-Egbema, polluted), Rivers (Eleme, polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = different concentrations of AMP used.*

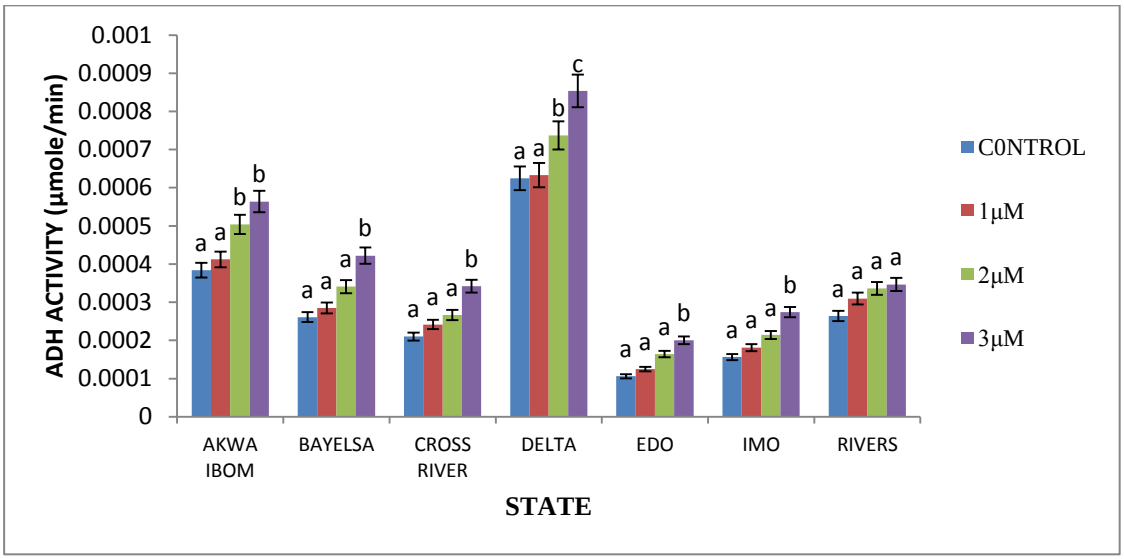

Figure 2. *AMP-mediated modulations of ADH activity of yeast isolated from fresh E. guineensis saps from some locations in Niger Delta region of Nigeria at 30°C, pH 8.0. Source of alcohol dehydrogenase: Akwa Ibom (Ibeno, polluted), Bayelsa (Imiringi, polluted), Cross River (Atimbo, not polluted), Delta (Alaja, polluted), Edo (NIFOR, not polluted), Imo (Ovuru-Mbaise not polluted), Rivers (Borokiri, not polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = different concentrations of AMP used.*

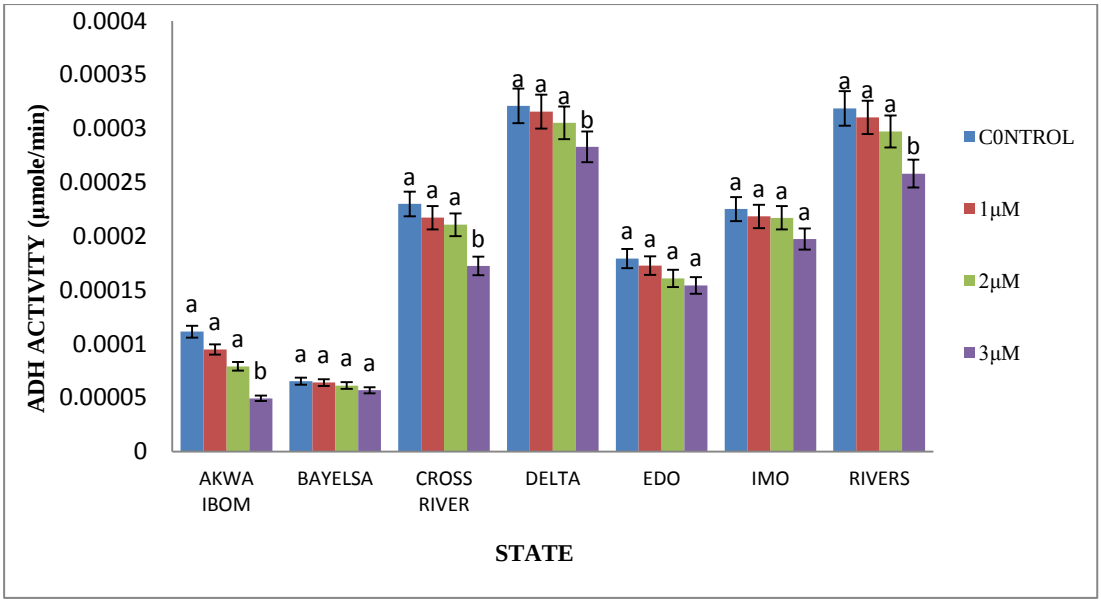

Figure 3: *Adenosine triphosphate (ATP)-mediated regulation of ADH activity of yeast isolated from R. hookeri saps from different locations in Niger Delta region 30^{0}C, pH 8.0. Source of alcohol dehydrogenase: Akwa Ibom (Metro Uyo, not polluted), Bayelsa(Tobia, not polluted), Cross River (Akamkpa, Polluted), Delta (Ovu, not polluted), Edo (Ikpoba Slope, polluted) Imo (Eboacha-Egbema, polluted), Rivers (Eleme, polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = different concentrations of ATP used.*

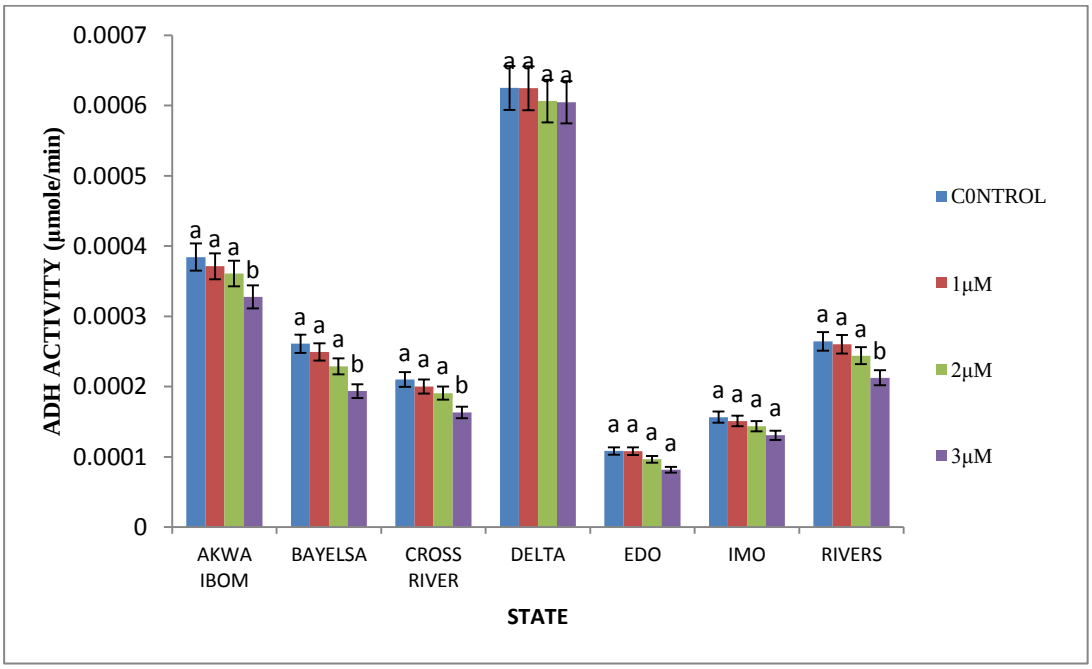

Figure 4: *Adenosine triphosphate (ATP)-mediated regulation of ADH activity of yeast isolated from E. guineensis saps from different locations in Niger Delta region 30^{0}C, pH 8.0. Source of alcohol dehydrogenase: Akwa Ibom (Ibeno, polluted), Bayelsa (Imiringi, polluted), Cross River (Atimbo, not polluted), Delta (Alaja, polluted), Edo (NIFOR,not polluted), Imo (Ovuru-Maise not polluted), Rivers (Borokiri, not polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = different concentrations of ATP used.*

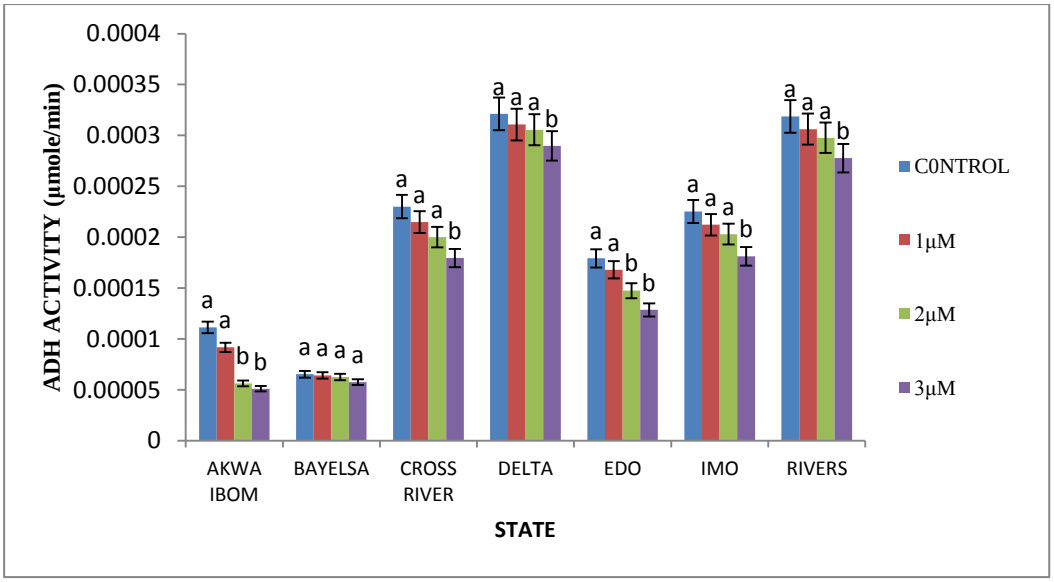

Figure 5:*.Effect of 2-mercaptoethanol-mediated regulation of yeast alcohol dehydrogenase activity isolated from fresh R. hookeri saps from some locations in Niger Delta region 30°C, pH 8. Source of alcohol dehydrogenase: Akwa Ibom (Metro Uyo, not polluted), Bayelsa(Tobia, not polluted), Cross River (Akamkpa, Polluted), Delta (Ovu, not polluted), Edo (Ikpoba Slope, polluted) Imo (Eboacha-Egbema, polluted), Rivers (Eleme, polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = different concentrations of 2-mercaptoethanol used.*

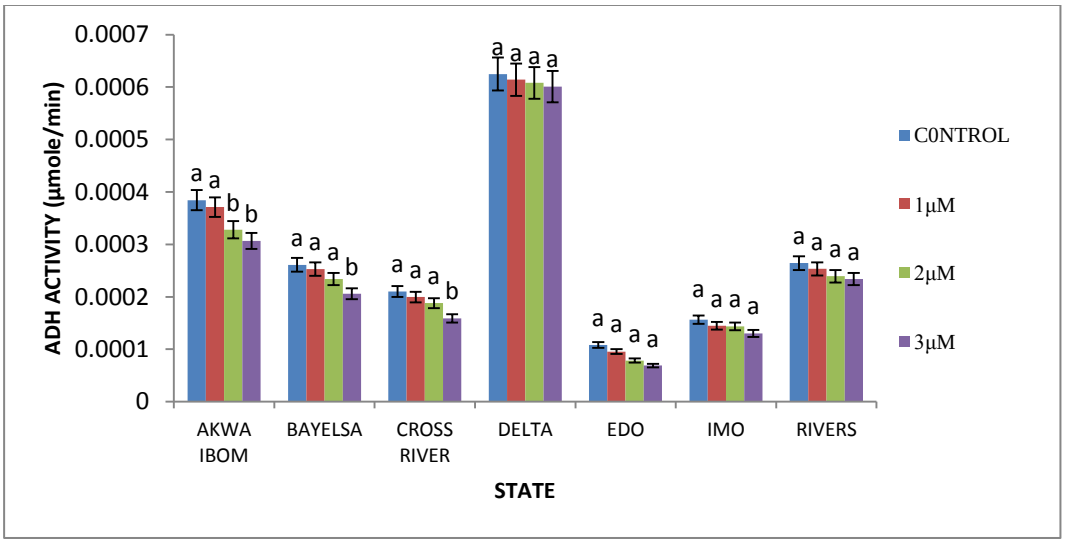

Figure 6: *Effect of 2-mercaptoethanol-mediated regulation of yeast alcohol dehydrogenase activity isolated from fresh E. guineensis saps from some locations in Niger Delta region 30°C, pH 8. Source of alcohol dehydrogenase: Akwa Ibom (Ibeno, polluted), Bayelsa (Imiringi, polluted), Cross River (Atimbo, not polluted), Delta (Alaja, polluted), Edo (NIFOR,not polluted), Imo (Ovuru-Maise not polluted), Rivers (Borokiri, not polluted). Same superscript = No significance difference and Different superscript = significantly different within a state. Legend: 1mM, 2mM and 3mM = Different concentrations of 2-mercaptoethanol used.*

The result of the effect of modulation of ADH activity by 2-mercaptoethanol is represented in figure 5. The result revealed significant decrease ($p < 0.05$) in ADH activity at 2 and 3mM concentration of 2-mercaptoethanol in Akwa Ibom and Edo while there was a significant decrease in activity at 3mM 2-mercaptoethanol in Cross River, Delta, Imo and Rivers when compared with their individual controls (Figure 5). Bayelsa demonstrated a non-significant decrease in ADH activity at all the concentrations investigated when compared with its control ($p>0.05$). The order of increase of percentage decrease in ADH activity at 3mM is Delta (8.0) > Rivers (10.5) > (11.30) > Edo (12.0) > Cross River (13.3) > Bayelsa (19.0) > Akwa Ibom (27.0).

The result of 2-mercaptoethanol on the ADH activity of *E. guineensis* (Figure 6) indicated significant decrease in activity at 2 and 3mM of 2-mercaptoethanol in Akwa Ibom and significant at 3mM Bayelsa and Cross River when compared with their individual controls ($p>0.05$). Delta, Edo, Imo and Rivers showed non-significant decrease in ADH activity at all the concentrations investigated. The order of increase of percentage decrease in ADH activity at 3mM is Delta (5.8) >Imo (6.8) > Rivers (7.8) > Edo (9.87) > Cross River (13.0) > Bayelsa (14.0) > Akwa Ibom (20.0).

The activation of several enzymes by AMP are well documented (21,22,23). Adenosine monophosphate (AMP) mediated ADH activity isolated from microorganisms were earlier reported (24). AMP activating AMP-activated protein kinase (AMPK) is previously documented (25, 26, 27). Since there are no previous literature reports on AMP regulating ADH activity of yeast obtained from other sources or yeast isolated from *R. hookeri* and *E. guineensis*, activation of ADH by AMP is probably due to allosteric binding to the enzyme.

Decrease in the activity of enzymes by ATP has been previously reported (21,22,23). Decrease in isocitrate dehydrogenase by ATP from human kidney mitochondria has been previously reported (28) .The decrease in activity observed in this study could be attributed to ATP allosterically binding to the enzyme thereby changing its conformation from active to inactive, preventing it from binding more of the substrate(ethanol).

Inhibition of enzymes by 2-mercaptoethanol has been well documented (29). Inhibition of horse liver arginase by 2-mercaptoethanol is well documented (30). However, there is no literature documentation of 2-mercaptoethanol inhibiting ADH activity. Inhibition of ADH by 2-mercaptoethanol may be due to the inhibitor competing with the substrate in binding to the active site since the inhibitor's structure resembles that of the substrate.

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