Original Research Article

Extraction, Purification and Kinetic Study of Lactate Dehydrogenase of Male Chicken from Ebocha-Oil Exploration Area, Nigeria

ABSTRACT

Aim: This study focused on the extraction, purification and kinetic studies of lactate dehydrogenase of male chickens from Ebocha oil exploration area, Imo state, Nigeria.

Methods: Twenty-one apparently healthy mature (6-9 months) male chickens (*Gallus domesticus*) from Ebocha oil exploration area, Imo State, Nigeria were screened for lactate dehydrogenase activity, thus accessing the level of chronic cell exposure to gas flaring. Their thigh muscle tissues were severed and investigated for lactate dehydrogenase activity using the standard method and sodium pyruvate as the substrate. Lactate dehydrogenase was isolated and purified by ammonium sulphate precipitation, desalted by dialysis and then gel filtration.

Results: The enzyme activity increased with advancement in the purification steps and was maximum using dialysis. The values for the lactate dehydrogenase activities were 103.43±3.27 U/L, 279.50±5.38 U/L, 318.16±13.08 U/L, 100.47±2.59 U/L, with a purification fold of 1, 3.7, 6.24 and 2.55 for the purification steps respectively. Also, the values of the protein concentrations were 0.071 mg/ml, 0.050 mg/ml, 0.035mg/ml and 0.027 mg/ml (values for the crude enzyme, ammonium sulphate precipitation, dialysis and gel filtration respectively). The enzyme showed optimal activity at pH range of 5.5-6.5 and temperature of 30°C-40°C. Using sodium pyruvate as the substrate, with a fixed enzyme volume, an increase in the concentration of substrate resulted in increase in enzyme activity until a saturation point 0.3mM was reached. The apparent Km and Vmax values obtained were 0.01 mM and 0.12 U/mg/min. The Lineweaver-burk plot of the partially purified enzyme gave real Km and Vmax values of 0.20mM and 0.16 U/mg/min respectively**.**

Conclusion: Partial purification procedures and biochemical properties of lactate dehydrogenase, from the muscle tissues of male chickens of Ebocha origin, gives room for more investigation on the metabolic shift caused by chronic exposure of the environment, humans and livestock to gas flaring and petroleum exploration.

Keywords: Lactate dehydrogenase, *Gallus domesticus*, purification, kinetic studies, Ebocha

1. INTRODUCTION

Nigeria is highly endowed with natural resources boasts of mineral deposits, natural gases and petroleum [1]. Natural resources have been a blessing in some cases, and in others it is nothing but a continuous source of pain and sorrow to the countries where these natural resources may be found. Some of these natural sources are a windfall for these nations, while others struggle to curb the resultant social consequences such as pollution, disease and environmental effects that come with the resources [2]. The extensive inventory of natural resources, which consists of natural gas and

petroleum, in Nigeria is located in a region referred to as the Niger Delta [3].

The role played by the oil and gas industries in improving the quality of life in Nigeria cannot be overemphasized. Alongside the good things that brought an enhancement in the standard of living was the emergence of deleterious substances in the environment following oil and gas activities [4]. The industrial pollutants, including carbon monoxide from exhausts of automobiles and other pollutants such as heavy metals, constitute an important source of environmental pollution. In oil drilling operations, crude oil, corrosive acid wastes, toxic chemicals and other harmful industrial wastes are intermittently discharged into the environment (air, soil and water). Sulphur and other toxic gases, generated by oil companies, are usually released into the atmosphere. These, together with the injected particulates and unburned hydrocarbons, undergo series of chemical reactions in the presence of sunlight, resulting in dense characteristic smog [4].

The environment provides and supports all life systems with water, air and land, including the materials for fulfilling all developmental aspirations of man. The Nigerian environment in this present time showcases a grim litany of woes [5].

Nigeria is a major producer of crude oil in the world. Pollution of the environment has steadily increased as a result of oil activities due to oil spillage. Crude oil production and export have brought tremendous financial benefits to the country, unfortunately, the oil industry has also brought serious pollution problems, resulting from numerous oil spillages, especially in the Niger Delta Area where the bulk of the oil exploration and exploitation are concentrated. In the Niger Delta Areas alone, there have been over 550 reported cases of crude oil spillage since 1976, releasing over 2.8 million barrels of crude oil into the environment [6,7].

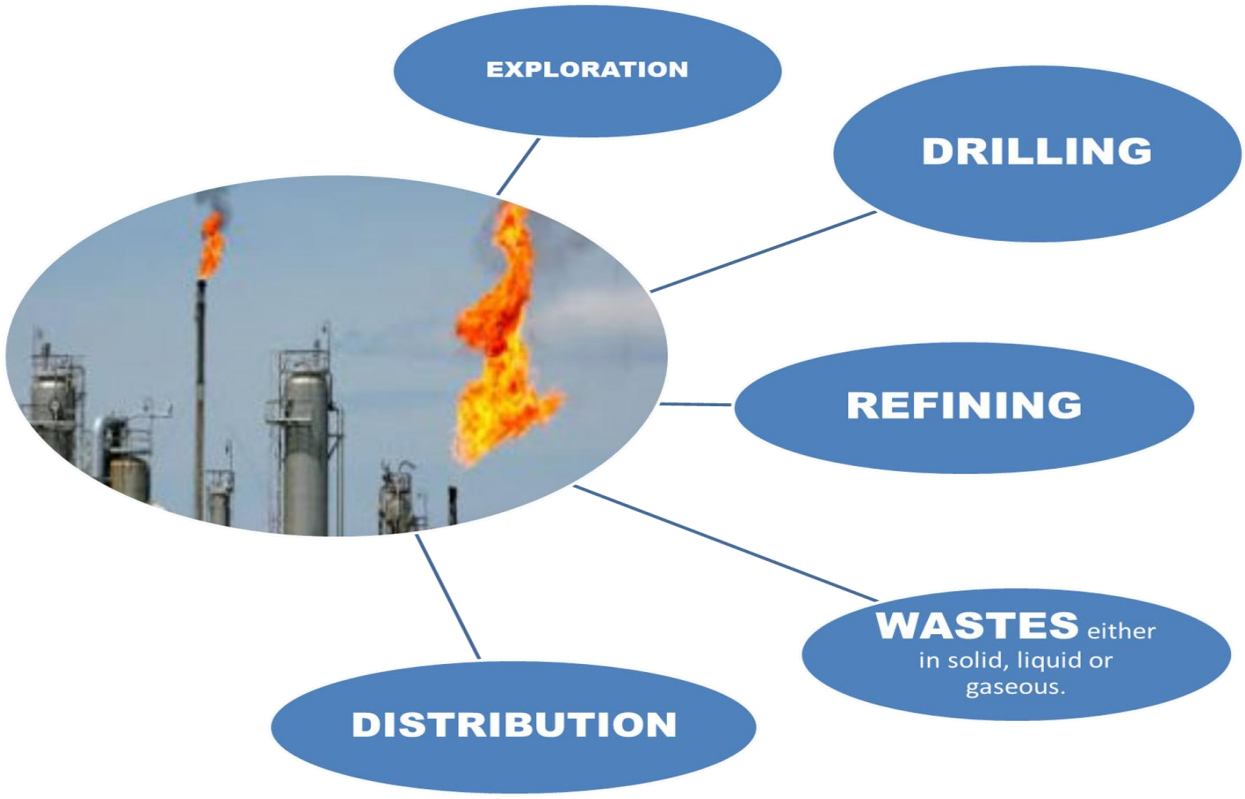

Fig. 1: Activities of a gas site/oil company

Ebocha-Egbema community in the Niger Delta of Nigeria has been faced with chronic and deplorable environmental, aquatic and human conditions due to the thick smoke and intense

heat emanating from the gas stations. Some research investigations reveal their soil and water are being polluted by gas flaring activities and oil spills which resulted in the damage of wildlife and vegetation. Sadly, most inhabitants of these communities complain of headaches, drowsiness, high body temperature, difficulty in breathing, asthma and other health issues. This research has been about critical evaluation of the impact of gas flaring to the metabolic activities in livestock in Nigeria. Oluwasoye *et al*. [8] defined climate risks as a scourge which unnoticeably thrives to harm the environment, affect health and safety, and practically affect businesses, local communities and nations negatively thus describe simply as a soft weapon of mass destruction. The effect of environmental pollution is enormous. Pollutants, when absorbed by living organisms, cause the release of reactive intermediates which induce changes in their tissues [9]. Several biochemical parameters have been analyzed to explain/understand the organism's attempt to cope with the effect of the pollution [10]. It was with a view to relating the composition of the serum proteins to disease that we have undertaken a study of a purified protein fraction (Lactate Dehydrogenase), on native chickens exposed to chronic gas flaring conditions in which it is well known that the serum proteins show great abnormality.

Lactate dehydrogenase is found in animal tissues, cells and fluids. It is well known that lactate dehydrogenase enzymes catalyze the inter-conversion of pyruvate and lactate with the concomitant oxidation/reduction of nicotinamide adenine dinucleotide hydrogen (NADH) to nicotinamide adenine dinucleotide (NAD^+) as cofactor in the reactions. Extracellular activity of lactate dehydrogenase increases under conditions of oxidative stress, since the cell's integrity could be disrupted during lipid peroxidation process [11]. It catalyzes the conversion of pyruvate, the final product of glycolysis, to lactate, when oxygen is absent or in limited supply. It also performs the reverse reaction during the Cori cycle in the liver; at high concentrations of lactate, the enzyme expresses feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased. It also catalyzes the dehydrogenation of 2-hydroxybutyrate, although it is a much poorer substrate than lactate. Lactate dehydrogenase is virtually present in a wide variety of organisms including animals and plants. Cellular enzymes in the extracellular space, although they have no further metabolic function in this space, are still of importance because they serve as indicators in cases of disturbances of the cell's integrity induced by pathologic conditions. LDH is a cytoplasmic enzyme present in essentially all major organ systems [11]. Recently, Airaodion et al. [12] reported that the hydrocarbon exposure significantly increased activity of lactate dehydrogenase when animals were fed with crude oil-treated diet.

2. MATERIALS AND METHODS

2.1 Study area

Ohaji-Egbema in Imo state was chosen for the study. Imo State is one of the nine states in the Niger Delta region of Nigeria. Egbema, an oil and gas producing community with active gas flaring for more than 45 years, is located in between many other active oil and gas flaring sites such as Izombe, Ossu and Oguta oil and gas fields operated by Addax, Akri and Ebocha oil and gas fields run by Nigeria Agip oil company. Therefore, the residents and animals are well exposed to the effects of gas flaring.

2.2 Experimental Animals

The native chickens (*Gallus domesticus)* were used for the study. The experimental chickens had their nativity in Egbema in the Niger Delta Area and their ancestors had existed in that petroleum hydrocarbon polluted area for over fifty years. Twenty one (21) apparently healthy male chickens (6-9 months old) were identified and used. Each chicken was dissected, the thigh muscle tissue extracted and stored in the refrigerator at 4ºC until needed for isolation.

2.3 Isolation of Lactate Dehydrogenase

Two hundred grams (200g) of the tissue was subjected to blending with 100ml of 50mM Tris-HCl buffer pH 7.5, 1 mM EDTA as described by Yannis [13]. The homogenate was separated by the use of a calico layer to trap the unbroken cells, nuclei and other cell debris.

2.4 Purification of Lactate Dehydrogenase

Precipitation was done by salting out with ammonium sulphate using the method described in Wingfield [14]. Dialysis was carried out by the method described by Brooks *et al*. [15]. Protein content of the enzyme extract was determined

according to Lowry method described by Danson and Eisenthal [16], using Bovine serum albumin (BSA) as the standard protein. At every purification step, the fractions collected were assayed for protein concentrations and the enzyme activity spectrophotometrically by measuring the first-order change in absorbance at 750 nm and 340 nm respectively as a result of oxidation of NADH or reduction of NAD^+.

The enzyme activity was calculated using this calculation:

$$\text{Activity (U/L)} = \frac{\Delta A \; x \; V \; x \; 1000}{d \; x \; t \; x \; 6.22 \; x \; v}$$

Where

V is the total volume of the reaction mixture in cm^3

v is the volume of added enzyme in cm^3,

ΔA is the change in absorbance for time t (min)

d is the cuvette size (1 cm).

2.5 Characterization of Lactate Dehydrogenase

After undergoing gel filtration chromatography, the enzyme was characterized. The optimum temperature was determined using the method of Bergmeyer [17]. The optimum pH of lactate dehydrogenase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method. Enzyme activity was assayed by the method of Bergmeyer [17] at different concentrations of the substrate using Tris-HCl buffer at pH 7.5.

3. RESULTS AND DISCUSSION

Table 1: Purification Table of Lactate Dehydrogenase Isolated from *G. Domesticus* Native to Ebocha

Purification Step	Volume (ml)	Enzyme Activity (U/L)	Protein Conc. (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Percentage Yield (%)
Crude enzyme	500	103.43±3.27	0.071±0.006	51718	1456.76	1	100
30% $(NH_4)_2SO4$	50	279.50±5.38	0.050±0.002	13975	5590.00	3.70	27.02
Dialysis	27	318.16±13.08	0.035±0.006	8590.32	9090.28	6.24	16.60
Gel filtration	20	100.47±2.59	0.027±0.004	2008.40	3721.11	2.55	3.88

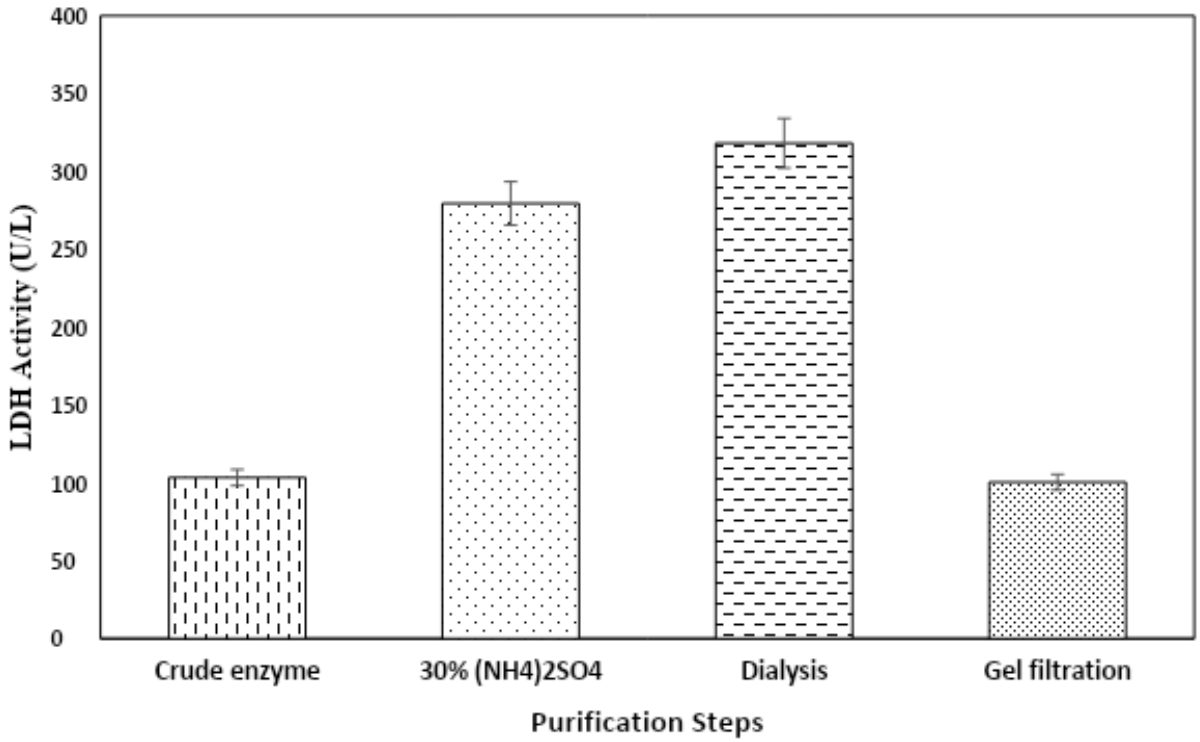

Fig. 2: Lactate Dehydrogenase Activity of the crude Enzyme

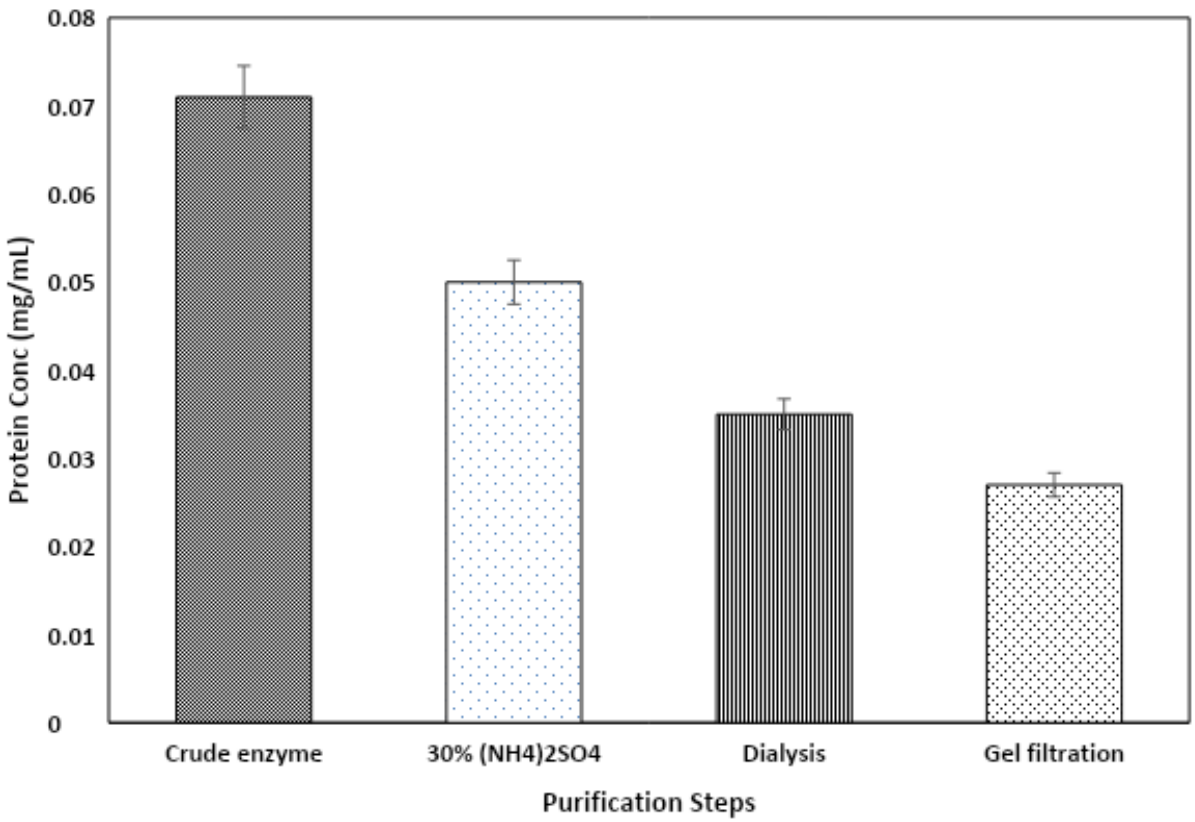

Fig 3: Protein Concentration (mg/mL) of the crude Enzyme

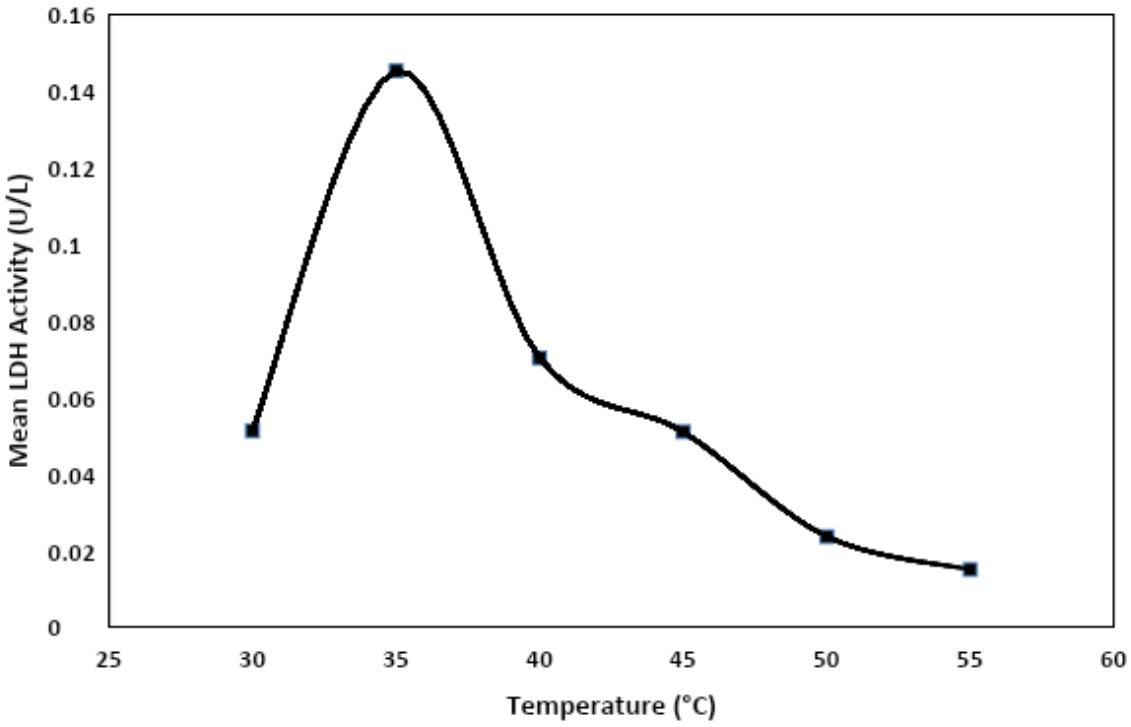

Fig. 4: Optimum Temperature values for Lactate Dehydrogenase Activity

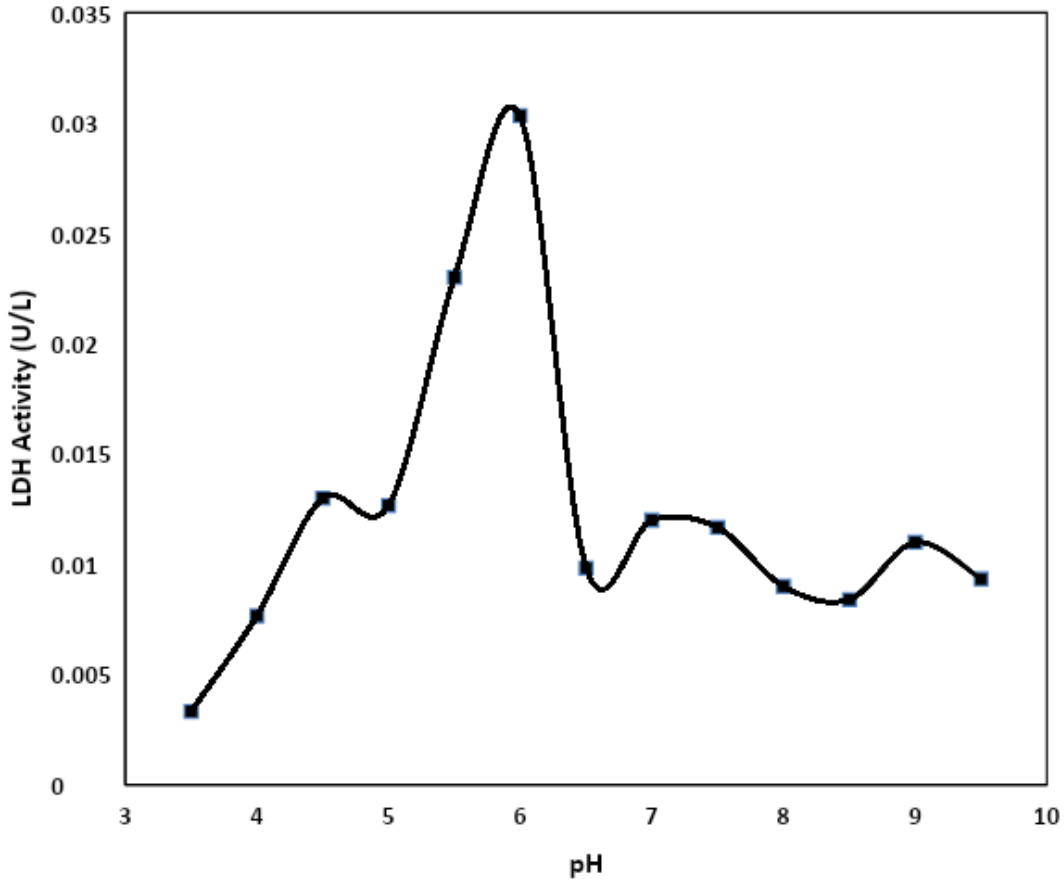

Fig 5: Optimum pH values for Lactate Dehydrogenase Activity

Table 2: Effect of Substrate concentrations on Lactate Dehydrogenase Activity

V	0.04	0.101	0.114	0.112	0.112	0.111	0.09	0.111	0.061
[S]	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9

The apparent Km and Vmax values obtained were 0.01 mM and 0.12 U/mg/min.

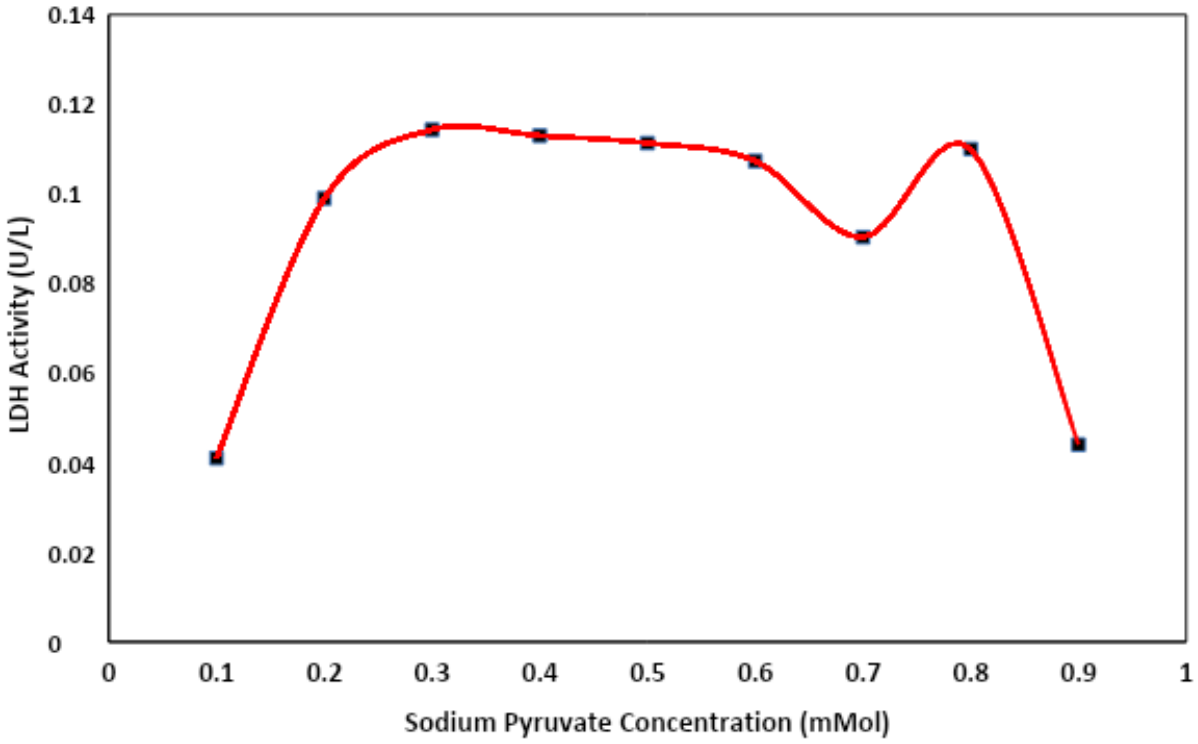

Fig 6: Lactate Dehydrogenase Activity against substrate concentration using Michalis-Menten plot

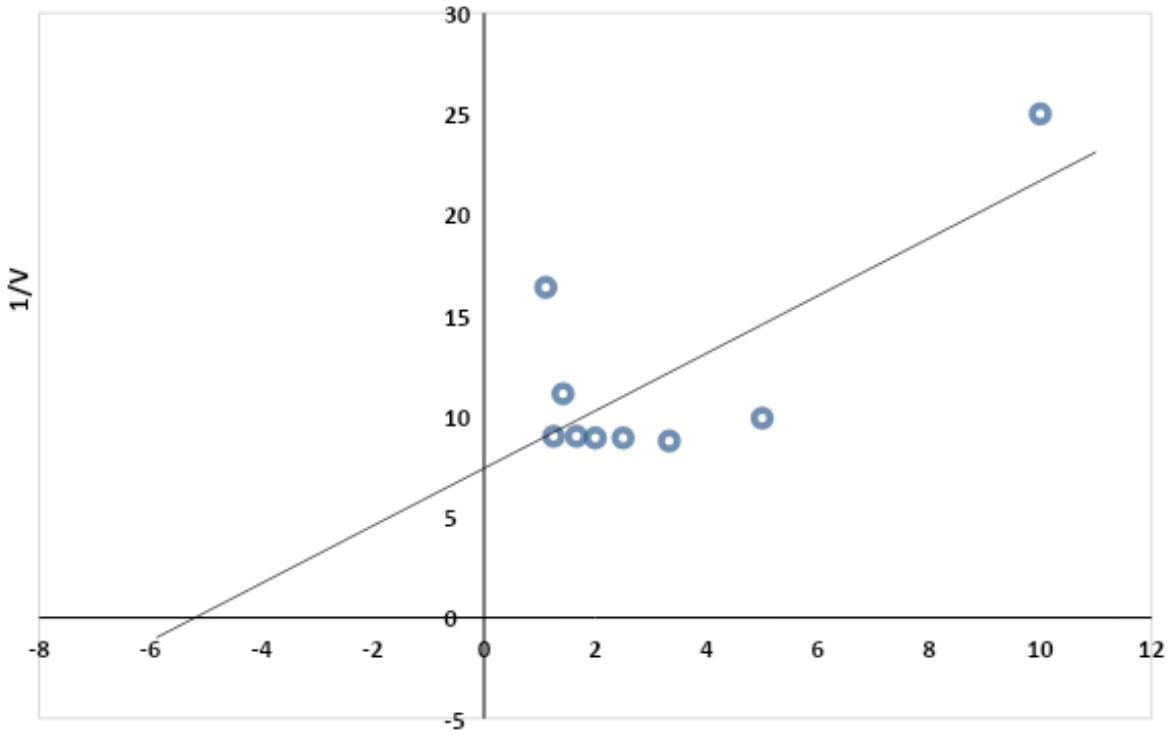

Fig 7: Lactate Dehydrogenase activity against substrate concentration using Lineweaver-Burk plot

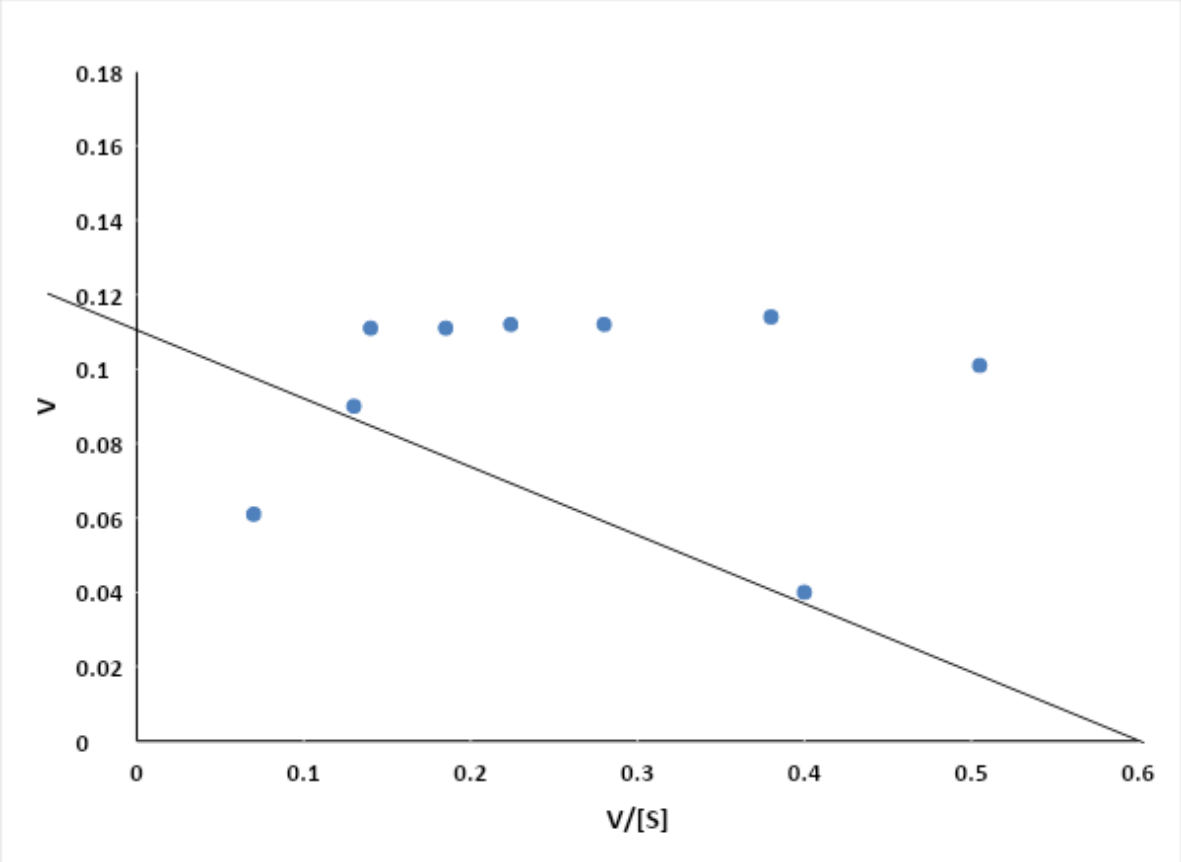

Fig 8: Lactate Dehydrogenase Activity against substrate concentration using Eadie-Hofstee Plot

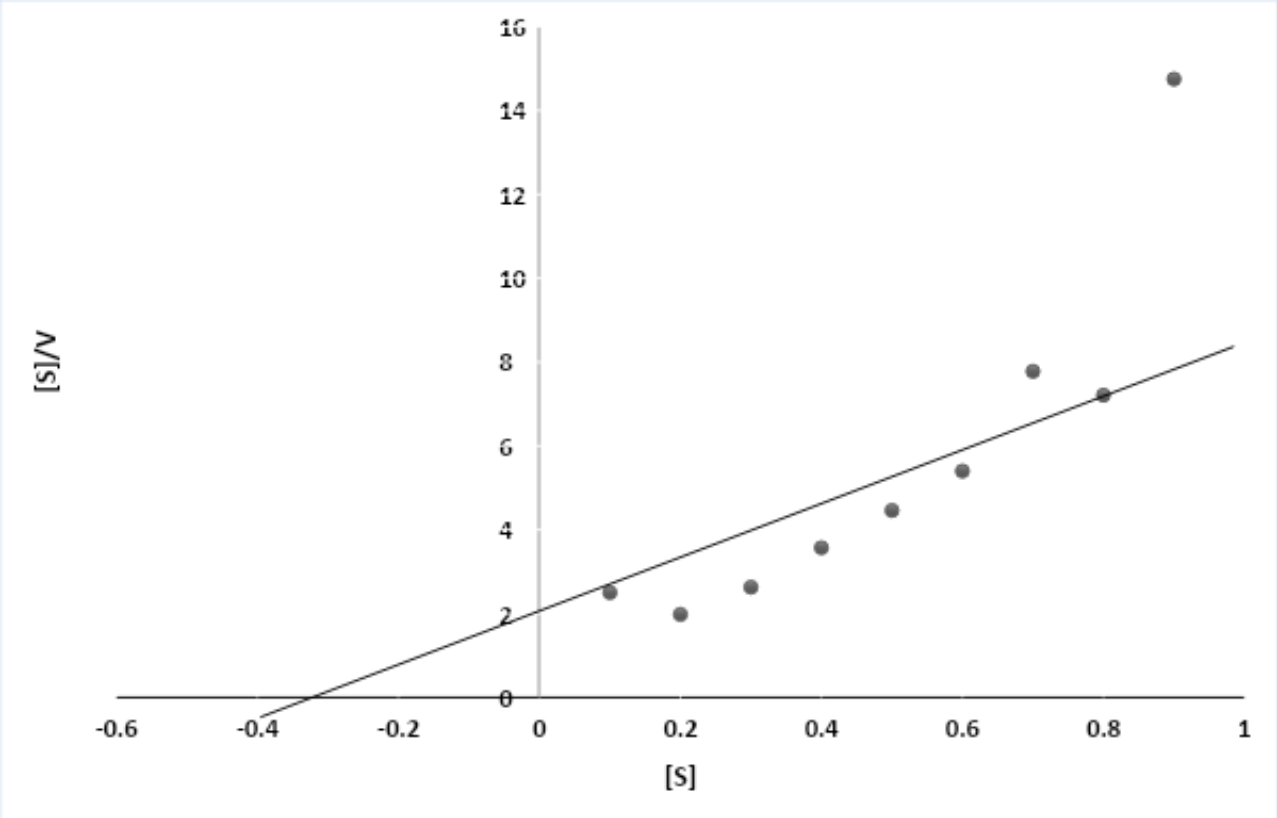

Fig 9: Lactate Dehydrogenase Activity against substrate concentration using Hanes-Wolf plot

One of the major challenges faced by oil producing communities is gas flaring and pollution, which affects the environment, inhabitants and aquatic life. This study sought to evaluate the metabolic alteration caused in the cells of the birds as a result of the gas flaring process. Lactate dehydrogenase (LDH) is an enzyme that catalyzes the inter-conversion of pyruvic acid and lactic acid. This enzyme is found in both plants and animals (humans) and it responds to cellular damage or injury, which manifests as an increase in its activity [18].

Specific activity is a term used to measure the rate of reaction of an enzyme with a substrate. It is a measure of purity of an enzyme solution and is quoted as units per milligram. The value becomes larger as the enzyme preparation becomes purer since the amount of protein (mg) is typically less, but the rate of reaction stays the same [19]. Specific activity is used in protein isolation methods to indicate the percentage of purification. When measuring enzyme kinetics (the rate of reaction of an enzyme with a substrate), specific activity is defined as the amount of substrate the enzyme converts per milligram protein in the enzyme purification, per unit time [19]. From the study, the crude enzyme yielded a mean concentration of 0.071mg/ml and enzyme specific activity of 1456.76U/mg.

Precipitation is an identified and useful method of enzyme isolation and purification. Lactate dehydrogenase isolated from Ebocha yielded mean protein concentration of 0.050 mg/ml and enzyme specific activity of 5590.00 U/mg. Ammonium sulphate precipitation is a useful technique as an initial step in protein purification because it enables quick, bulk precipitation of cellular proteins [20]. It is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration [20]. Studies from Diao *et al*. [21], Zia *et al*. [22] and Pandey *et al*. [23] had reported the use of precipitating enzymes from plant sources. The result of this study is in accordance with the report of Yannis [13], after he isolated lactate dehydrogenase from a pig's heart and purified same. However, the limitations of this method is that oftentimes

different substances can precipitate along with the protein, hence, other purification techniques were performed, such as dialysis and gel filtration [24].

Further purification of the lactate dehydrogenase isolated from Ebocha (Dialysis and gel filtration) yielded mean protein concentrations and specific activities of 0.035mg/ml and 9090.28 U/mg; 0.027 mg/ml and 3721.11 U/mg respectively.

From the study, the mean enzyme activity for Lactate dehydrogenase isolated from the Ebocha male chickens were 103.43±3.27 U/L, 279.50±5.38 U/L, 318.16±13.08 U/L, 100.47±2.59 U/L (values for the crude enzyme, ammonium sulphate precipitaton, dialysis and gel filtration respectively).

Fig. 4 shows the results of the effect of temperature on lactate dehydrogenase activity of chickens from Ebocha. Extremely high or low temperature values generally result in complete loss of the activity for most enzymes which may be due to ionization of amino acids in the active site of the enzyme lactate dehydrogenase [25]. Also, extremely high temperature leads to the denaturating (alteration) of protein structure resulting from the breakdown of the weak ionic and hydrogen bonding that stabilize the three-dimensional structure of the enzyme active site [26].

From this study, the optimum temperature on lactate dehydrogenase activity for chickens from Ebocha was 37 °C. This result collaborates with reports of Onyeze *et al* [25], who worked on chronic gas flaring pollution-induced alterations in kinetic parameters of lactate dehydrogenase in *Gallus Domesticus* Native to Ebocha, Niger Delta, Nigeria.

Enzymes are affected by changes in pH. The most favourable pH value- the point where the enzyme is most active is known as the optimum pH. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme, there is also a region of pH optimal stability [27].

Research works on effects of pH on lactate dehydrogenase by Mitchell *et al*. [28], Yang *et al*. [29], Onyeze *et al*., [25] and Masood *et al.* [30], when they isolated lactate dehydrogenase from psychrophilic marine bacterium; pig heart; blood and serum of *G. domesticus;* and *varanus* liver respectively, suggested an optimum pH range of 7.0-8.0 for lactate dehydrogenase. Interestingly, the optimum pH on lactate dehydrogenase activity for chickens in Ebocha was 6.0, suggesting the enzyme's high substrate inhibition in chickens native to Ebocha. This reconciles the findings of Arriaga *et al* [31], who reported that at pH values below 7.2 (pH 5.0-6.8), lactate dehydrogenase showed high substrate inhibition and by way of this inhibition, pH regulated, primarily, lactate dehydrogenase activity to pyruvate in *Phycomyces blakesleeanus*.

At Tris-HCl buffer of pH 7.5, lactate dehydrogenase isolated from *G. domesticus* native to Ebocha (optimum pH 6.0) could have been grossly inhibited in its interaction with sodium pyruvate, thus reached its saturation at 0.3 mM. This suggests that the active site of the enzyme might have been compromised by heavy metal constituents of the flared gas. Kinetic studies have been reported by Armstrong *et al.* [32] that heavy metals inhibit lactate dehydrogenase activity isolated from Baker's yeast, thus substantiating the claim.

This is attributable to reports from Cox *et al*. [33], claiming that when animal tissues cannot be supplied with sufficient oxygen to support aerobic oxidation of the pyruvate and NADH produced in glycolysis, NAD^+ is regenerated from NADH by the reduction of pyruvate to lactate. Excess production of lactate (lactic acid) causes acidosis, thus reducing the pH of the cell.

Onyeze *et al*. [25] reported that for an enzyme catalyzed reaction, there is usually a hyperbolic relationship between the rate of reaction and the concentration of substrate. This is because: at low concentration of substrate, there is a steep decrease in the rate of reaction with decreasing substrate concentration. The rate of reaction when the enzyme is saturated with a substrate is the maximum rate of reaction, V_{max}. The relationship between the rate of catalysis for a substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the Km (Michaelis Constant) of the enzyme; an inverse measure of affinity. The apparent K_m and V_{max} values were 0.01mM and 0.12 U/mg/min respectively.

Using Lineweaver-Burk's plot (Fig. 7), lactate dehydrogenase isolated from chickens native to Ebocha had real Km value of 0.20mM and real Vmax value of 0.16 U/mg/min. Alteration in the Km value may reflect structural modifications in enzyme at the active site or rearrangement of the subunit composition as lactate dehydrogenase, lactate dehydrogenase being an isoenzyme [25]. This implies that pollution due to petroleum hydrocarbon (gas flaring) affected the enzyme's active site by causing a decrease in LDH activity.

Clearly, gas flaring constitutes climate risks in the Niger Delta regions of Nigeria and causes a number of health conditions such as: noise and air pollution, increase in atmospheric temperature, acid rain, roof corrosion and respiratory diseases. The extent of human vulnerability to these conditions often depends on the proximity of the human settlements to the gas flaring stations [8].

4. CONCLUSION

This study has shown that gas flaring activities in the Niger-Delta region of Nigeria still impact negatively on the environments of its host communities. This inference was drawn from the results obtained in this study, which indicate that gas flaring activities could have a long term effect on the enzymatic level of domestic birds.

Ethical Approval:

As per international standard standard written ethical approval has been collected and preserved by the author(s).

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