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EXTRACTION AND BIOCHEMICAL CHARACTERIZATION OF A HIGH POTENTIAL IRON-SULFUR PROTEIN (HIPIP) FROM *ACIDTHIOBACILLUS* SPECIES ISOLATED FROM AGBAJA IRON MINES OF KOGI STATE, NIGERIA

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ABSTRACT: *A high potential iron-Sulphur protein (HiPIP) Iron Oxidase (E.C 1.9.3.1) was isolated from Acidithiobacillus species obtained from Ironstones of Agbaja Mines of Kogi State, Nigeria. The aim was to study the biochemical characteristics of the enzyme so as to find out its catalytic contribution to the rate of bioleaching of the studied organism. A simple partial purification profile consisting of cell homogenization, ammonium sulphate precipitation, Ion-exchange using Carboxyl methyl cellulose, and sephadex G-200 chromatography were used. The biochemical and kinetic properties of the enzyme were thereafter studied. The Iron oxidase had its highest specific activity of 14.70 µmole/ml/mg of protein and yield of 89% with a purification fold of 1.43 after size exclusion chromatography. Optimum temperature and pH were found to be 50°C and 6.0 with Km and Vmax of 0.03mM and 12.mMole/min respectively. Inhibition studies conducted shows that tween 20 and SDS had the maximum rates. Other characteristics of the enzyme shows that it might be a novel member of HiPIP coming from the isolated Acidithiobacillus of Agbaja Iron ore Mines.*

KEYWORDS: HiPIP, Iron Oxidase, Acidithiobacillus, Bioleaching, Biochemical Characteristics

INTRODUCTION

The Agbaja mine is a large iron mine located in the central Nigerian state of Kogi. Agbaja represents one of the largest iron ore reserves in Nigeria and the world having estimated reserves of about 1.25 billion tonnes of ore capable of yielding about 48% of iron metal. (Alafara, 2003). There are a number of researches ongoing on the best mining option to be applied in order to fully maximize this vast deposits and bioleaching is one of such options. Bioleaching is a process described as the dissolution of metals from their minerals sources by certain naturally occurring microorganisms or the use of microorganisms to transform elements so that the elements can be extracted from a material when water is filtered through it (Lundgren, 1983). Generally bioleaching refers to the conversion of solid metal values into their water soluble forms by the use of microorganisms. Example in the case of copper or Iron, their sulphides are microbially oxidized to sulphates and metal values present in the aqueous phases are discarded (Rawlings, 2002). These microorganisms act as catalysts that speed up natural processes in the ore. The types of bacteria most often used in this processes include *Leptospirillum ferrooxidans, Thiobacillus ferrooxidans*, certain species of *Pseudomonas, Acidianus*, *Sulfolobus* and *Sulfobacillus species*. In direct bioleaching, minerals which are susceptible to oxidation undergoes direct enzymatic attack by the microorganisms.

The chemolithoautotrophic bacterium, *Acidithiobacillus ferrooxidans* and other similar species is of great importance in biomining or bioleaching operations. Among the bacteria that thrive

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in acidic mine drainage, it is one of the most commonly studied. A. ferrooxidans obtains its energy through oxidation of Fe (II) to Fe (III), with O_2 as the terminal electron acceptor. The oxidation mechanism of Fe (II) has been well studied, and the key components of this system have been identified. In the first step, ferrous ion is oxidized by iron oxidase, and reduced iron oxidase then transfers electrons to cytochrome c552. Cytochrome c552 transfers electrons to cytochrome c oxidase, and, finally, the electrons are transferred to molecular oxygen by the oxidase (Zeng, *et a*l, 2010). Iron oxidase is a member of the high redox potential iron–sulfur proteins (HiPIP), which generally function in electron transport (Cavazza *et al*, 1995). HiPIPs are usually isolated from purple phototrophic bacteria and form a class of small proteins (6–10 kDa) containing a $[Fe_4S_4]$ cluster. This cluster undergoes a one-electron transfer reaction between $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^{3+}$ (Cowan, *et al,* 1998). The use of *Acidothiobacillus* ferroxidans in the isolation/metal extractions including iron in different media have been extensively reported (Bartels, *et al*, 1989). Some researchers previously investigated the simultaneous enzymatic leaching of metal oxides and sulphides. Therefore, the aim of this current work is to investigate the biochemical properties of a partially purified iron oxidase from an *Acidothiobacillus species* isolated from Agbaja Mines of Kogi State.

MATERIALS AND METHOD

All reagents and chemicals used in this study were of high analytical grade. Some of the equipment used include Intelligent Thermostatic Shake Cultivation Cabinet (incubator shaker) made in England, Vertical Heating Pressure Steam Sterilizer (autoclave),Colony Counter, General Laboratory Incubator, Atomic Absorption Spectrophotometer, etc.

Sample collection:

Crushed iron ore samples at different locations within Agbaja Iron ore mining site were collected from drilled pits 4 and 5 at about 3-5m depth where most of the microbial activity takes place, and thus where most of the bacteria population is concentrated. Iron ore samples were collected into clean dry and sterile polythene bags using sterile spatula. Fifty gram of the iron ore samples were dissolved in 100ml of distilled water to make iron ore suspensions and left on an incubator shaker for 7 days.

Isolation of bacteria

Fifty grams of crushed Agbaja iron ore sample was weighed out into a conical flask containing 100ml distilled water. The pH of the distilled water was adjusted to 2.0 using 0.3ml of concentrated H_2SO_4. The conical flasks were plugged with cotton wool. Thereafter the suspension was homogenized and incubated for 7 days at room temperature (25-30^oC). On the seventh day, 250ml nutrient agar was prepared enriched with the following basal salts; 0.8% Urea, 0.1% K_2HPO_4, 0.025% $MgSO_4.H_2O$, 0.02% yeast extract and 10% glucose and the pH of the media was adjusted to 7.5 using H_2SO_4. The media was sterilized at 121^oC for 15 minutes.

Sample Inoculation

1ml of the suspension from the conical flask was inoculated into each petri-dish and the media dispensed into the petri-dishes by pour plate method and allowed to gel and incubated at 37^oC for 48 hours. Bacteria that grew were sub-cultured into plates containing nutrient agar by

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streaking in order to obtain pure culture and were incubated at 37ºC for 24 hours. After which pure colonies were sub-cultured and enzyme extraction was carried out subsequently.

Extraction and assay of crude enzyme activity (Guowei *et al*, 2013)

The already isolated and identified *Acidithiobacillus* species from the culture above was centrifuged at 14,000 rpm for 15 minutes to remove cell debris from the cell free extract. The oxidation assay were performed by the colorimetric method modified from Harvey et al (1955). About 1g of $FeSO_4.7H_2O$ was dissolved in distilled water at a concentration of 0.025mg/ml and used as substrate. Reaction mixture consist of 10mM of KHP (potassium hydrogen phthalate) buffer at a pH of 3.5, 5mM of ammonium peroxide sulphate, 0.025mg/ml $FeSO_4.7H_2O$ and 0.25mg/ml of enzyme solution. The final volume of the reaction was adjusted to 0.45ml with distilled water. After incubation at room temperature for 5 minutes, bromophenol blue was added to the reaction mixture and the enzyme activity was measured at 480nm in a spectrophotometer. Enzyme activity was measured from the formular below:

$$\text{Activity (U/ml)} = \frac{\text{Abs Blank- Abs Sample x 1000x total volume of enzyme mixture}}{\text{Extinction coefficient of } Fe_2 \text{ x Time of incubation}}$$

Ammonium sulphate precipitation

Solid ammonium sulphate was added to the supernatant obtained and 80% saturation was achieved by mixing well for 30 minutes and centrifuging at 10,000 xg. The solid ammonium sulphate was slowly added to the crude extract with constant stirring beginning from 10% until 80% saturation was achieved. The extract was allowed to stand for 30 minutes in a freezer and pellets were recovered by centrifuging at 20,000xg for 10 minutes and re suspended in the extraction buffer for further purification.

Protein content determination

Protein determination was done using the Biuret method. Bovine serum albumin (BSA) was used as the protein standard. The principle is based on copper ions in the Biuret reagent which complexes with amide groups in the proteins in an alkaline environment to create a blue colour whose absorbance was 540 nm. The amount of blue colour that forms is directly proportional to the quantity of proteins in the samples.

Carboxyl methyl cellulose chromatography /Gel filtration chromatography using sephadex G-200

CM- cellulose chromatographic column pre-equilibrated with 10mM of KHP (potassium hydrogen phthalate) buffer at a pH of 3.5 containing 0.01M NaCl was loaded onto a column (1.5 x 20) with 5ml of the crude enzyme and the flow monitored with about 15 fractions collected. The column was washed with 5-10 volume of 10mM of KHP (potassium hydrogen phthalate) buffer + 0.5M NaCl and eluted with 0.01M of KHP (potassium hydrogen phthalate) buffer (pH 3.5). Fractions were collected and assayed for Iron oxidase activity. Also, gel filtration chromatography with sephadex G-200 was performed for further purification. All the fractions were measured using a UV-Spectrophotometer.

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Determination of optimum temperature

The iron oxidase activity of the partially purified enzyme (pH 6.0) at various temperature was determined by incubating the reaction mixture at different temperature from 10-70°C for 60 minutes at 37°C and the reaction stopped by adding bromophenol blue in order to get the optimum Temperature.

Determination of optimum pH

The iron oxidase activity of the partially purified enzyme solution at room temperature was measured at different pH values: 2, 3, 4, 5, 6, 7, and 8. The pH was adjusted with 10mM of KHP (potassium hydrogen phthalate) buffer, and phosphate buffers respectively. Reaction mixtures were incubated at 37°C for 60 minutes to measure activity.

Thermostability studies of partialy purified Iron Oxidase from *Acidithiobacillus spp*

Equal volumes (1.0ml) of the enzyme extract were incubated at intervals between 15-30 minutes at 10°C-80°C in a waterbath. The test tubes were cooled to room temperature and treated as described previously for the determination of enzyme activity. Residual activity of the partially purified enzyme was also determined.

Determination of kinetic constants (K_M and Vmax)

The substrate ($FeSO_4.7H_2O$) concentration was varied over a range of 0.02 - 0.10mM and incubated with the enzyme solution and activity determined for each substrate concentration in triplicate. The kinetic constants were determined from Line Weaver-Burk's (double-reciprocal) plot.

Effect of Inhibitors

The effect of various inhibitors on the Iron oxidase activity of *Acidithiobacillus species* were determined at room temperature and p H 6.0 using $FeSO_4.7H_2O$ as substrate. Enzyme solutions were pre-incubated with 1% each of Absolute ethanol, isopropanol, EDTA, SDS, and triton X-100.

Activation Energy

The activation energy was measured for the activity against reciprocal of temperature at 10 to 50 ^oC in 20 mM phosphate buffer (pH 7.8). Activation energy was estimated according to the formula: Log k = -Ea/RT + Log V; where R = gas constant, T = absolute temperature, k = equilibrium constant, A = frequency factor, E_a = Activation energy. The activation energy was determined from the slope of a plot of Log V against 1/T.

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Results and Discussion

Table 1: Purification profile of iron oxidase from *Acidithiobacillus species*

Experiment	Activity(U) (µmole/ml/min)	Protein (mg/ml)	Specific activity(U/mg protein)	Purification fold	Yield (%)
Crude	1.65	0.16	10.31	1.00	100
$(NH_4)_2SO_4$	1.57	0.15	10.47	1.02	95
CMC	1.53	0.13	11.77	1.14	93
G-200	1.47	0.10	14.70	1.43	89

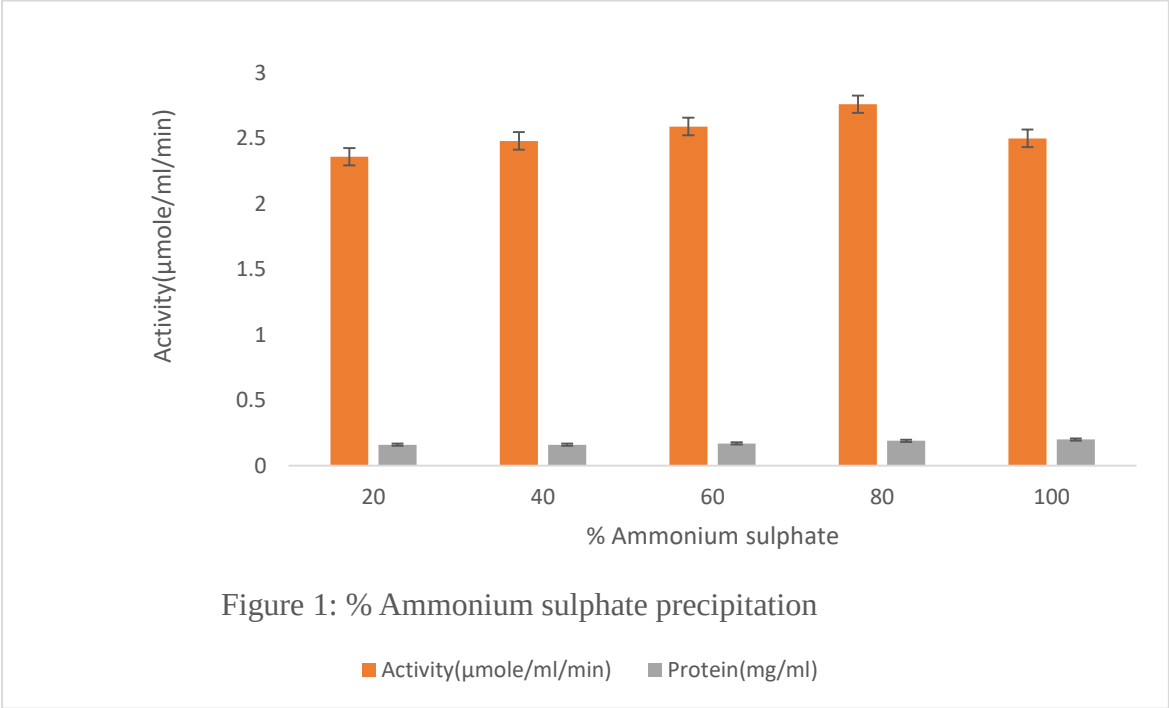

Figure 1: % Ammonium sulphate precipitation

Figure 2: Elution profile of Iron oxidase from *Acidithiobacillus spp* using CMC

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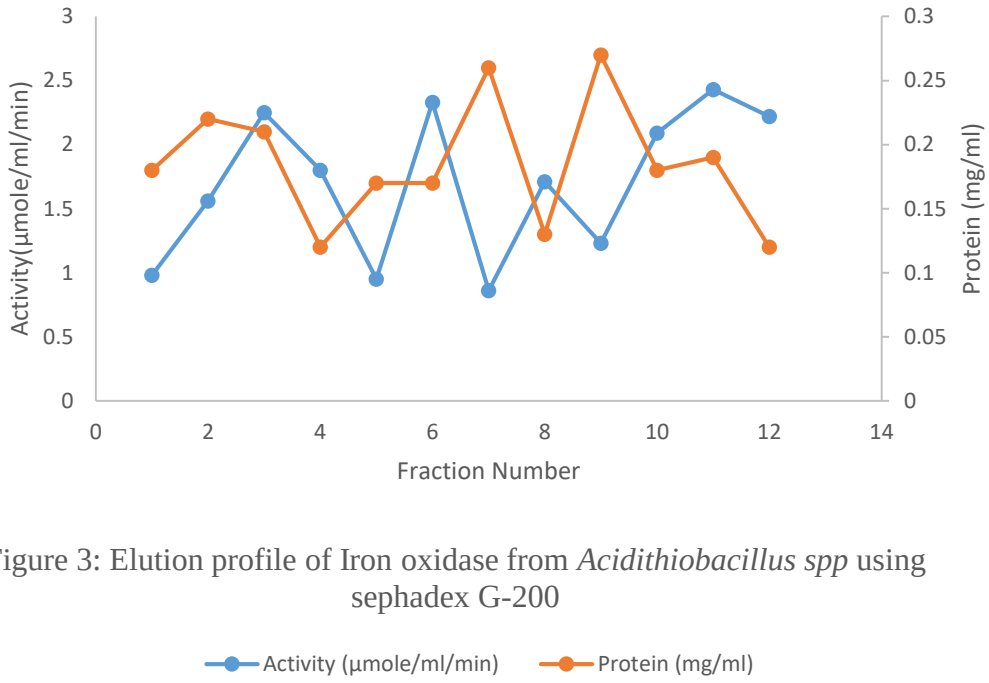

Figure 3: Elution profile of Iron oxidase from *Acidithiobacillus spp* using sephadex G-200

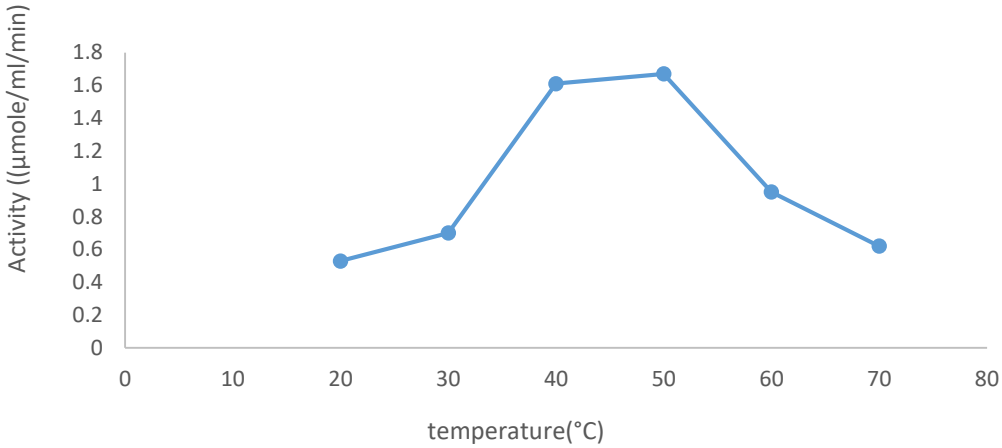

Figure 4: Effect of temperature on partially purified Iron Oxidase from *Acidithiobacillus spp*

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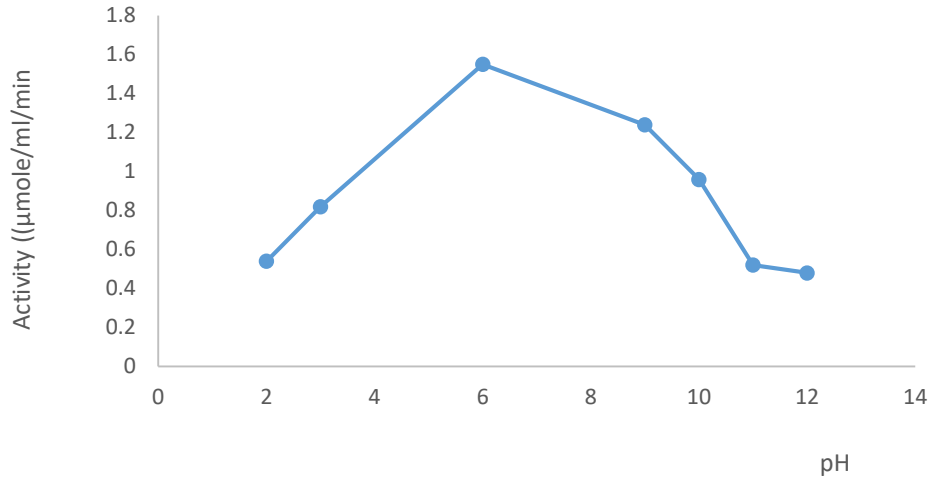

Figure 5: Effect of pH on the Activity on iron oxidase from *Acidithiobacillus spp*

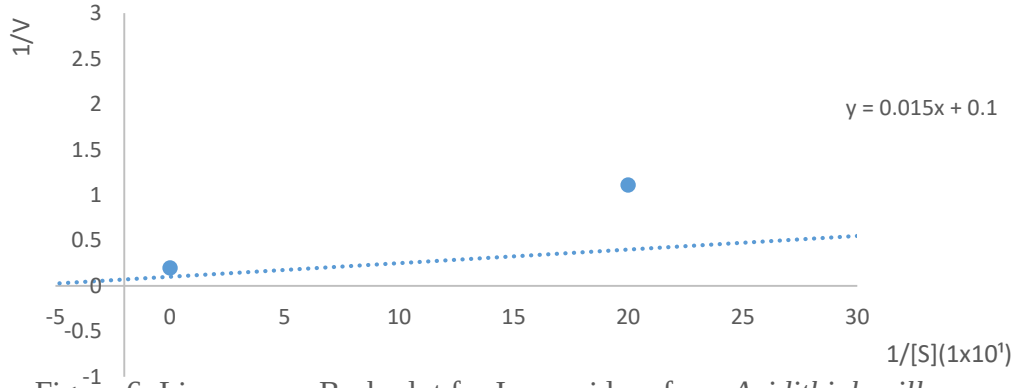

Figure 6: Lineweaver-Burk plot for Iron oxidase from *Acidithiobacillus spp*

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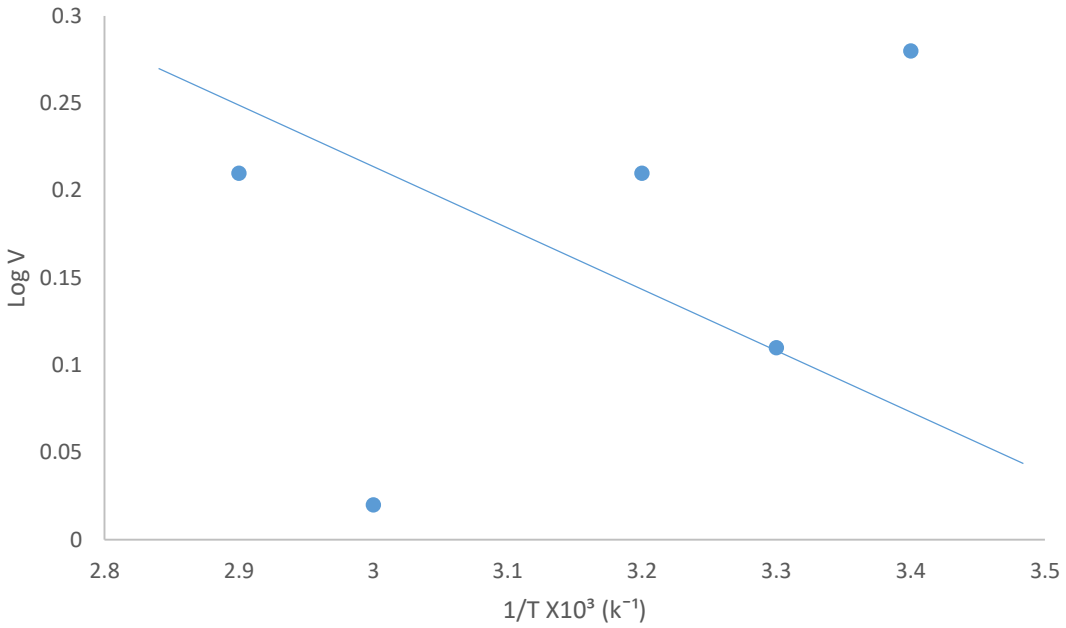

Figure 7: Arrhenius plot of partially purified iron oxidase from *Acidithiobacillus spp*

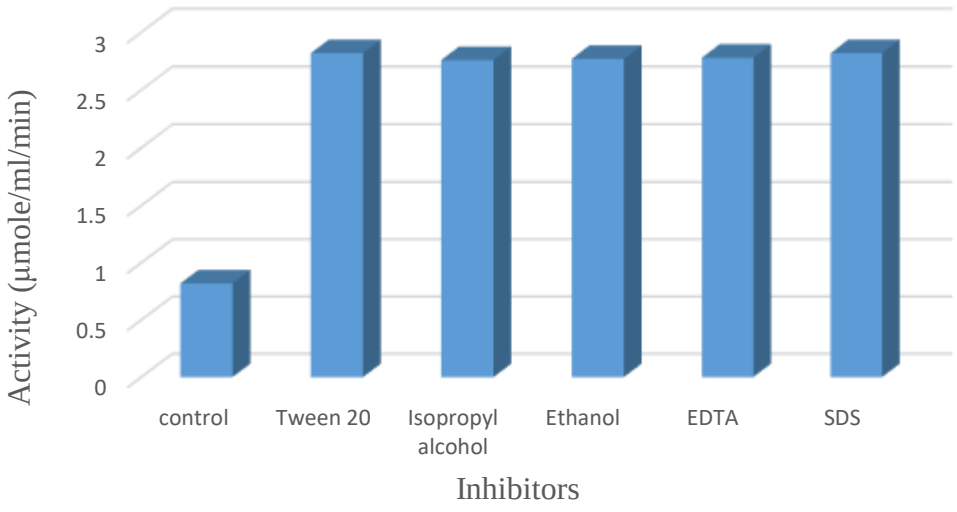

Figure 8: Effect of inhibitors on enzyme activity

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DISCUSSION

The effect of temperature on Iron Oxidase activity is shown in figure 4. Optimum temperature was recorded as 50^0C.This is principally attributed to the fact that Iron Oxidase from Iron mine bacteria in most cases as moderate to extreme thermophiles (Joseph *et al*, 1999). In addition, most mine bacterial active Iron Oxidases possess optimum temperature between 37- 70^0C. Generally, the effect of temperature on Enzyme-catalyzed reactions are extremely complex. This is because a change in the observed rate may be due to a variety of causes like stability of the enzyme protein, pH of the buffer system, Km, Vmax and a host of other factors. Thermo stability applied to enzymes usually refers to the degree to which the secondary, tertiary, and quaternary structure (active sites) of the enzyme are affected by an external pertubing factor (Maes *et al*, 1999). Most useful Iron Oxidases are usually stable up to 80^0C depending on the type of mine they were isolated from. Enzymes also vary widely in their susceptibility to heat treatment. Two factors that in general seem to have predictive value regarding the stability of an enzyme are: i) The structure complexity of the enzyme and ii) The purity of the enzyme preparation. Theoretically, large polymeric enzymes are likely to be less heat stable than the lower molecular weight single enzyme proteins with some disulphide bonds. Also crude enzyme preparations are known to be more heat-stable than purified enzymes for the simple reason that impure enzymes are well protected from direct heat by the presence of extraneous proteins, carbohydrates and lipids in the system (Takumi *et al*, 2004).

Figure 7 Shows Arrhenius plot for Iron Oxidase from *Acidithiobacillus species*. From extrapolated values, the Ea (activation energy) was calculated as 15.2 KJ/mole. It will be recalled that, the collision theory of chemical reactions explains the Arrhenius relationship and shed more light on the effect of temperature on the rate of chemical reactions occurring in solution. As such, the Arrhenius empirical equation and the collision theory of reaction rate represent an attempt, though incomplete to provide an explanation for the effect of temperature on the rates of chemical and enzymatic reactions. It is generally considered that a reaction is a gradual process and that before molecules of reactants react, they must pass through a configuration known as the activated complex which has energy content greater than either the product or reactants and separated by an energy hill, the height of which is equal to the energy of activation, Ea. The effect of pH on enzyme activity is shown in Figure 5. The pH optimum for the partially purified Iron Oxidase was found to be 6.0. This is in agreement with a number of pH optimums from various Iron Oxidases from diverse sources (Douglas, 2005). Another reason for these observed optimum pH values are due to the fact that Iron Ore Oxidases normally act within acidic to near physiological pH i.e. around pH 2.0-6.8. Two principal ways in which pH affect enzyme reaction includes: Change in the state of the ionisable groups at the active site. Such changes affect the binding of the substrates and/or subsequent decomposition of the enzyme substrate complex. Changes in overall protein structure: changes in protein structure can alter configuration of the active site. In some case it can lead to complete denaturation of the subunits. Therefore, the observed bell-shaped curve could be due to the unfolding and subsequent denaturation of the enzyme molecule outside the pH region in which the enzyme is stable and active. Also, the pH variation may have caused alterations in the ratio of free hydrogen ions and hydrogen ions bound to the enzyme or substrate.

Elution profile of partially purified Iron Oxidase from Carboxy methyl cellulose (CMC) is shown in Figure 2. Three very conspicuous peaks were observed. The first major peak of fractions 5 and 7 gave specific activity of 11.77 µmole/m/min/mg (Table 1). Second peak (Fraction 9-10) gave almost similar values like the first suggesting that the CMC purification

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step was not conclusive enough. In all, about 15 fractions were collected and fractions with higher specific activities were collected, pooled together and eluted using Sephadex G-200. Elution using sephadex G-200 gave a specific activity of 14.70 µmole /ml/min/mg (Table 1, fig 3). The purification fold rose up to 1.43 and yield was recorded as 89%. About 14 fractions were collected for the gel filtration chromatography. Molecular weight determinations using the sephadex G-200 gave an average value of 26KDa (done as a pilot study elsewhere) for the partially purified Iron Oxidase. Figure 1 shows the $(NH4)_2SO_4$ precipitation for the crude enzyme. This was run as a pilot study to determine suitable concentrations that will give better activities with minimal loss of enzyme solution. Concentrations of 60% and 80% were found to be most suitable. This is not far from precipitations using $(NH4)_2$ SO_4 for precipitating enzyme from microbial sources. Most of the useful precipitation are achieved between 35-70% (Jun *et al*, 2005). This stage of purification gave an activity of µmole/ml/min and a purification of 1.81 with the highest yield of 95% (Table 1). This simply suggest that at this stage, a lot of other extraneous proteins and other macromolecules were still present in the enzyme solution Hence the need for further purification.

Effect of substrate Concentration on enzyme activity is shown in figure 6. When the Lineweaver Burk's plot of the determination was done, Km and Vmax were gotten as 0.03mM and 12.7 µmole/ml respectively. These values are in line with those of other very useful Iron Oxidases from Iron stones (Turk, 2016). Generally, for industrial applications, low Km and high Vmax values are preferable for enzymes (Turk, 2016). This simply suggest that this isolated iron Oxidase might have some industrial uses. All the inhibitors used for the inhibition studies were found to significantly affect the activity of the enzyme. This might be probably due to the highly sensitive nature of Iron oxidase and its role in the electron transport chain system. Detergents like tween -20 and SDS usually reduces disulphide bonds by breaking S-S groups (Ayorinde *et al*, 2000). The loss of activity upon additions of some of the other inhibitors indicates that this enzyme may be made up of disulphide linkages.

The procedures developed for purification of this Iron Oxidase was found to be rapid and simple, providing partially pure enzyme in high yield. The purified enzyme possess long term stability on storage, maximal activity at a physiological pH, and a Km/Vmax for substrate suitable for Industrial application. The enzyme can be purified partially with overall yield of 89% by a simple protocol involving ammonium sulphate fractionation, ion exchange and gel filtration chromatographic steps. The enzyme also catalyzes the hydrolysis of hydrated iron sulphate very well. The pH optima of 6.0 and temperature optima of 50ºC suggest that the enzyme could have been isolated from a moderately thermophilic *Acidithiobacillus species*. In this work, inhibition from detergents like SDS shows that the enzyme's active site may be rich in cysteine residue. In conclusion, this work has shown that microbial sources of this partially purified Iron Oxidases from Agbaja Iron Ore mines could open new insight into the metabolism of bioleaching bacteria and ultimately leads to the cloning of very efficient strains for the Biomining Industry.

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