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Bioefficiency of Indigenous Microbial Rhodanese in Clean-up of Cyanide Contaminated Stream in Modakeke, Ile-Ife, Osun State, Nigeria

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Abstract

Cyanide pollution of aquatic environment has become a great concern in Nigeria because of the increase in cassava cultivation. In Nigeria, cassava processing milling plants are usually situated around streams or rivers such that the waste from each stages of processing easily find their way into these water bodies as effluents and waste waters. Extracellular rhodanese of *Klebsiella edwardsii* isolated from Atutulala stream, Modakeke, where cassava is being processed, was assessed for its bioremediation potential. Cyanide concentration of the stream was analysed for six months. Four bacterial isolates were screened for their ability to degrade free cyanide and the best strain was further screened for rhodanese producing ability. The enzyme was purified by 85% ammonium sulphate precipitation and diethyl aminoethyl-cellulose ion-exchange chromatography. The pure enzyme had a specific activity of 0.0473 Rhodanese Unit mg-1 with a purification fold of 4.56 and a percentage yield of 30.30%. The enzyme demonstrated a broad pH range but the optimum pH was at 6.0 while the optimum temperature was 60°C. The bioremediation potential of the enzyme was assessed under various conditions such as the field pH and temperature as well as optimum pH and temperature using the cyanide contaminated water as substrate source in a typical assay protocol. The enzyme was able to convert 1.6481 µmol of cyanide to thiocyanate in the water sample at optimum pH and temperature of the enzyme. It could be concluded from the study that at optimum pH and temperature, rhodanese exhibited remediation activity in cyanide contaminated aquatic ecosystems and thus, can be used for its restoration.

Keywords: Bioremediation; Rhodanese; Cyanide; *Klebsiella edwardsii;* Atutulala stream

Introduction

Cyanide, a compound containing CN– as its functional group is highly toxic and an important constituent of industrial effluents [1]. In the biosphere, plants, bacteria and fungi are the major sources of cyanide [2]. It is found naturally in cassava roots, potato like tubers grown in tropical countries in the form of cyanogenic glucosides (the precursors of HCN) in various concentrations depending on the variety and growth condition [3]. Cassava (*Manihot esculenta*) is known to be the third most important source of calories in the tropics, after rice and corn [4]. Depending on the absence or presence of toxic levels of cyanogenic glucosides, cassava varieties are often categorized as either sweet or bitter. The sweet cultivars are able to produce as little as 20 milligrams of cyanide (CN) per kilogram of fresh roots, while the bitter ones may produce more than 50 times as much (1 g/kg) [5]. These cyanides which exist as cyanogenic glycosides are hydrolyzed into the toxic compound hydrogen cyanide during processing [5,6]. In South Western Nigeria, cassava processing milling plants are usually situated around running streams or rivers such that the waste from each stages of processing easily find their way into these water bodies as effluents and waste waters [6]. The cassava are even washed and rinsed in these water bodies before and after peeling. In south-western Nigeria especially, local farmers detoxify their cassava tubers/roots by steeping/retting, soaking and wetting or moistening the whole peeled or unpeeled tubers/roots in these running streams for about 3-5 days; after which the skins of the unpeeled roots peel off easily [7-9]. Subsequent washing and sieving with baskets which separate the fibres from the starch are also done in the water bodies. The cassava is now largely detoxified by the prolonged soaking in the running water. All these activities contribute to the release of cyanogenic glycosides from enzymatic degradation following maceration to produce hydrogen cyanide, which may result in acute cyanide poisoning [10]. Cyanide binds and inactivate several enzymes, particularly those with iron in the ferric (Fe^{3+}) state and cobalt [11]. It binds to the active site of cytochrome c oxidase, the terminal protein in the electron transport chain located within mitochondrial membranes where it exerts its ultimate lethal effect of histotoxic anoxia [12]. The binding of cyanide ion to cytochrome hinders the transport of electrons from cytochrome c oxidase to oxygen, a process which prevents the cells from utilizing the oxygen present in the bloodstream, resulting in cytotoxic hypoxia or cellular asphyxiation [13]. Many methods are available for the treatment of cyanide such as ferrocyanide precipitation, prussion-blue and copper catalyzed H_2O_2 precipitation, ozonation, incineration, alkaline chlorination and electrolysis [14]. These methods remediate cyanide contaminated wastewaters with attendant high reagent costs and may often not achieve complete breakdown of some cyanide complexes [15]. Biological method for cyanide removal from industrial wastewaters often termed bioremediation, is cost effective and environmentally friendly [16-18]. Certain microorganisms such as bacteria and fungi are capable of metabolizing cyanide to produce non-toxic end products using the cyanide as the sole nitrogen and carbon sources under aerobic and anaerobic environments [19]. Some species of Bacillus and Klebsiella are resistant to cyanide even at concentrations higher than 1 mM as the enzymes within them called rhodanese have

been reported to be involved in cyanide detoxification [20-23]. Rhodanese is a ubiquitous enzyme that is known to be responsible for the biotransformation of cyanide to less toxic thiocyanate using thiosulphate as the donor substrate [24-27]. The use of enzymatic proteins may represent a good alternative for overcoming most disadvantages related to the use of microorganisms [28,29]. They are not inhibited by inhibitors of microbial metabolism such as occur in industrial wastewaters, such as cassava mill wastewater with low nitrogen (N) and high chemical oxygen demand (COD), leading to a nutritionally imbalanced wastewater [30]. Moreover, they can be used at extreme conditions limiting microbial activities and are effective at low pollutant concentrations. This study involved the isolation, partial purification and characterization of microbial rhodanese isolated from Atutulala stream with further assessment of the bioremediation potential of the enzyme.

Materials and Methods

Sampling area and water sample collections

Water samples were collected from Atutulala Stream also called Odo-Isale, Kola Village, Modakeke, Ile-Ife city in Osun State, South-western, Nigeria. The village is made up of predominantly farmers. Atutulala lies within Latitude 7°46'17.5"N and Longitude 4°49'54.5" E. It is used by the villagers for domestic purposes and also for cassava processing as villagers wash and soak already peeled cassava in the stream for days before milling into fufu and gari products. Water samples were collected from three different designated points for six months from July to December 2015 in sample bottles using grab method. The samples were then brought to the laboratory for cyanide analysis (Figure 1).

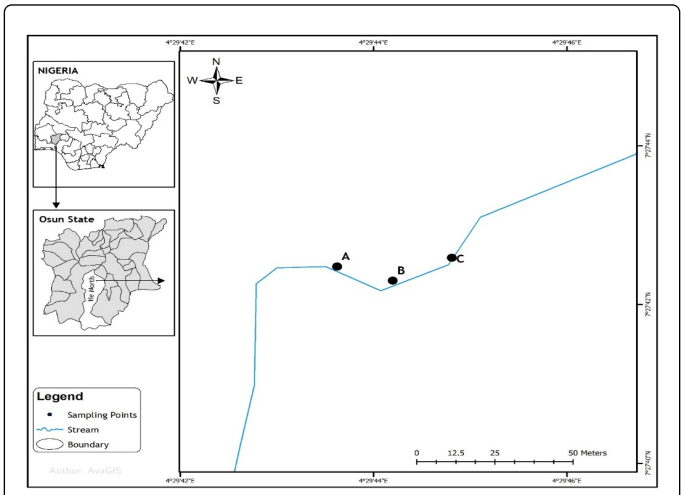

Figure 1: Map Showing the Study Area and Sampling Points.

Cyanide analysis

The cyanide content of the water samples was determined to ascertain the presence of cyanide in the water samples by adding 4 ml alkaline picrate to 1 ml of the sample filtrate in a corked test tube and incubated in a water bath for 5 mins. After colour development (reddish brown colour), the absorbance of the corked test tube was measured in spectrophotometer at 490 nm. The absorbance of the blank containing only 1 ml distilled water and 4 ml alkaline picrate solution was also read. The cyanide content was extrapolated from a cyanide standard curve prepared by measuring different concentrations of KCN solution containing 5 to 50 µg cyanide and 25 ml of 1 N HCl. The different concentrations were used to prepare the cyanide standard curve.

Isolation, characterization and identification of rhodanese producing bacterium

One millilitre (1 ml) of the water sample was taken from each of the sample bottle into a test tube containing 9 ml of sterile distilled water. Serial dilution was carried out to thin out the load of microorganisms to be plated. Serial dilution up to 10^{-6} was carried out and dilution 1, 2, 4, 6 were pour plated with a composed basal medium containing agar agar (1.5% w/v), peptone (1% w/v), yeast extract (0.5% w/v), sodium chloride (0.5% w/v), potassium cyanide (0.3% w/v) and were incubated in duplicates at 37°C for 48 h. After incubation, plates were examined and representative colonies were isolated from each plate for further identification. The representative colonies were purified by repeated streaking on the same medium and were then maintained on nutrient agar slants at 4°C for further study.

The different isolates were grown separately in composed broth medium basal broth containing peptone (1% w/v), yeast extract (0.5% w/), sodium chloride (0.5% w/v), potassium cyanide (0.3% w/v) dissolved in deionized water. The initial pH was adjusted to 9.5. MacFarland standards (0.5) of the isolates were prepared and used for inoculation of the media. The media were incubated at 37°C on a rotary shaker at 230 rpm for 48 h. Each of the culture media was then assayed for rhodanese production.

Four bacterial isolates were selected because they showed appreciable rhodanese activities and were screened for their ability to degrade free cyanide. The best strain with the highest rhodanese activity was selected for further study and characterized. The selected rhodanese-producing strain was identified based on its cell morphology, cultural and biochemical characteristics [31,32].

Enzyme production and partial purification

The time course of the enzyme production was determined and compared with growth. This was done using Zlosnik and Williams [33] growth methods with appropriate modifications by inoculating a 100 ml of the sterile basal broth with 1 ml standard inoculum of 0.5 McFarland standard in an Erlenmeyer flask and incubated at 37°C for 48 hr with agitation at 140 rpm. At intervals of 3 hr, 5 ml of samples were collected aseptically for a period of 48 hr, the optical density of each sample was checked at 630 nm using a colorimeter and recorded as the cell optical density. The samples each at the different time intervals was further centrifuged at 12,000 rpm for 30 mins to separate the cell from supernatant. The supernatant which served as the crude enzyme was checked for rhodanese activity at 460 nm according to the principles of the colorimetric determination of thiocyanate formation by Lee et al. [34], the reaction mixture consists of 0.5 ml of 50 mM Borate buffer (pH 9.4), 0.2 ml of 250 mM KCN, 0.2 ml of 250 mM $Na_2S_2O_3$, and 0.1 ml of the enzyme solution in a total of volume of 1.0 ml. The mixture was incubated for 1 min at room 37°C and the reaction was stopped by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent. The absorbance was taken at 460 nm. One Rhodanese Unit (RU) was defined as the amount of the enzyme that will convert one micro-mole (1 µmol) of cyanide to thiocyanide in one minute at 37°C [35]. Protein concentration was

determined by using Bovine Serum Albumin (BSA) as the standard, where the protein absorbance was extrapolated from the standard curve [36]. The absorbance was read at 595 nm.

In order to achieve partial purification of the enzyme, the supernatant obtained from the centrifugation of the crude homogenate was brought to 85% ammonium sulphate saturation i.e., 55.6 g of ammonium sulphate (analytical grade) was added to 200 ml of crude enzyme and stirred slowly for 1 hr until all the salt had dissolved completely in the supernatant. The mixture was left overnight in the fridge, followed by centrifugation at 4,000 rpm for 30 min at 10°C. The supernatant was discarded and precipitate was collected and suspended in 0.1 M Phosphate buffer pH 7.2. It was evaluated for rhodanese activity and the protein content was determined. The ammonium sulphate precipitate was dialysed against several changes of 0.1 M solution of phosphate buffer (pH 7.2) at 4°C for 18 hr. The dialysate was centrifuged at 4,000 rpm at 10°C for 30 mins to remove insoluble materials and the supernatant was assayed for rhodanese activity and protein. The yield and fold of purifications were calculated. The enzyme was further purified using DEAE-Cellulose cation exchanger equilibrated with 0.1 M phosphate buffer (pH 7.2) followed by elution with 1.0 M NaCl in the same buffer. Fractions of 3 ml were collected from the column at a rate of 128.57 ml per hr. The fractions were assayed for rhodanese activity and protein profile. The active fractions from the column were pooled and dialyzed against 50% glycerol in 0.1 M phosphate buffer, pH 7.2. The yield and fold of purifications were calculated [37].

Characterization of the enzyme

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentrations of KCN between 0.05 M and 0.005 M at fixed concentration of 0.25 M $Na_2S_2O_3$. Also, the concentration of $Na_2S_2O_3$ was varied between 0.05 M and 0.005 M at fixed concentration of 0.25 M KCN. Plots of the reciprocal of initial reaction velocity (1/V) versus reciprocal of the varied substrates 1/[S] at each fixed concentrations of the other substrate were made [38]. Lines through the points were drawn by using the method of regression.

To determine the effect of pH on the activity of the enzyme, the enzyme was assayed using 50 mM of citrate buffer (pH 3-6); 50mM phosphate buffer (7-8) and 50mM borate buffer (pH 9-11) in place of the reaction buffer of the rhodanese assay [37].

The optimum temperature of the enzyme was investigated by assaying the enzyme at temperatures between 30°C and 80°C at an interval of 10°C. The assay mixture was first incubated at the indicated temperature for 10 min before addition of an aliquot of the enzyme which had been equilibrated at the same temperature.

The substrate specificity of the enzyme was determined by using different sulphur compounds such as sodium sulphite, 2-mercaptoethanol, ammonium persulphate, ammonium sulphate and sodium metabisulphite in a typical rhodanese assay mixture. The different sulphur compounds were substituted in place of sodium thiosulphate. The activity was taken and expressed as a percentage activity of the enzyme using sodium thiosulphate as the control [37].

Assessing the enzyme for bioremediation potential

The cyanide content of the water sample from Atutulala stream was determined to ascertain the presence of cyanide in it. The pH and temperature of the water sample were also determined. The assay protocol of the enzyme was set up at varying conditions such as pH of the water sample (pH=6.5), the optimum pH of the enzyme (pH=6.0), temperature of the room (37°C), the optimum temperature of the enzyme (60°C), presence or absence of buffer (50mM Citrate Buffer pH=6.0) in the protocol, introduction of sulphur compounds (250mM sodium thiosulphate) and the use of the water sample as the sulphur source with the water samples as the cyanide source to be remediated by the enzyme. Eight different conditions were set up in the assay protocol assessment of the remediation potential of the rhodanese enzyme as stated below:

1. pH=6.5; Temperature=37°C; Water sample as Sulphur source; No Buffer
2. pH=6.5; Temperature=60°C; 250mM of $Na_2S_2O_3$; No Buffer
3. pH=6.0; Temperature=60°C; Water sample as Sulphur source; No Buffer
4. pH=6.0; Temperature=60°C; 250mM of $Na_2S_2O_3$; No Buffer
5. 50mM Citrate Buffer (pH=6.0); Temperature=37°C; Water sample as Sulphur source
6. 50mM Citrate Buffer (pH=6.0); Temperature=37°C; 250mM of $Na_2S_2O_3$
7. 50mM Citrate Buffer (pH=6.0); Temperature=60°C; 250mM of $Na_2S_2O_3$
8. 50mM Citrate Buffer (pH=6.0); Temperature=60°C; Water sample as Sulphur source

Each of the assay protocols have control which follows the same protocol as the test except that the 0.5 ml of the purified enzyme solution was added after all the other reagents have been added and incubated for 1 min at the appropriate temperatures. The amount of cyanide converted to thiocyanide was calculated by finding the difference between the test and the control.

Results and Discussion

The cyanide concentrations for the six months (July-December) were all above World Health Organisation (WHO) and Nigeria International Standard (NIS) limits of 0.07 mg/l and 0.01 mg/l for cyanide in drinking water respectively (Table 1). This study agrees with Akinpelu et al. [39] who reported high levels of cyanide from cassava production effluent and further stated that intake of much of hydrogen cyanide could damage body cells.

	July	Aug	Sept	Oct	Nov	Dec	WHO (2003)	NIS (2007)
Cyanide	1.44	3.84	2.67	0.71	1.6	2.11	0.07	0.01
(mg/l)	± 0.95	± 2.13	± 0.91	± 0.36	± 1.14	± 1.25		

Table 1: Cyanide Concentration (Mean ± SD) of Atutulala Stream from July-Dec, 2015.

A total of four bacterial strains were isolated from Atutulala stream, out of which one was selected for further study based on its appreciable rhodanese production. Cultural, morphological, biochemical and physiological characteristics of the isolate was examined and they were identified as a strain of *Klebsiella edwardsii. Klebsiella edwardsii* rhodanese production was maximum at 48 h in the death phase and this may be due to the inability of the bacteria to adapt as early as possible to the environmental conditions such as pH, temperature (Figure 2). This has been noted by Goyal et al. [40] and Prakash et al. [41], who stated that inhibition of enzyme production may occur as a result of catabolic repression by metabolizable monosaccharides such as glucose, increase in concentration of protease and rapid change in pH with the aim of conserving energy by bacteria.

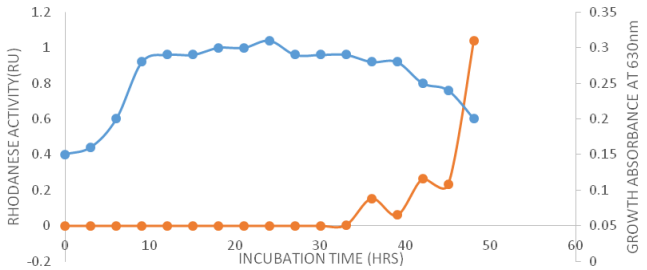

Figure 2: Growth and Rhodanese activity of *Klebsiella edwardsii* isolated from Atutulala Stream.

The enzyme from *K. edwardsii* was purified 2.26 folds using ammonium sulphate precipitation at 85% saturation and 4.56 folds through DEAE-Cellulose with a yield of 30.3% (Table 2). The chromatography result showed a specific activity of 0.047 Rhodanese Unit/mg with a percentage recovery of 30.3, the activity increased dramatically because the bounded proteins were released and increased the volume of the enzyme during ion-exchange chromatography.

Purification Steps	Total Activity (RU)	Total Protein (mg/ml)	Specific Activity (RU/mg)	Fold	Yield
Crude Extract	58.34	562.5	0.01037	1	100
Ammonium Sulphate Precipitation	4.802	205.625	0.0234	2.26	36.56
Ion Exchange on DEAE Cellulose	8.07	170.625	0.0473	4.56	30.3

Table 2: Summary of Partial Purification Process of Crude Rhodanese Obtained from *K. edwardsii.*

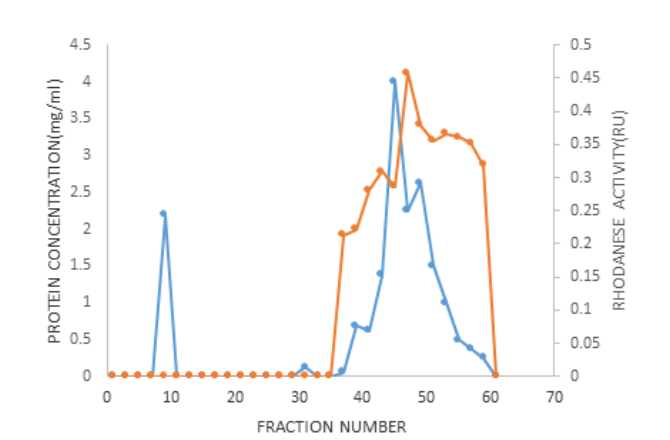

Figure 3: Elution Profile of Rhodanese of *K. edwardsii* on DEAE-Cellulose.

The kinetic parameters (K_m and V_{max}) of *Klebsiella edwardsii* rhodanese for potassium cyanide are 50 mM, 0.56 RU/mg and for sodium thiosulphate are 200 mM, 1 RU/mg respectively (Figures 4 and 5). This indicate great affinity of the *Klebsiella edwardsii* rhodanese for these substrates and that it would catalyze the detoxification reaction with great efficiency.

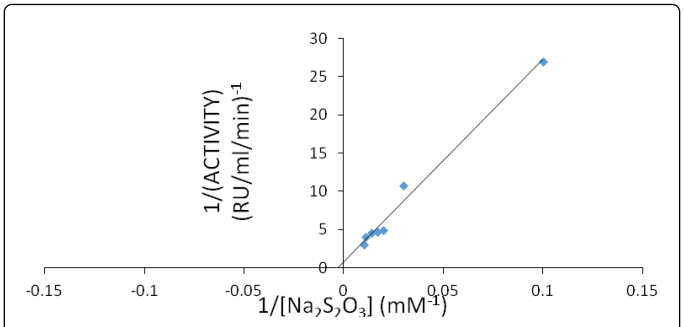

Figure 4: Lineweaver-Burk Plot for Varying Concentration of Sodium Thiosulphate.

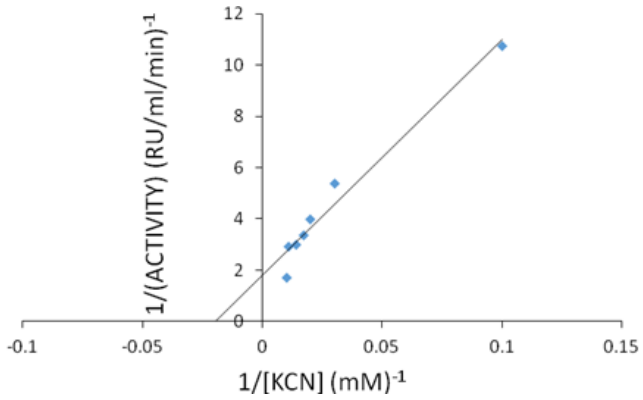

Figure 5: Lineweaver-Burk Plot for Varying Concentration of Potassium Cyanide.

The optimum pH obtained for *Klebsiella edwardsii* rhodanese was 6 (Figure 6) which is similar to the work of Oyedeji et al. [19] who reported an optimum pH of 6 for rhodanese from Pseudomonas aeruginosa isolated from the soil of cassava processing site. The optimum temperature for *Klebsiella edwardsii* rhodanese is 60°C in this study (Figure 7) as reported by Ehigie et al. [42] for rhodanese isolated from the leaves of *Mormodica charantia*.

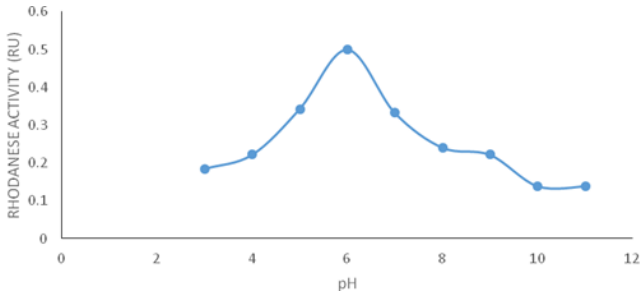

Figure 6: Effect of pH on Partially Purified Rhodanese Activity from *K. edwardsii.*

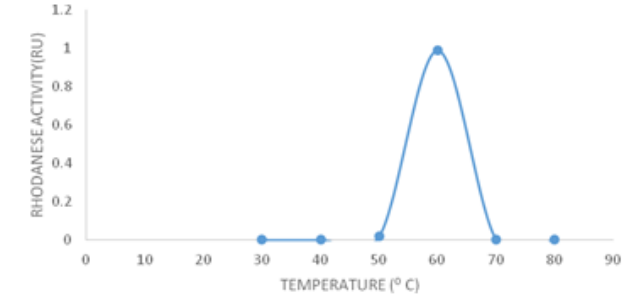

Figure 7: Effect of Temperature on Partially Purified Rhodanese Activity from *K. edwardsii.*

The substrate specificity study of rhodanese isolated from *Klebsiella edwardsii* has preference for sodium thiosulphate but also showed higher activity with ammonium sulphate and ammonium persulphate (Table 3). The higher activities shown by the enzyme for ammonium sulphate (122.89%) and ammonium persulphate (178.31%) could be as a result of their concentrations in the native environment. The substrate specificity result also showed that sodium sulphite, sodium metabissulphite, and 2-mercaptoethanol can also be used for its catalytic activities.

Substrate	% Residual Activity
Sodium Thiosulphate	100
Sodium Sulphite	78.31
Ammonium Sulphate	178.31 (>100)
Ammonium per Sulphate	122.89 (>100)
2-Mercaptoethanol	33.7
Sodium Metabisulphite	67.5

Table 3: Effect of Different substrates on Partially Purified Rhodanese from *K. edwardsii.*

The bioremediation potential of the purified rhodanese on Atutulala water sample showed that the enzyme had highest activity of 1.6481 Rhodanese Unit (C) when the water sample was used as the sulphur source at the optimum pH and temperature of the enzyme (Table 4). This value corresponds to the amount of cyanide in µmol that was reduced to a less toxic thiocyanate in the water sample tested. This lends credence to the effect of temperature and pH of the enzyme studied. The enzyme also showed higher activity of 1.0741 Rhodanese Unit (A) as compared to the remaining experiments, when the water sample (pH 6.5) was used as the sulphur source at 37°C. This may be due to the presence of sulphur in the water sample which was just sufficient for the enzyme to act. The activities of the enzyme in the presence of citrate buffer (pH 6.0) when water sample was used as sulphur source at 60°C (G); when sodium thiosulphate was used as sulphur source at 60°C (H); and when sodium thiosulphate was used as sulphur source at 37°C are 0.8148 Rhodanese Unit, 0.5000 Rhodanese Unit and 0.4259 Rhodanese Unit respectively (Table 4). Other conditions (B, D, E and F) also resulted in low activity of the enzyme in converting cyanide to thiocyanate in the water sample probably because of the presence of sulphur compound just sufficient enough in the sampled water for the enzyme to act; and the addition of more sulphur from sodium thiosulphate might have reduced the activity as the enzyme might have reached its maximum velocity irrespective of the optimum temperature and pH.

Conditions	Rhodanese Activity (Rhodanese Unit)
A) pH=6.5; Temperature=37°C; Water sample as Sulphur source	1.0741
B) pH=6.5; Temperature=37°C; 250mM of $Na_2S_2O_3$	-0.6482
C) pH=6.0; Temperature=60°C; Water sample as Sulphur source	1.6481
D) pH=6.0; Temperature=60°C; 250mM of $Na_2S_2O_3$	-0.9259
E) 50mM Citrate Buffer (pH=6.0); Temperature=37°C; Water sample as Sulphur source	-0.7592

F) 50mM Citrate Buffer (pH=6.0); Temperature=37°C; 250mM of $Na_2S_2O_3$	0.4259
G) 50mM Citrate Buffer (pH=6.0); Temperature=60°C; 250mM of $Na_2S_2O_3$	0.8148
H) 50mM Citrate Buffer (pH=6.0); Temperature=60°C; Water sample as Sulphur source	0.5

Table 4: Bioremediation Potential of *K. edwardsii* Rhodanese.

Conclusion

The presence of rhodanese in *Klebsiella edwardsii* suggested that the enzyme may possess functional cyanide detoxification mechanism necessary for the survival of the organism in Atutulala stream in which cassava tubers are soaked during processing. The water sample was confirmed to contain cyanide beyond the permissible limits of WHO and NIS. The isolated enzyme was also effective at a high temperature, a very unique and important property for detoxification of cyanide. It can then be concluded from this study that the rhodanese enzyme assessed under various conditions was found to be suitable to be adopted for clean-up of cyanide polluted water bodies at the optimum pH and temperature recorded by this study. Further studies of the biochemical parameters of the enzyme is therefore recommended to understand the appropriate applications of the enzyme in the bioremediation procedure.

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