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Full Length Research Paper

Characterization of rhodanese produced by *Pseudomonas aeruginosa* and *Bacillus brevis* isolated from soil of cassava processing site

Olaoluwa Oyedeji[1]*, Kehinde O. Awojobi[1], Raphael E. Okonji[2] and Olufemi O. Olusola[1]

[1]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

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Wastewater from numerous cassava processing industries contains cyanide which is toxic to several forms of life. This effluent constitutes serious hazards to the environment and aquatic life in receiving water bodies. Enzymatic remediation of polluted environment presents advantages over traditional technologies and also over microbial remediation. Extracellular rhodanese of strains of *Pseudomonas aerugionosa* and *Bacillus brevis* isolated from soil of cassava processing site were studied. Biochemical characteristics of the purified enzymes, including pH and temperature profiles were also determined. Production of rhodanese correlated with the rate of bacterial growth at the exponential phase for both strains. Optimum incubation time for maximum enzyme production was 20 h in both cases. The enzyme was purified 9.72-fold with a yield of 37.8% and specific activity of 6.32 mg^{-1} protein in the case of *P. aeruginosa*. The enzyme from *B. brevis* was purified 7.44-fold with a yield of 22.6% and specific activity of 5.21 mg^{-1} protein. The enzyme generally demonstrated a broad pH range but optimum pH was 6.0 and 7.0 for *P. aeruginosa* and *B. brevis* rhodanese activity respectively. Optimum temperatures for *P. aeruginosa* and *B. brevis* rhodanese were 50 and 40°C, respectively, with both enzymes retaining activity up to 70°C. The Km values for KCN and $Na_2S_2O_3$ as substrates for *P. aeruginosa* rhodanese were 12.5 and 0.0066 mM, respectively, while the Km values for the same substrates for *B. brevis* rhodanese were 3.12 and 11.1 mM, respectively. Cations, Hg^{2+}, Ba^{2+}, and Co^{2+} all inhibited the enzyme activities of both bacteria. The bacteria rhodaneses have potential for effective remediation of cyanide-polluted environments, ultimately leading to improvement of fish and other aquatic organisms in receiving water bodies.

Key words: Cyanide, cyanogenic glycosides, cassava mill wastewater, rhodanese, *Pseudomonas aeruginosa*, *Bacillus brevis.*

INTRODUCTION

Wastes generated by cassava processing pose serious environmental pollution especially with increased industrial production of cassava flour and starch (Goodley, 2004). These wastes such as peelings, fibrous by-products and wastewater effluents are indiscriminately disposed into the environment without prior treatment to reduce the volume, toxicity or mobility of the hazardous substances. Cassava (*Manihot esculenta* Crantz) is a woody shrub widely cultivated in tropical and subtropical areas of the world for its edible roots (Burrell, 2003). In Africa and Latin America, cassava is mostly used for human consumption while in Asia and parts of Latin America, it is used commercially for the production of

*Corresponding author. E-mail: laoluoyedeji@gmail.com. Telephone: +234 8033745381.

animal feeds and starch-based products (FAO, 1991). Cassava contains cyanogenic glycosides that are easily hydrolysed into the toxic compound hydrogen cyanide during processing (Oti, 2002; Abiona et al., 2005). Cyanide is toxic to several forms of life because it binds to key enzymes of importance in aerobic respiration such as cytochrome oxidase, leading to inhibition of respiration (Chen and Liu, 1999; Cipollone et al., 2007).

Numerous human disorders such as development of goitre, tropical ataxic neuropathy, paralysis and death are associated with consumption of improperly processed cassava (Osuntokun, 1994; Oluwole et al., 2000; Ernesto et al., 2002; Siritunga and Sayre, 2003). Cassava is normally processed before consumption as a means of detoxification, preservation and modification (Oyewole, 1991). The extraction of starch from the root requires large amounts of water and the residual water after separation of starch and fibre contains small amounts of starch, proteins and hydrocyanic acid. When the effluent is released directly or indirectly into streams and rivers, it may lead to detrimental effects on fish and other aquatic organisms (Oti, 2002; Oboh and Akindahunsi, 2003; Oboh, 2004). Cassava processing-related water pollution problems have been reported as serious in many countries such as Thailand (Kiranwanich, 1977). The continuous indiscriminate discharges of untreated effluents constitute danger to the environment, especially to water sources used for cassava processing.

Wastewater containing cyanide must therefore be treated before been discharged into the environment. Chemical oxidation methods such as the addition of sodium hypochlorite and hydrogen peroxide are currently used to remediate cyanide contaminated wastewaters with attendant high reagent costs associated with the use of these chemical techniques. Also, complete breakdown of some cyanide complexes may not be achieved (Yanase et al., 2000). Biological treatments, however, offer a cost effective and environmentally friendly acceptable method for cyanide removal from industrial wastewaters (Watanabe et al., 1998; Akcil et al., 2003; Siriantapiboon and Chuamkaew, 2007). Certain microorganisms such as bacteria and fungi can metabolize cyanide to produce non-toxic end products using the cyanide as the sole nitrogen and carbon sources under aerobic and anaerobic environments. Such organisms which include species of *Bacillus* and *Klebsiella* are resistant to cyanide even at concentrations higher than 1 mM (Chen and Liu, 1999; Kao et al., 2003; Ebbs, 2004). The enzymes within these strains of micro-organisms' rhodaneses are reported to be involved in cyanide detoxification (Raybuck, 1992; Colnaghi et al., 1996).

The use of enzymatic proteins may represent a good alternative for overcoming most disadvantages related to the use of microorganisms (Gianfreda et al., 2004; Gianfreda et al., 2010). 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 (Metcalf and Eddy, 1991). Moreover, they can be used at extreme conditions limiting microbial activities and are effective at low pollutant concentrations. Bioremediation through enzymatic techniques are therefore environmentally friendly processes with capability of remediation of many compounds that are unfriendly to the environment by ecological standards of our societies. Rhodanese, a thiosuphate: cyanide sulphurtransferase (EC 2.8.1.1), is an enzyme that catalyses the transfer of the sulphane sulphur from thiosulphate to cyanide, forming the less toxic thiocyanate and sulphite (Westley, 1981; Westley et al., 1983).

$$SSO_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^-$$

This study aimed to characterize rhodaneses produced by bacteria isolated from soil of cassava-processing site with the objective of evaluating potentials for applications in remediation of cyanide-contaminated cassava mill wastewater dumping site.

MATERIALS AND METHODS

Collection of soil samples

Soil sample was collected from a cassava processing site located at new market area of Ile Ife city in Osun Sate, Nigeria. The upper layer of the soil was removed and the next layer of the soil (1 to 2 inches below the surface) was collected in polythene bags using sterile spatula. The samples were then brought to the laboratory for analysis.

Isolation of bacteria

10 g of soil sample was weighed and mixed vigorously in 100 mL sterile distilled water inside a conical flask. The mixture was serially diluted from 10^{-1} to 10^{-9} and the dilutions 10^{-7}, 10^{-8} and 10^{-9} were then plated on Luria-Bertani (LB) agar medium. The plates were incubated in duplicates at 37°C for 24 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 LB agar plates. They were then maintained on LB agar slants at 4°C for further study.

Screening for rhodanese production

The different isolates were grown separately in LB broth medium supplemented with 50 mM potassium cyanide to serve as inducer. 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.

Characterization and identification of isolates

Eight bacterial isolates were screened for their ability to degrade free cyanide. The two best strains were selected for further study

and characterized. The two selected rhodanese-producing strains were identified based on their cell morphology, cultural and biochemical characteristics (Cappuccino and Sherman, 1996).

Growth and enzyme production

The time course of the enzyme production was determined and compared with growth. This was done using growth methods described by De Souza and Martins (2001) with appropriate modifications. Aqueous suspension (5 ml) of 24 h old pure culture of test isolates was made in sterile distilled water and compared with 0.5 McFarland standards with initial absorbance at 680 nm of at least 0.1. The aqueous suspension was used to inoculate 45 ml of sterile LB medium contained in a 250 ml tightly closed Erlenmeyer flask and incubated for 24 h. This was in turn used to inoculate 450 ml of sterile LB medium contained in a 450 ml Erlenmeyer flask to give the same absorbance of 0.1. The culture was incubated at 37°C on a rotary shaker at 100 g for 72 h during which samples (5 ml) were aseptically collected at 4 h intervals for turbidity measurement to monitor the bacterial growth. Turbidity was determined by measuring the increase in optical density (OD) at 680 nm with a colorimeter. After turbidity measurements, sample was centrifuged at 6 000 *g* for 15 min and the clear supernatant were collected as the crude enzyme and used for rhodanese assay.

Enzyme assay

Rhodanese activity was measured according to the method of Lee et al. (1995). The reaction mixture consisted of 0.5 ml of 50 mM borate buffer (pH 9.4), 0.2 ml of 0.25 M KCN, 0.2 ml of 0.25 M $Na_2S_2O_3$ and 20 µL of enzyme in a total volume of 1.0 ml. The mixtures were incubated for 1 min at room temperature and stopped by the addition of 0.5 ml of 15% formaldehyde. After the addition of 1.5 ml of ferric nitrate solution (10g Fe $(NO_3)_2$. $9H_2O$, 20 ml HNO_3 and distilled water to 100 ml that is sorbo reagent (Sorbo, 1953), absorbance was read at 460 nm. The unit of activity was expressed in rhodanese unit (RU). One rhodanese unit was taken as the amount of enzyme, which under the given condition produced an optical density reading of 1.08 at OD 460 nm (Sorbo, 1951).

Protein concentrations were determined by the method of Lowry et al., (1951), using crystalline bovine serum as standard.

Enzyme purification

The crude enzyme extract was subjected to 65% ammonium sulphate saturation (430 g L^{-1}) by the addition of solid ammonium sulphate over a period of 1 h with continuous stirring. The solution was then kept at 4°C overnight. The solution was centrifuged at 10 000 *g* for 15 min and the precipitate obtained was dialysed against several changes of a 50 mM citrate buffer, pH 5.0. Protein content was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as standard. The partially purified enzyme was also assayed for rhodanese activity. Preswollen CM Sepharose CL-6B was packed into a 2.5 × 40 column and equilibrated with 200 ml of 50 mM citrate buffer, pH 5.0. The ammonium sulphate and dialyzed sample from the preceding step was layered onto the column and eluted with 250 ml linear gradient of 0 to 0.5 M KCl in citrate buffer, pH 5.0. 3 ml fractions were collected at a flow rate of 36 ml/h. The protein concentration was monitored by measuring absorbance at 280 nm. The fractions were also assayed for enzyme activity and the fractions with high activities were pooled together.

Determination of kinetic parameters

The kinetic parameters, Km and Vmax of the enzyme were determined according to the method of Lee et al. (1995). The Km for KCN was determined by varying the concentration of KCN between 10 mM and 100 mM at 50 mM $Na_2S_2O_3$. Also, Km of $Na_2S_2O_3$ was determined by varying $Na_2S_2O_3$ concentration from 50 to 500 mM at a fixed concentration of 50 mM KCN. The apparent kinetic parameters were determined from the double reciprocal plots (Lineweaver-Burk plots) (Lineweaver and Burk, 1934). Lines through the points were drawn by using the method of regression.

Effect of temperature on enzyme activity

To determine the effect of temperature on enzyme activity, 20 µL of enzyme was assayed at temperatures between 30 and 80°C. The assay mixture was first incubated at the indicated temperature for 10 min before initiating reaction by the addition of an aliquot of the enzyme, which had been equilibrated at the same temperature.

Effect of pH on enzyme activity

The effect of pH on the rhodanese activity was performed by assaying the enzyme using the following three buffers of different pH values: 0.2 M citrate buffer (pH 5.0 to 6.5), 0.2 M phosphate buffer (pH 6.5 to 8.5) and 0.2 M borate buffer (pH 8.5 to 11.0).

Effect of cations on enzyme activity

The effect of various cations on rhodanese activity was also studied. The salts included mercuric chloride ($HgCl_2$), barium chloride ($BaCl_2$), calcium chloride ($CaCl_2$), cobalt chloride ($CoCl_2$), magnesium chloride ($MgCl_2$) and manganese chloride ($MnCl_2$) at 0.05 and 1.0 mM. The final concentration of the salt in the assay mixture was either 250 µM or 500 µM.

RESULTS

A total of eight bacterial strains were isolated from the soil of a cassava- processing site out of which two were selected for further study based on their appreciable rhodanese production. Cultural, morphological, biochemical and physiological characteristics of the two isolates were examined and they were identified as a strain each of *Pseudomonas aeruginosa* and *Bacillus brevis*. The two isolates were subjected to growth and rhodanese activity studies and it was found that the enzyme activity correlates with the rate of growth in each case (Figure 1a and b). The optimum incubation time for maximum rhodanese activity by *P. aeruginosa* was 20 h. The organism also had a lag phase of about 4 h followed by an exponential phase of about 32 h. (Figure 1a). *B. brevis* had a similar growth pattern with *P. aeruginosa* with an exponential phase of about 40 h. Optimum incubation times for enzyme production was also 20 h. Tables 1 and 2 show the purification steps for rhodanese from the two bacterial strains isolated from soil of cassava processing site. The enzyme from *P. aeruginosa*

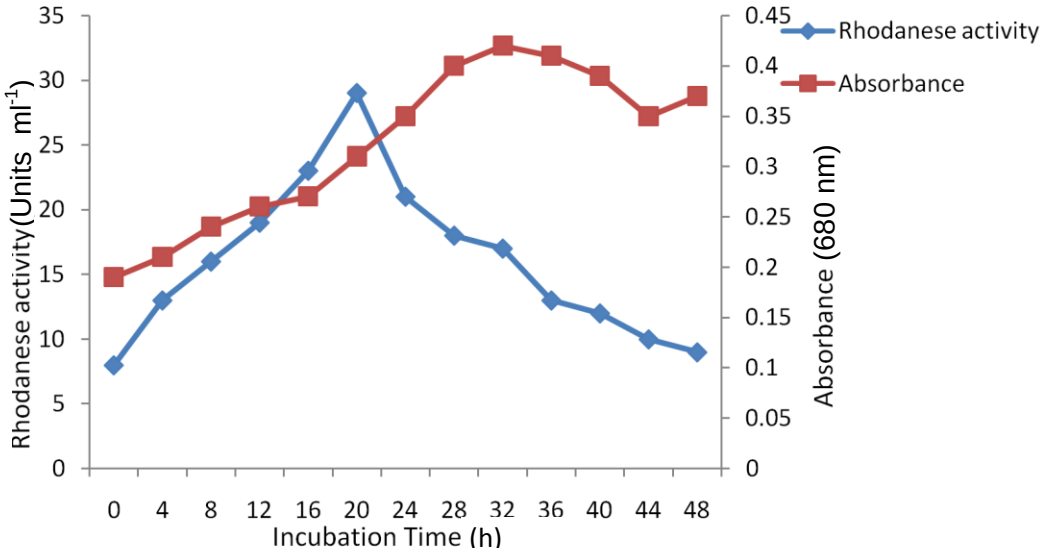

Figure 1a. Growth and rhodanese activity of *P. aeruginosa* isolated from soil of cassava processing site.

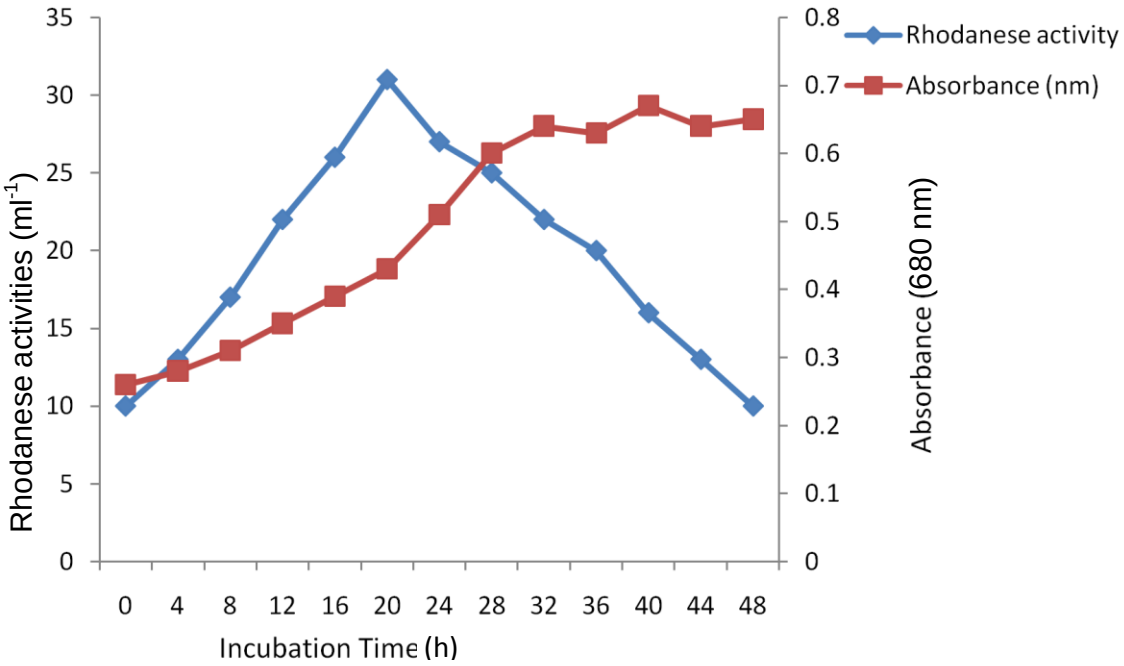

Figure 1b. Growth and rhodanese activity of *B. brevis* isolated from soil of cassava processing site.

was purified 1.54 folds using ammonium sulphate precipitation at 65% saturation and 9.72 folds through CM Sepharose CL-6B. The enzyme from *B. brevis* was purified 1.6 folds using ammonium sulphate precipitation at 65% saturation and 7.44 folds through CM Sepharose CL-6B. The elution profile of rhodanese from *P. aeruginosa* and *B. brevis* are shown in Figure 2a and b, respectively. A single active peak was observed in each case. The effect of temperature between 30 and 80°C on the rhodanese activity of *P. aeruginosa* and *B. brevis* showed that optimum temperature for activity were 50 and 40°C respectively (Figure 3a and b). The enzyme from *P. aeruginosa* retained activity up to 70°C but above this there was no detectable rhodanese activity. The enzyme from *B. brevis* also retained activity up to 80°C beyond which no activity was detected. The effect of pH

Table 1. Summary of purification of rhodanese obtained from *Pseudomonas aeruginosa* isolated from soil of cassava processing site.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude enzyme	78.6	120.4	0.65	100	1
Ammonium sulphate precipitation	42.9	42.8	1.00	54.6	1.83
Ion exchange chromatography on CM sepharose CL-6B	29.7	4.7	6.32	37.8	9.72

Table 2. Summary of purification of rhodanese obtained from *Bacillus brevis* isolated from soil of cassava processing site.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude enzyme	64.6	92.8	0.70	100	1
Ammonium sulphate	33.9	30.4	1.12	52.5	1.60
Ion exchange chromatography on CM Sepharose CL-6B	14.6	2.8	5.21	22.6	7.44

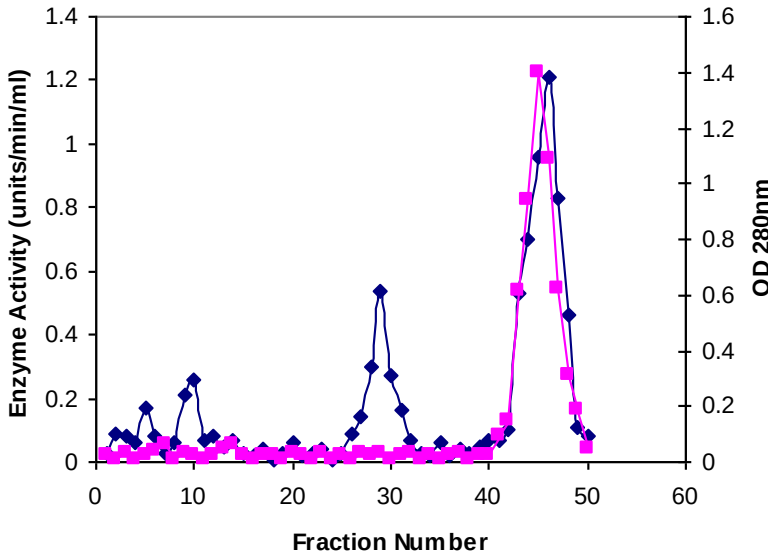

Figure 2a. Elution profile of rhodanese of *P. aeruginosa* on CM Sepharose CL-6B. The column was equilibriated with 200 ml of 50 mM citrate buffer, pH 5.0 and then eluted with 250 ml linear gradient of 0-0.5 M KCl in 50 nM citrate buffer, pH 5.0. Fractions of 3 ml were collected at a flow rate of 36 ml/h. -♦-♦-, Enzyme activity; -■-■-, protein profile.

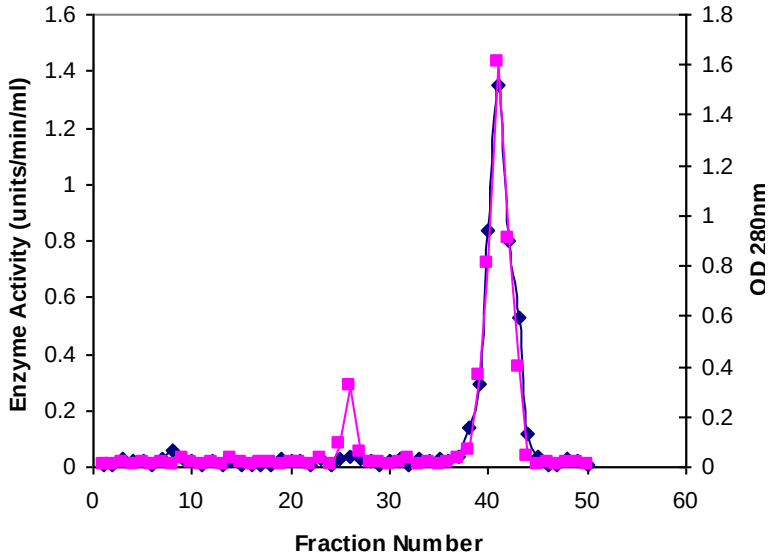

Figure 2b. Elution profile of rhodanese of *B. brevis* on CM Sepharose CL-6B. The column was equilibriated with 200 ml of 50 mM citrate buffer, pH 5.0 and then eluted with 250 ml linear gradient of 0-0.5 M KCl in 50 nM citrate buffer, pH 5.0. Fractions of 3 ml were collected at a flow rate of 36 ml/h. -♦-♦-, Enzyme activity; -■-■-, protein profile.

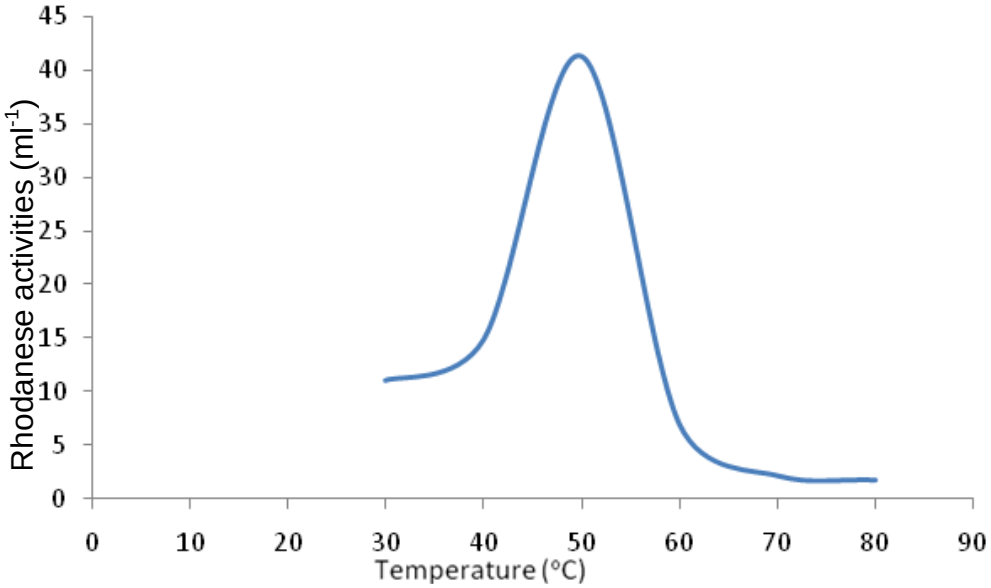

Figure 3a. Effect of temperature on the activity of rhodanese of *P. aeruginosa* isolated from soil of cassava processing site.

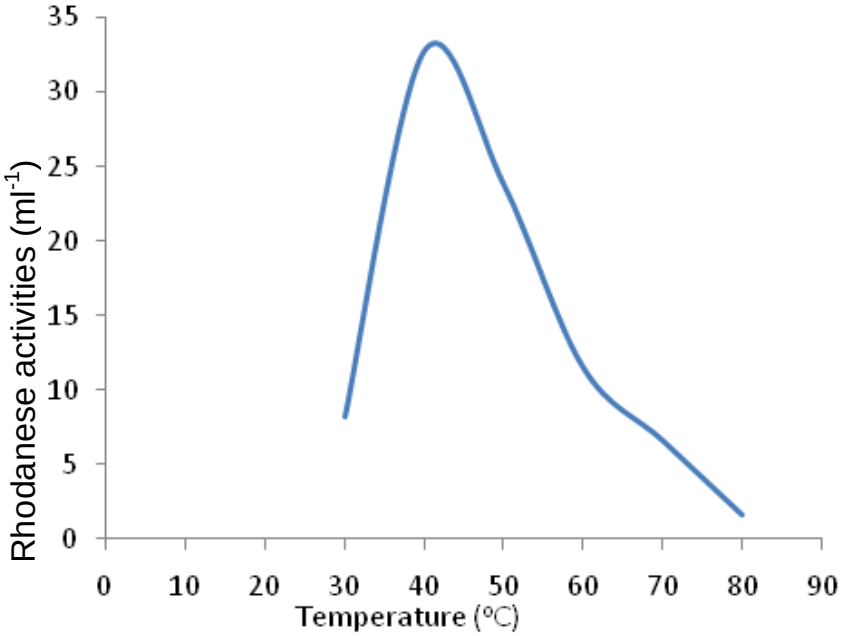

Figure 3b. Effect of temperature on the activity of rhodanese of *B. brevis* isolated from soil of cassava processing site.

on activity of rhodanese from *P. aeruginosa* and *B. brevis* are shown in Figure 4a and b. Activity was found in the pH range 5.5 to 9.0 in both cases but the optimum pH was 6.0 for rhodanese of *P. aeruginosa* while it was 7.0 for rhodanese from *B. brevis*.

Michaelis-Menten constants (Km) and maximum reaction velocities (Vmax) of the rhodanese from the two bacterial strains were determined using Lineweaver-Burk plot under optimum conditions. For the enzyme from *P. aeruginosa*, the Km values for KCN and $Na_2S_2O_3$ were 12.5 and 0.066 mM, respectively while the Vmax values for the two substrates (KCN and $Na_2S_2O_3$) were 62.5 and 14.7, units respectively (Figure 5a and b). For the enzyme from *B. brevis*, the Km values for KCN and

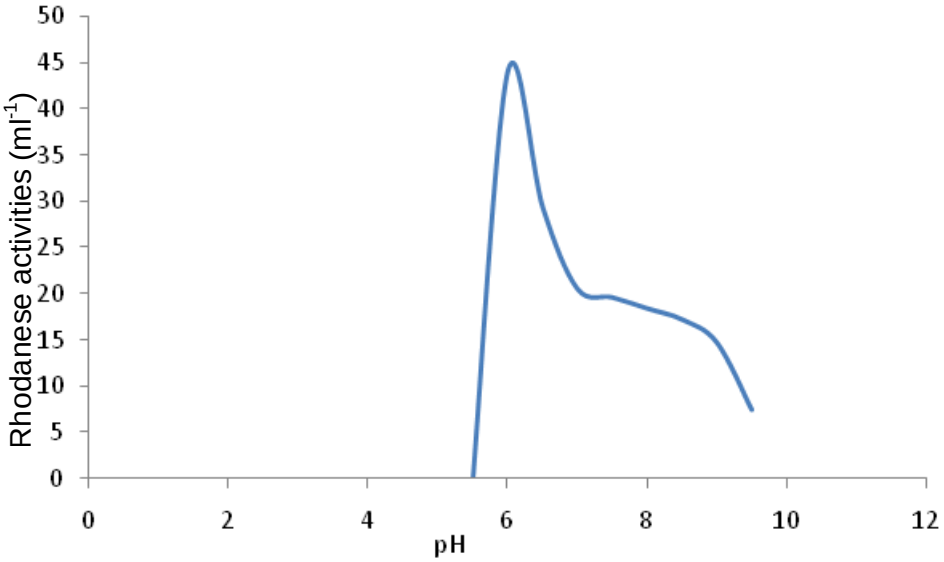

Figure 4a. Effect of pH on the activity of rhodanese of *P. aeruginosa* isolated from soil of cassava processing site.

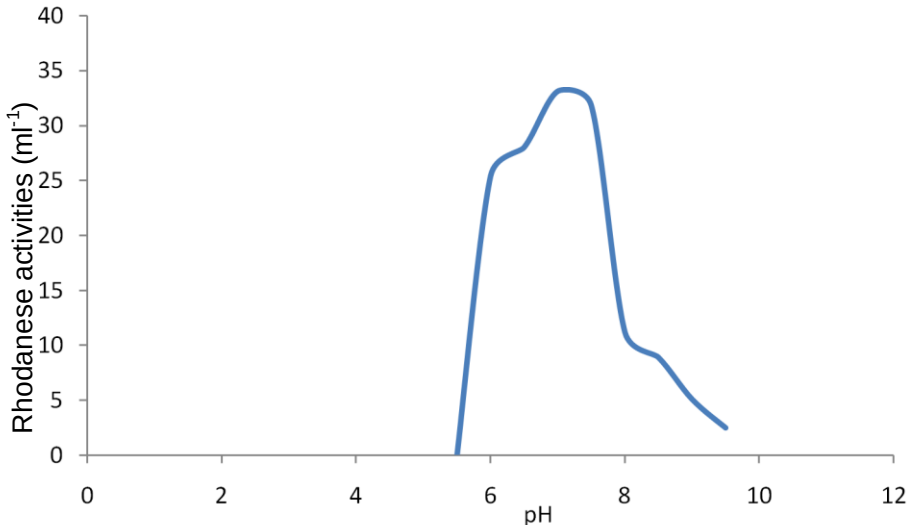

Figure 4b. Effect of pH on the activity of rhodanese of *B. brevis* isolated from soil of cassava processing site

$Na_2S_2O_3$ were 3.12 and 11.1 mM, respectively while the Vmax values for the two substrates (KCN and $Na_2S_2O_3$) were 24.3 and 43.4 units, respectively (Figures 6a and b). All the metallic ions tested were observed to negatively affect the activity of rhodanese from both isolated strains of bacteria.

DISCUSSION

Cyanide is a potent cytotoxic agent that kills the cell by inhibiting cytochrome oxidase of the electron transport chain. It is an inorganic pollutant present in the effluent from numerous cassava-processing industries and constitutes hazards to the environment especially the aquatic ecosystems (Arguedes and Cooke, 1982; Abiona et al., 2005, Arimoro et al., 2008). Rhodanese is an enzyme present in the three evolutionary lineages – eubacteria, archaea and eucaryotes, where it is believed to play central role in cyanide detoxification (Aminlari and Shahbazi, 1994; Bordo and Bork, 2002). However, there is evidence that other functions, such as participation in formation of iron-sulphur centres and regulation of energy metabolism are also performed by rhodanese (Toohey,

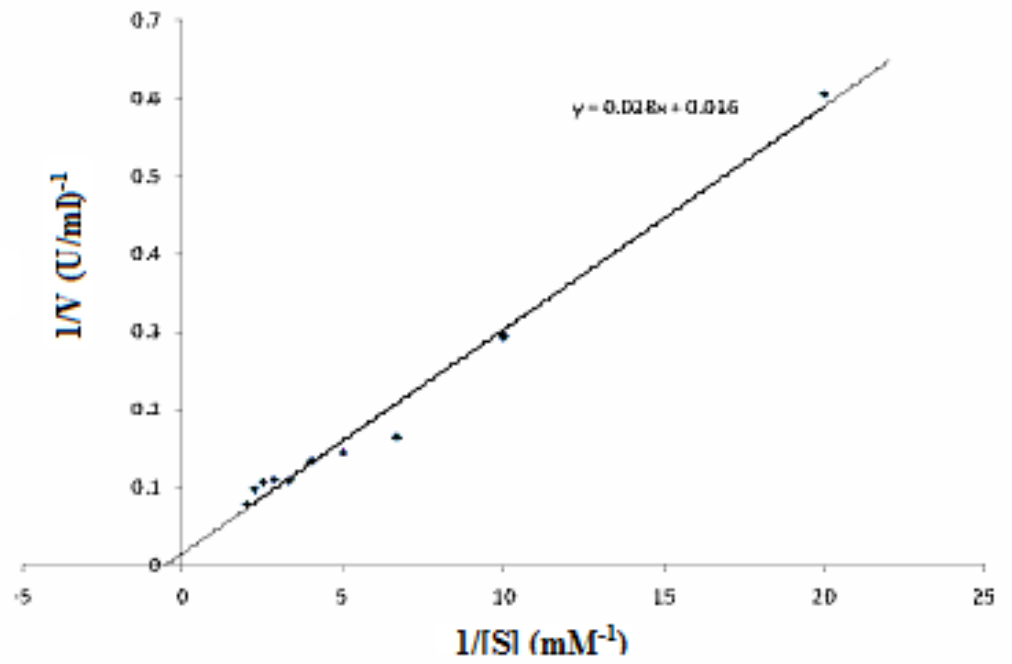

Figure 5a. Lineweaver-Burk plot (1/V versus 1/[S]) for the determination of kinetic parameters of the rhodanese of *P. aeruginosa.* Enzyme was assayed at varying concentrations of KCN at 50 mM $Na_2S_2O_3$.

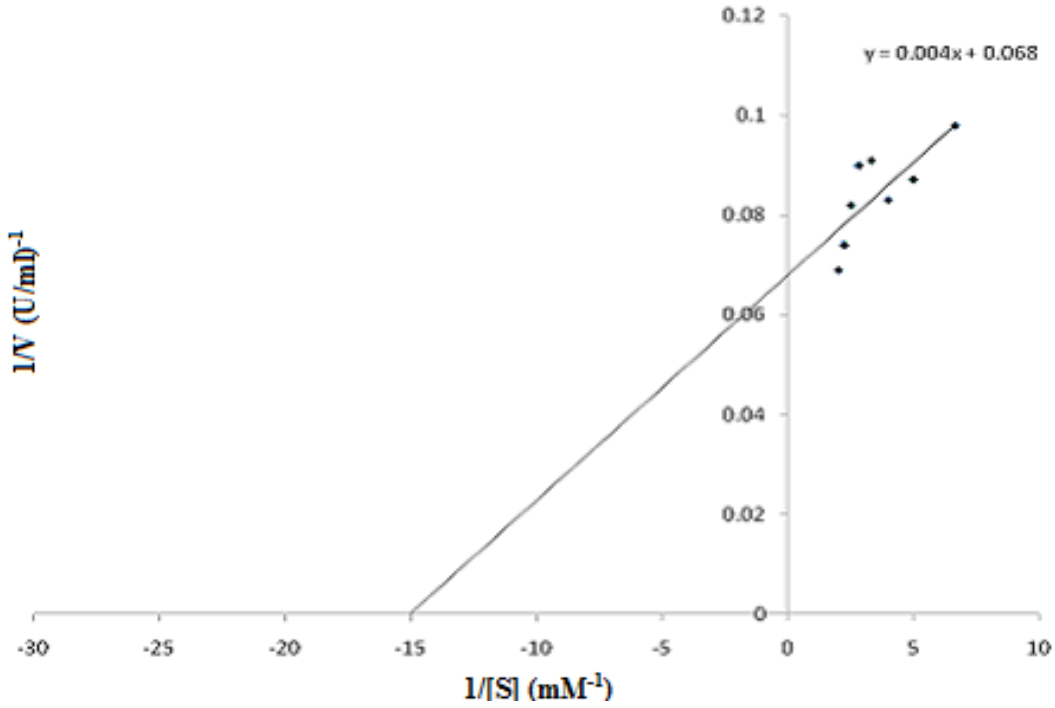

Figure 5b. Lineweaver-Burk plot (1/V versus 1/[S]) for the determination of kinetic parameters of the rhodanese of *P. aeruginosa.* Enzyme was assayed at varying concentrations of $Na_2S_2O_3$ at 50 mM KCN .

1989; Aminlari et al., 1994). Out of eight bacterial strains isolated from soil of cassava processing site, two were selected based on their appreciable rhodanese production. These were identified based on cultural,

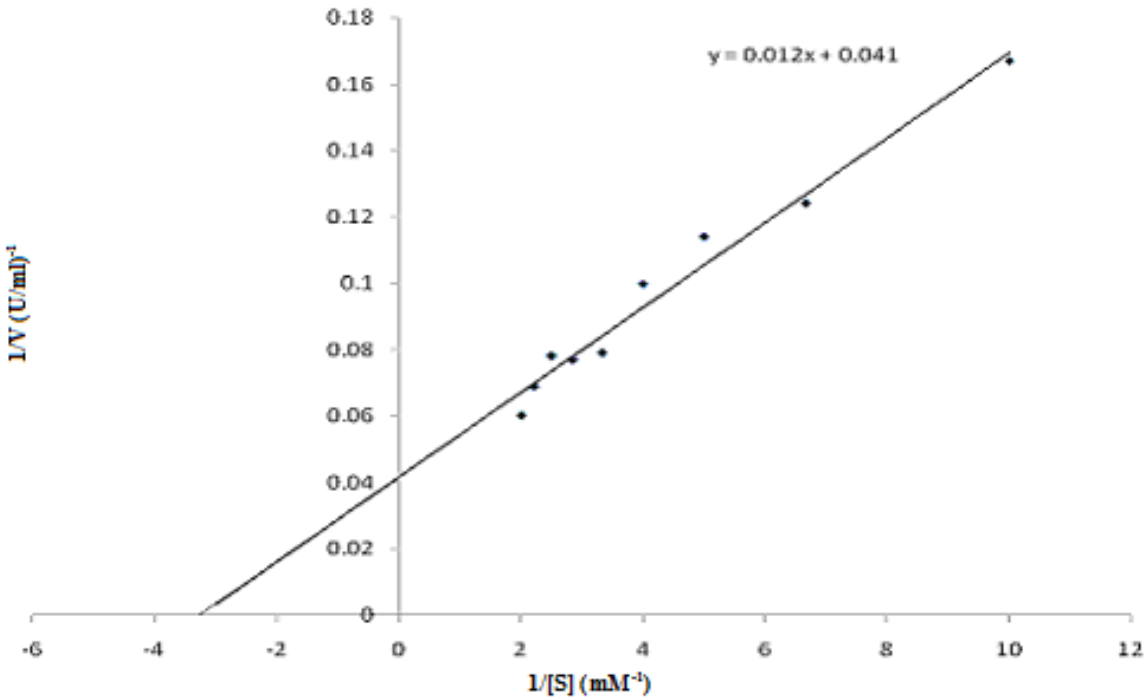

Figure 6a. Lineweaver-Burk plot (1/V versus 1/[S]) for the determination of kinetic parameters of the rhodanese of *B. brevis.* Enzyme was assayed at varying concentrations of KCN at 50 mM $Na_2S_2O_3$.

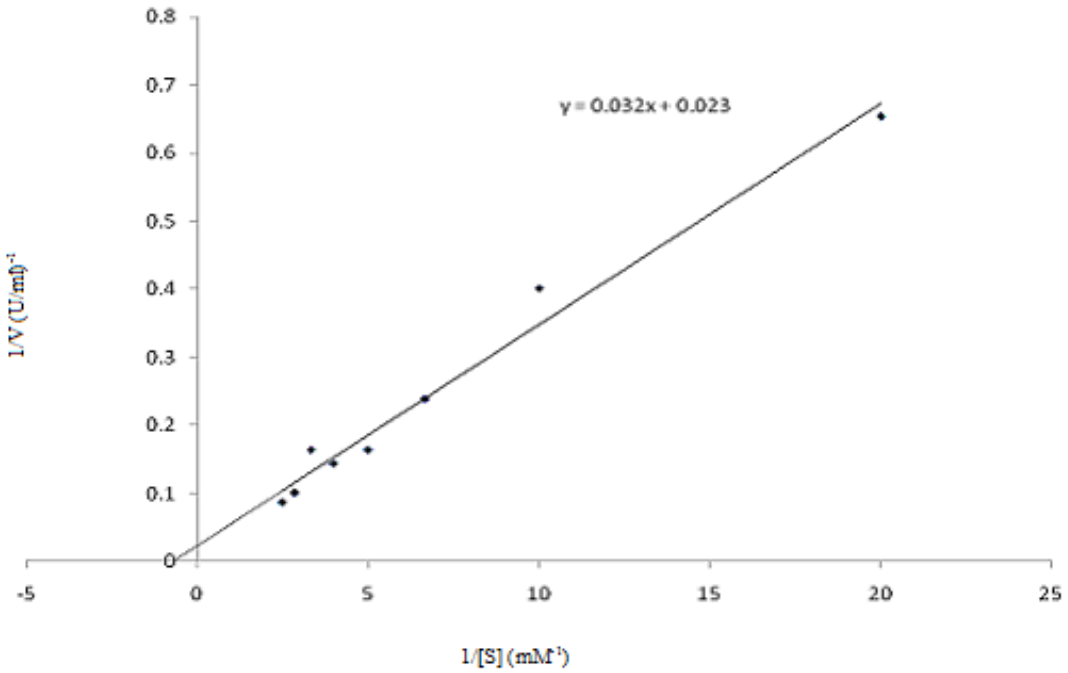

Figure 6b. Lineweaver-Burk plot (1/V versus 1/[S]) for the determination of kinetic parameters of the rhodanese of *B. brevis.* Enzyme was assayed at varying concentrations of $Na_2S_2O_3$ at 50 mM KCN .

biochemical and physiological characteristics to be a strain each of *Pseudomonas aeruginosa* and *Bacillus brevis.* Rhodanese have been identified in a variety of bacterial species such as *Escherichia coli* (Alexander and Volini, 1987; Ray et al., 2000), *Azotobacter vinelandii* (Chiu et al., 2009) and *P. aeruginosa* (Ryan and Tilton,

1977; Cipollone et al., 2004). The two bacterial strains produced a similar pattern of growth and rhodanese activity similar to that reported by Hoster et al. (2001). Rhodanese activity increased rapidly with increase in bacterial cell growth and peaked at the exponential phase followed by a slow decrease in enzyme activity as the cell aged to the stationary phase.

Rhodanese produced by the two bacterial strains were purified and characterized. The optimum temperature and pH for the activity of enzyme from *P. aeruginosa* were 50 and 6.0°C, respectively while the optimum temperature and pH for the activity of enzyme from *B. brevis* were 40 and 7.0°C, respectively. The pH ranges for the activity of *P. aeruginosa* and *B. brevis* rhodanese were 5.5 to 9.5 and 5.5 to 9.0, respectively. These results are in agreement with results reported for rhodaneses from different sources. Sorbo (1953) reported an optimum temperature of 50°C for bovine liver rhodanese, Ezzi et al. (2003) obtained a wide temperature optimum of 35 to 55°C for rhodanese of different strains of *Trichoderma* while Okonji et al. (2011) obtained an optimum temperature of 50°C for mudskipper liver rhodanese. Different optimum pH values in the range of 8.0 to 11.0 have been reported for different organisms (Lee et al., 1995; Saidu, 2004). The values obtained for the rhodanese from the two isolated bacterial strains underscore their versatility in detoxification of cyanide at varying physicochemical conditions.

The apparent Km values, as determined by Lineweaver-Burk plots for KCN and $Na_2S_2O_3$ were 12.5 and 0.066 mM, respectively in the case of rhodanese of *P. aeruginosa* while it were 3.12 and 11.1 mM, respectively in the case of rhodanese from *B. brevis*. According to Ezzi et al. (2003), the Km^{CN} values for rhodanese ranged from 7 to 16 mM between selected strains of *Trichoderma* while Cipollone et al. (2004) reported Km values for cyanide and thiosulphate binding to a strain of *Pseudomonas* rhodanese as 1.0 and 14 mM, respectively. The values obtained for the bacterial enzymes studied show that they have better affinity for these substrates and will catalyse detoxification reaction more efficiently compared to enzymes from other sources. All the metallic ions tested were observed to negatively affect the activity of rhodanese enzyme from both strains of isolated bacteria. The inhibition of bacterial rhodanese by these cations may be a result of interaction of these metal ions with sulphydryl groups at the enzyme catalytic site or induction of changes in the secondary and tertiary structure of enzymes. Such structural changes have been reported after incubation of protein with metal ions (Tayefi-Nasrabadi et al., 2006).

Cyanide concentration in cassava mill wastewater has been reported to contain as high as 200 mg L^{-1} depending on the cyanoglycoside content of the cassava varieties (Siller and Winter, 1998). The resulting wastewater from cassava starch processing is therefore toxic and can pose a serious threat to the environment and aquatic life in the receiving waters. Arimoro et al. (2008) reported the harmful effect of cassava effluent discharges into a stream in the Niger delta area of Nigeria on the benthic macro invertebrate inhabitants of this aquatic ecosystem. Also, Ehiagbonare et al. (2009) reported various undesirable consequences of cassava processing effluent discharges into Okada water which serve as a fishing site and source of water for various communities. Wastewater containing cyanide must therefore be treated before discharging into the environment. Current treatment strategies for cyanide removal employ chemical and physical methods which are often expensive and involve use of additional hazardous reagents (chlorine and sodium hypochlorite or hydrogen peroxide) with attendant high reagent costs associated with the use of these chemical techniques (Chiu et al., 2009).

Enzymatic treatment of cassava effluent before discharges into the environment is recommended in order to preserve the environment and prevent the extinction of sensitive species exposed to the toxicity of untreated effluents from cassava processing. Enzymes may also present advantages over traditional technologies and also over microbial remediation. They are not inhibited by inhibitors of microbial metabolism, can be used at extreme conditions limiting microbial activities and are effective at low pollutant concentrations. Rhodaneses produced by indigenous bacteria of cassava impacted site was characterized in this study and found to possess the ability for efficient detoxification of cyanide to less toxic thiocyanate. Further studies of the biochemical parameters of the enzyme is required to understand the appropriate applications of the enzyme in the bioremediation of cyanide-contaminated sites ultimately leading to improvement of fish and other aquatic organisms in cassava processing effluent-receiving water bodies.

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