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# Table of Content

**Studies on some physicochemical properties of Rhodanese synthesized by  
Bacillus cereus isolated from the effluents of iron and steel smelting industry**  
Babamotemi Oluwasola Itakorode, Raphael Emuebie Okonji, Odunayo Adedeji,  
Nkem Torimiro, Chinwe Onwudiegwu and Ademakinwa Oluwaseyi

*Full Length Research Paper*

# **Studies on some physicochemical properties of Rhodanese synthesized by *Bacillus cereus* isolated from the effluents of iron and steel smelting industry**

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The study purified and investigated the physicochemical properties of rhodanese (a cyanide detoxifying enzyme) synthesized by *Bacillus cereus*. This was with a view to producing an industrially important enzyme. The bacterial strain was identified as *B. cereus* by sequencing of its 16SrRNA gene. *B. cereus* rhodanese was purified with a fold of 3.53, yield of 36.80% and specific activity of 25.30  $\mu\text{mol}/\text{min}/\text{mg}$  protein. The molecular weight determined on SDS-PAGE was 33.800 kDa. The enzyme exhibited maximum activity at 9.0 pH and 50°C. The  $K_m$ s of *B. cereus* rhodanese for sodium thiosulphate and potassium cyanide were  $19.9 \pm 1.05$  and  $31.4 \pm 1.55$  mM respectively, while  $V_{\text{max}}$  were  $6.19 \pm 0.40$  and  $4.83 \pm 0.93$  RU/ml respectively. The substrate specificity study using different sulphur compounds showed that the enzyme prefers sodium thiosulphate. The enzyme showed stability at a temperature range of 40-50°C. At 10 mM concentration, metals such as ( $\text{BaCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{MnCl}_2$ , and  $\text{SnCl}_2$ ) had little influence on the enzyme activity while NaCl and  $\text{HgCl}_2$  inhibited enzyme activity. The presence and biochemical properties of *B. cereus* rhodanese suggest its possible application in cyanide detoxification.

**Key words:** *Bacillus cereus*, isolate, cyanide, purification, rhodanese, kinetics.

## **INTRODUCTION**

Cyanide strong affinity for metal cations makes it a favourable agent in the mining and electroplating industries (Dash et al., 2009). It is highly toxic to living

organisms (Dursun et al., 1999). Cyanide, a potent inhibitor of cytochrome oxidase binds tightly to terminal oxidase (Porter et al., 1983). The release of cyanide to

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the environment can cause rapid breathing, nerve damage and other neurological effects (Department of Interior U.S Cyanide Fact Sheet, 2002). Industrial activities such as metal extraction, metal plating, and synthetic fibres production can increase the concentration of cyanide in the environment (Campos et al., 2006). Free and complex cyanide compounds are found in both environmental matrices and waste streams. The high metabolic inhibition potential of free cyanide classified it as the most toxic form (Knowles and Bunch, 1986). The oxidative product of cyanide such as cyanate (OCN<sup>-</sup>) can be produced during treatment of cyanide-containing effluents. It has been estimated that the release of cyanide from industrial processes is above 14 million kg/year and these effluents contain a high concentration of cyanide (Gurbuz et al., 2009).

Physical and chemical processes have been used in the treatment of effluents. However, the reagents and chemicals used in such treatment process are toxic and the end products of such processes required additional treatment prior to disposal (Behnamfard and Salarirad, 2009). Biodegradation method of cyanides removal is more efficient because it is more economical and has less operative cost (Akcil, 2003; Desai and Ramakrishna, 1998; Young and Jordan, 1995). Microorganisms can degrade cyanide into non-toxic products (thiocyanate). Organisms such as *Bacillus sp.* are resistant to cyanide even at high concentrations (Chen, 2003; Kao et al., 2003; Ebbs, 2004).

Rhodanese is an enzyme reported to be involved in cyanide detoxification in microorganisms (Raybuck, 1992; Colnaghi et al., 1996). Enzymatic bioremediation maybe prefers to the use of microorganism because proteins are not affected by inhibitors of microbial metabolism (Gianfreda and Rao, 2010; Rao et al., 2010). Rhodanese is one of the enzymes reported to be involved in cyanide detoxification (Westley, 1981; Agboola and Okonji, 2004). Proper treatment of cyanide-containing effluent is necessary to protect the environment and water bodies from cyanide toxicity. The presence and need for the cyanide detoxifying enzyme, such as rhodanese can therefore not be overemphasized. This study, therefore, aimed to purify and characterize rhodanese produced by a bacterium isolated from the effluents of Iron and Steel Smelting Company with the view to evaluating its potentials for applications in bioremediation of the cyanide-stressed environment.

## MATERIALS AND METHODS

### Collection of samples

Effluents were gotten from Iron and Steel Smelting Company located at Fashina, Ile-Ife, Osun State, Nigeria (7°29'37"N 4°28'33"E) using a 200 ml sterile bottle by submerging the bottle to a depth of about 20 cm, with the mouth facing slightly upwards below the surface of the water. The samples were labelled, put in ice packs, transported to the Laboratory and analysed within 1 h.

### Isolation and screening for Rhodanese production

One millilitre of the water sample was serially diluted and a loopful of dilution 10<sup>-8</sup> was plated on modified Bushnell Hass agar and incubated inverted at 37°C for 96 h to select for cyanide degrading bacteria. To screen for rhodanese production, the method of Zlosnik and Williams (2004) was used. Briefly, the isolates were grown in 100ml of a growth medium containing 0.3% KCN, 1% bacteriological peptone, 0.5 % NaCl and 0.5 % yeast extract, at pH of 9.5. The growth media prepared were inoculated with 1 ml of 0.5 McFarland standardized cells suspension. After incubation for 48 h at 30°C, the culture media were checked for rhodanese activity.

### Isolates identification and characterization

The different isolates obtained were screened for their cyanide degrading abilities. The most productive strain was selected, identified using Bergey's manual of determinative bacteriology and by sequencing its 16S rRNA gene. The molecular analyses were carried out using molecular techniques and equipment available at the Bioscience Centre of the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. Extraction of bacteria DNA was carried out using modified method of Trindade et al. (2007).

### Polymerase chain reaction (PCR) amplifications

Assay mixture for PCR amplification consists of 4 µl of the DNA solution, 0.4 µl of 10 mM dNTPs, 2 µl of 25 mM MgCl<sub>2</sub>, 1 µl of 10 pmol each of primer (Forward 5'- CCAGCAGCCGCGGTAATACG - 3' and Reverse 5'-ATCGGCTACCTTGTTACGACTTC -3'), 0.24 µl of Taq polymerase (1 U/µl) (Promega USA) and the 5 µl of 5x PCR buffer. Sterile DNase free water was added to make a volume of 25 µl. The PCR amplicons were visualized using 1.5% agarose gel electrophoresis. Sequence similarity search of the GenBank data was done using the National Centre for Biotechnology and Information (NCBI) Basic Alignment Search Tool (BLAST) program.

### Enzyme assay

Rhodanese activity was measured using sodium thiosulphate and potassium cyanide as substrates. The assay mixture consists of borate buffer (pH 9.4, 50 mM), 0.25 M KCN, 0.25 M sodium thiosulphate and 0.1 ml of the enzyme in a final volume of 1 ml. After 1 min of incubation at 37°C, the reaction was terminated by adding 0.5 ml 15% formaldehyde. Concentration of thiocyanate produced was determined by the addition of 1.5 ml Sorbo reagent (which is made up of 10 g Fe (NO<sub>3</sub>)<sub>2</sub> · 9H<sub>2</sub>O, 20 ml HNO<sub>3</sub> and 80 ml distilled water) (Sorbo, 1953b). The absorbance of the reaction medium was taken at 460 nm. The unit of rhodanese activity (RU) is defined as the micromoles of product (thiocyanate) formed in one minute. The protein concentration was determined by the method of Bradford (1976), standard used was bovine serum albumin (BSA).

### Rhodanese purification on CM-Sephadex C-50 and Biogel P-100

To purify rhodanese produced, all the cultures were pooled together at 39<sup>th</sup> h of incubation. Bacterial cells were removed by centrifugation. The filtrate was subjected to 80% ammonium sulphate saturation and left in the refrigerator overnight. 5 ml of the concentrated enzyme was layered on CM-Sephadex c-50 column. Fractions were collected at a flow rate of 36 ml/h. Protein and

rhodanese activity in the fractions were determined. Pooled active fractions were dialyzed against 50% glycerol in 100 mM phosphate buffer pH 6.5. 5 ml of the active fraction was layered on Biogel P-100 column equilibrated with 100 mM phosphate buffer pH 6.5. Fractions of 2 ml were collected from the column at 20 ml/h and monitored for protein and rhodanese activity.

### Molecular weight determination

The molecular weight of native rhodanese was determined on Sephadex G-100 (2.5 x 90 cm) using the marker proteins Lysozyme (14.5kDa),  $\alpha$  Chymotrypsinogen (25 kDa), Ovalbumin (45 kDa) and BSA (66 kDa). The elution volume ( $V_e$ ) of Blue Dextran (2 mg/ml) was used to estimate the void volume ( $V_o$ ) of the column. The elution volume of rhodanese was estimated by layering 10 ml of the enzyme solution on the same column.

### Determination of Subunit Molecular Weight using SDS-PAGE

The method of Weber and Osborn (1975) was used to determine the subunit molecular weight of the purified enzyme on a 10% slab gel apparatus with a notched glass plate. Gels of 1.5 mm thickness were prepared using perplex spacers of the same size.

### Kinetic studies

The purified enzyme kinetics parameters ( $K_m$  and  $V_{max}$ ) were determined using KCN and  $Na_2S_2O_3$  as substrates.  $K_m$  and  $V_{max}$  values for KCN were determined using Lineweaver and Burk plot (1934) obtained by varying the concentration of KCN in the reaction medium between 20 mM and 100 mM at a fixed concentration of  $Na_2S_2O_3$ . Concentration of  $Na_2S_2O_3$  was varied between 20 mM and 100 mM at a fixed concentration of KCN.

### Substrate specificity of the purified enzyme

Different sulphur containing compounds were used as substrates in a typical rhodanese assay mixture. Rhodanese activity was measured as earlier described. The relative activities were compared with sodium thiosulphate which was taken as control.

### Effect of pH and temperature on the purified enzyme

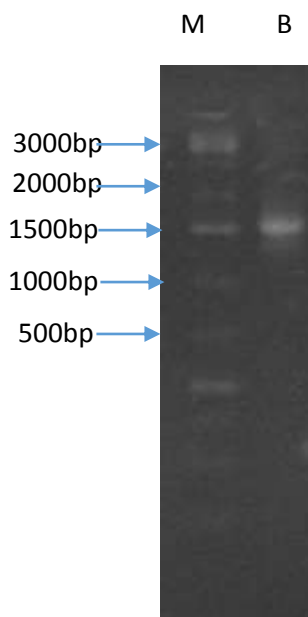
The influence of pH on the enzyme activity was determined by assaying for the enzyme in different buffer solutions. Also, the effect of temperature was checked by varying the temperature of the assay mixture between 30 and 80°C at 10°C interval.

### Heat stability of the purified enzyme

Thermal stability of the enzyme was tested by incubating aliquot of the enzyme at a particular temperature for 60 min, at 10 min interval, the enzyme solution was assayed for residual activity.

### Effect of salts on the purified enzyme activity

The influence of various salts on *B. cereus* rhodanese was investigated. The tested salts were  $BaCl_2$ ,  $CaCl_2$ ,  $HgCl_2$ ,  $MnCl_2$ ,  $SnCl_2$ , KCl and NaCl at 1 mM and 10 mM in assay mixture.



**Figure 1.** Agarose gel showing 16S rRNA amplicon bands of the Isolate. Lane M shows the standard DNA ladder, Lane B shows the 1500 bp of the 16S rRNA amplicon bands of the isolate.

## RESULTS AND DISCUSSION

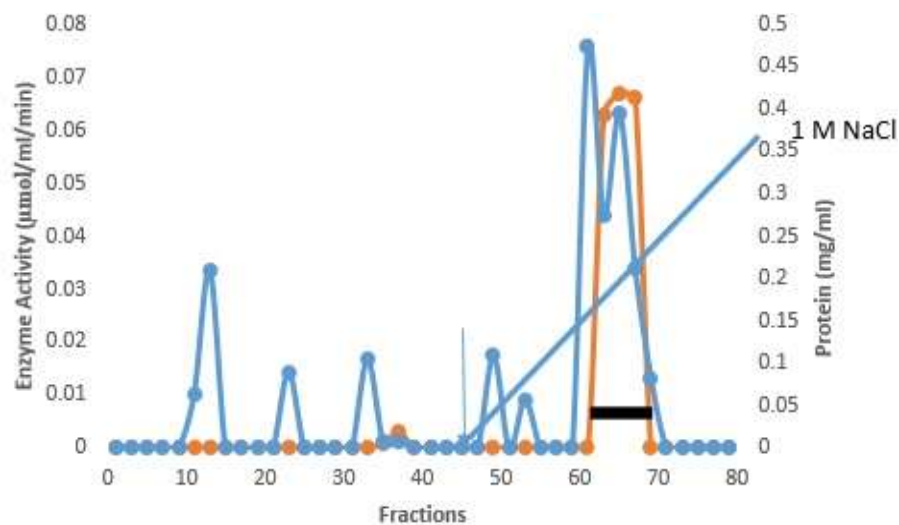
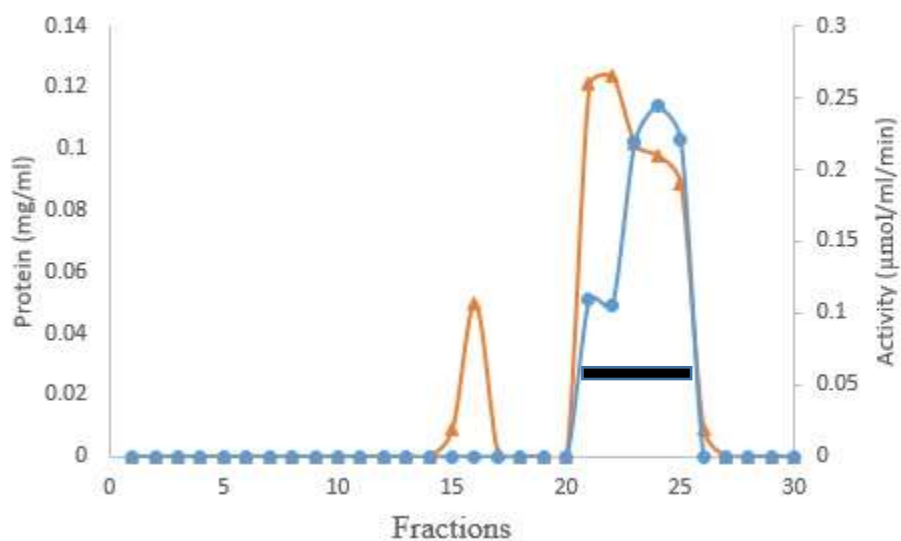
The isolated strain, *B. cereus*, share a maximum of 98% homology with *B. cereus* KX65992 and 97% homology with *B. cereus* KF973315. The agarose gel of 16S rRNA amplicon band of isolate is shown in Figure 1. The presence of rhodanese has been identified in different species of bacteria such as *Escherichia coli* (Ray et al., 2000), *Azotobacter vinelandii* (Kaewkannetra et al., 2009), *Bacillus brivis* (Oyedjeji et al., 2013) and *P. aeruginosa* (Cipollone et al., 2008). *B. cereus* rhodanese was purified with a fold of 3.53, yield of 36.80% and a specific activity of 25.30  $\mu\text{mol}/\text{mg}$  of protein. A summary of purification results is presented in Table 1. The elution profiles for CM Sephadex c-50 ion-exchange chromatography and Biogel P-100 are shown in Figures 2 and 3, respectively. The specific activity of Rhodanese as determined by different researchers varied between 5.21 to 131 RU/mg (Fagbounka et al., 2004; Ehigie et al., 2015).

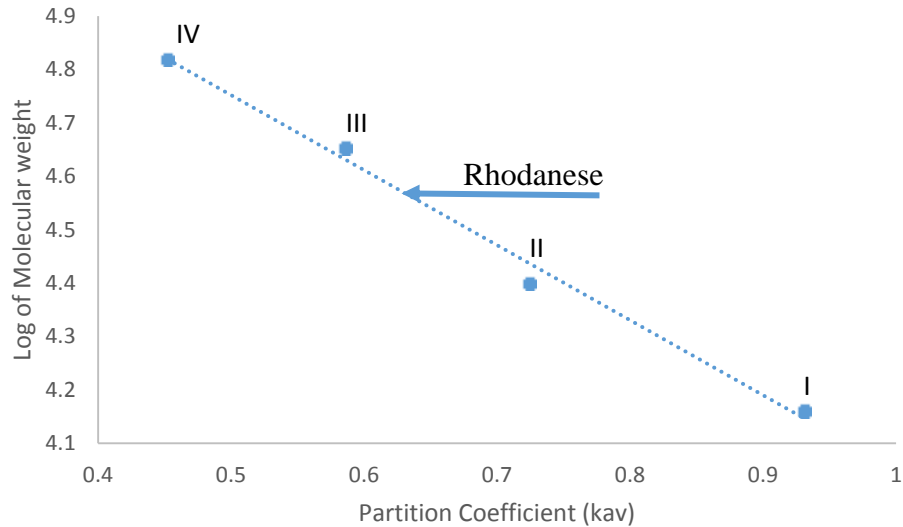
Enzyme activity and protein concentration were determined after each step. Activity was determined using 0.25 mM KCN, 0.25 mM  $Na_2S_2O_3$  and 50 mM Borate buffer pH 9.4. Protein was determined using Bradford's method.

The molecular weight of native and denatured rhodanese were determined using the marker proteins

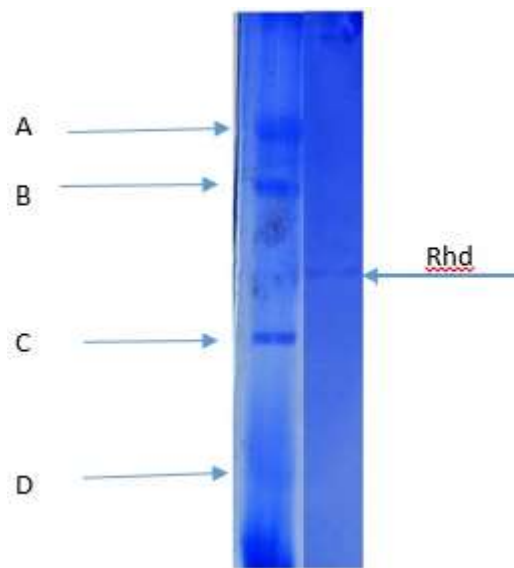
**Table 1.** Summary of Purification Process for *B. cereus* rhodanese.

Purification Steps	Total Protein (mg)	Total Activity (RU)	Specific Activity (RU/mg)	Yield (%)	Purification Fold
Crude Sample	12.845	91.904	7.154	100	1
80% Ammonium precipitation	6.053	52.095	8.74	57.61	1.230
Ion exchange Chromatography	2.271	52.400	23.08	57.00	3.22
Biogel P-100	1.339	33.88	25.30	36.80	3.53

**Figure 2.** Ion-exchange chromatography of rhodanese from *B. cereus* on CM-Sephadex c-50. —○—, Enzyme activity at 460 nm; —●—, Protein concentration at 595 nm; —, pooled fractions**Figure 3.** Gel-filtration chromatography of *B. cereus* rhodanese on Biogel P-100. —●—, Enzyme activity at 460 nm; —◇—, Protein concentration at 595 nm; —, pooled fractions



**Figure 4.** Calibration curve for the determination of native molecular weight of *B. cereus* rhodanese.

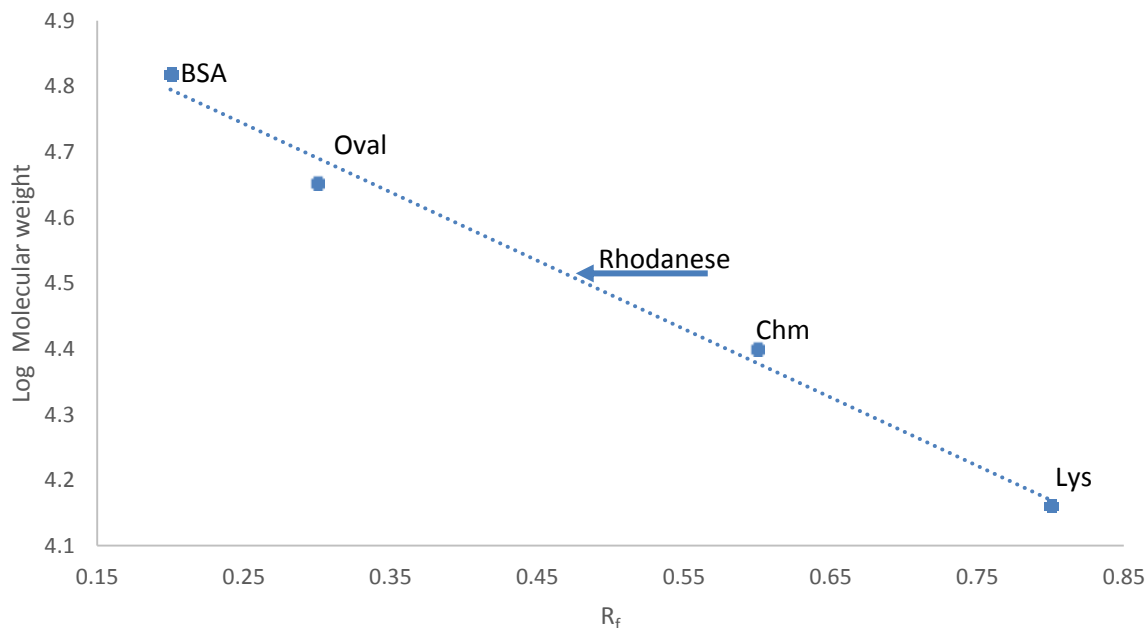


**Figure 5.** SDS-PAGE of purified *B. cereus* rhodanese.

Lysozyme (14.5kDa),  $\alpha$  Chymotrypsinogen (25 kDa), Ovalbumin (45 kDa) and BSA (66 kDa) to be about 34,000 Da by Gel filtration and SDS PAGE, respectively indicating that the enzyme is of monomer in nature. The calibration curve for the determination of the native molecular weight of *B. cereus* rhodanese is shown in Figure 4 while the electrophoresis pictogram and calibration curve obtained for the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) are shown in Figures 5 and 6, respectively. Studies have

shown that rhodanese molecular weight falls between 31 to 37 kDa (Akinkunle et al., 2010; Saidu, 2004; Lee et al., 1995; Okonji et al., 2015). A molecular weight of 34,800 and 35,700 Dalton were reported for rhodanese isolated from mouse and fruit bat (*Eidolon helvum*, Kerr) liver respectively (Lee et al., 1995). Akinkunle et al. (2010) reported a weight of 34.5 and 36.8 kDa for African catfish liver rhodanese.

The molecular weight of rhodanese from *B. cereus* was determined on Sephadex G-100 Column (2.5 × 90 cm).



**Figure 6.** Calibration curve for the determination of subunit molecular weight of *B. cereus* rhodanese.

**Table 2.** Summary of enzyme kinetics of *B. cereus* Rhodanese with KCN and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> Substrates.

Substrate	K <sub>m</sub> (mM)	V <sub>max</sub> (RU/ml)
KCN	31.4 ± 2.08	4.83 ± 0.05
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	19.9 ± 1.05	6.19 ± 0.08

The standard proteins were: I = Lysozyme (14.5kDa), II = α Chymotrypsinogen (25 kDa), III = Ovalbumin (45 kDa) and IV = BSA (66 kDa)

The standard molecular weight markers used included: D = Lysozyme (14.5 kDa), C = α-Chymotrypsin (25.0 kDa), B = Ovalbumin (45.0 kDa), and A = BSA (66.0 kD). Rhd = Purified *B. cereus* rhodanese.

The standard molecular weight markers include: Lysozyme (14.3kDa), α-Chymotrypsin (20.0 kDa), Ovalbumin (45.0 kDa) and BSA (66.0 kDa).

The K<sub>m</sub> values of *B. cereus* rhodanese for both substrates were 31.4 ± 2.08 and 19.9 ± 1.05 mM for KCN and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> respectively. The kinetic data are summarised in Table 2. The K<sub>m</sub> values for *B. cereus* rhodanese falls within the range reported from other sources. K<sub>m</sub> values as determined by different researches were varied between 13.5 to 78 mM (Keith and Volini, 2000; Hossein and Reza, 2011; Agboola and Okonji, 2004). Apparent K<sub>m</sub> values of 36.81 and 19.84 mM were reported for rhodanese in Liver of Rainbow (Hossein and Reza, 2011). *Escherichia coli* rhodanese was reported to have apparent K<sub>m</sub> values of 78 mM and 17 mM for KCN and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> respectively (Keith and Volini, 2000). K<sub>m</sub>

**Table 3.** Relative enzyme activity for different sulphur donor compounds.

Sulphur compounds	% Specificity
Sodium thiosulphate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> )	100
Sodium Sulphite	90
Ammonium persulphate ((NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub> )	65
2-Mercaptoethanol (CH <sub>2</sub> (SH)CH <sub>2</sub> (OH))	53
Sodium metabisulfite (Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub> )	36.8

values obtained in this study indicate that the enzyme has an affinity for thiosulphate and this could clarify how the organism is able to thrive in the cyanogenic environment.

Table 3 summarizes the results of percentage residual activity of the different sulphur containing compounds as substrates. The substrate specificity study showed that rhodanese from *B. cereus* had a preference for thiosulphate for its catalytic activity. *B. cereus* rhodanese was inactivated by 2-mercaptoethanol and sodium metabisulfite. Rhodanese ability to use different sulphur-containing compounds was reported by Okonji et al. (2008). The use of sulphane sulphur in rhodanese cyanide detoxification mechanism was reported by Westley (1981). Chew and Boey (1972) reported the inactivation of rhodanese from tapioca leaf by 2-mercaptoethanol.

Optimum *B. cereus* rhodanese activity was obtained at pH 9.0 and 50°C (Figures 7 and 8). The enzyme retained

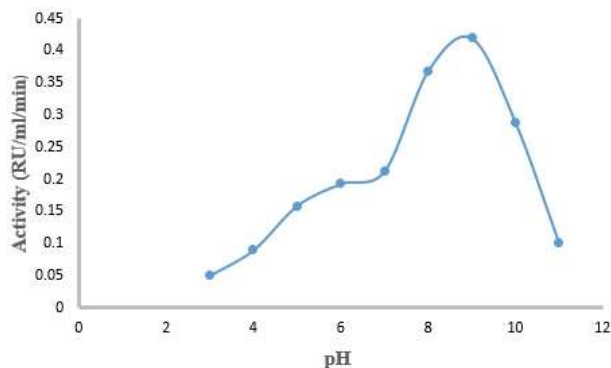


Figure 7. Effect of pH on *B. cereus* rhodanese activity.

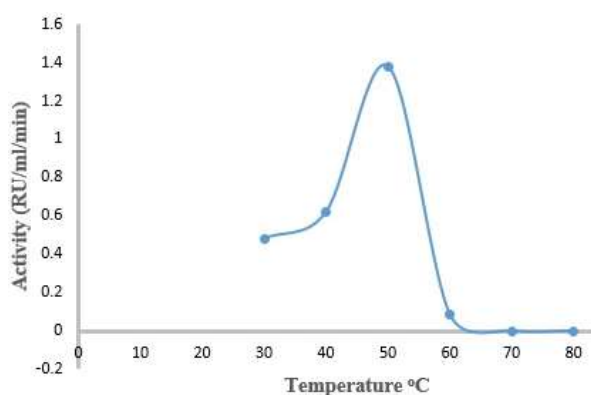


Figure 8. Effect of temperature on *B. cereus* rhodanese activity.

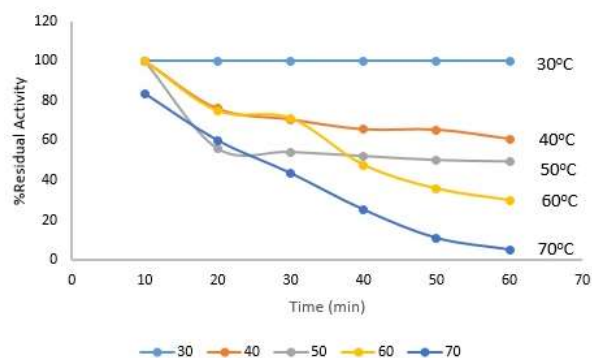


Figure 9. Thermal stability of *B. cereus* rhodanese.

activity up to 50°C for 20 min (Figure 9). The pH value obtained in this study is similar to rhodanese from other sources. pH range of 8.0-11.0 has been reported by different researchers (Lee et al., 1995; Hossein and Reza, 2011). Oyedeji et al. (2013) reported an optimum pH range of 5.5-9.5 for rhodanese from *Pseudomonas*

Table 4. Effect of Salts on *B. cereus* rhodanese activity.

Salt	Enzyme activity (%)	
	1 mM	10 mM
NaCl	58.9 ± 1.08	20.27 ± 0.48
KCl	88.79 ± 2.01	80.44 ± 1.48
HgCl <sub>2</sub>	85.85 ± 0.56	64.52 ± 0.82
CaCl <sub>2</sub>	69.58 ± 0.95	85.75 ± 2.08
BaCl <sub>2</sub>	67.45 ± 1.38	92.73 ± 1.98
SnCl <sub>2</sub>	82.11 ± 2.88	98.32 ± 3.38
MnCl <sub>2</sub>	98.50 ± 2.33	99.72 ± 2.98

The salts were used at different concentrations in the reaction mixture.

*aeruginosa* and *Bacillus brevis* respectively. Optimum temperature of 50 and 40°C were reported for the *P. aeruginosa* and *B. brevis* rhodanese respectively. At 60 and 70°C, the enzyme lost about 50 and 80% of its activity respectively. This value is in agreement with heat stability study of rhodanese from other sources.

Metals salts such as BaCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub>, KCl, and SnCl<sub>2</sub> showed little effect at 1 mM and 10 mM concentration while NaCl and HgCl<sub>2</sub> inhibited the enzyme at 10 mM concentration (Table 4). The observed activity suggests consistent exposure to these metals by the organism. The inhibition of *B. cereus* rhodanese by Hg<sup>2+</sup> and Na<sup>+</sup> was likely because of the reaction of these metal ions with the active site residues of the enzyme which may prompt the change in the functional structure of the enzyme. The influence of metal ions on enzyme structures has been reported (Tayefi-Nasrabadi et al., 2006). Agboola and Okonji (2004) reported the inhibition of rhodanese from fruit bat liver by Hg<sup>2+</sup> ion. Several other researchers have reported the influence of metal ions on rhodanese (Fagbounka et al., 2004).

## Conclusion

Industrial effluents contain a high concentration of cyanide which is toxic to the aquatic life if discharged to the environment without proper treatment. In this study, a cyanide degrading bacterium was isolated from an industrial effluents and subjected to rhodanese production. The biochemical and catalytic properties of rhodanese produced suggests the involvement of the enzyme in cyanide degradation mechanism of the organism, a function that can be employed in the treatment of cyanide-containing effluents.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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