

Isolation and Characterization of Rhodanese from *Bacillus licheniformis* Obtained from Industrial Effluents of Steel Rolling Industry in Ilorin

Adeola Folashade Ehigie¹, Raphael Emuebi Okonji², Wasiu Ayinde Oladiran³ and Ona Leonard Ehigie^{4#}

^{1,3,4}Department of Biochemistry, College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

²Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria

corresponding author.

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ABSTRACT

Pollution from industrial effluents and anthropogenic sources is becoming a very big ecological problem in Nigeria. A typical example of such pollutants is cyanide which can be degraded to a less toxic compound by some microorganism. Some facultative anaerobic bacteria such as *Bacillus licheniformis* utilizes cyanide as a substrate for survival in industrial contaminated and polluted ecosystem. This study screened for bacterial from industrial effluents in Ilorin that can synthesize rhodanese, an enzyme that can degrade cyanide to less toxic substance.

Bacillus licheniformis was isolated and biochemically characterized. Rhodanese was partially purified using 85% ammonium sulphate precipitation, ion exchange chromatography on Sephadex CM-50. The molecular weight of the enzyme was determined on a gel filtration column (Sephadex G-100) and finally on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The kinetic parameters (K_m and V_{max}) were determined. Statistical analysis was performed using one-way analysis of variance and results were considered significant at $p < 0.05$.

The result of this study showed that the optimum growth temperature for *B. licheniformis* was 35°C at a pH 9.0 while the highest duration time for the synthesis of rhodanese was 39 hours. Potassium cyanide (KCN) and casein were the best carbon and nitrogen sources. The *B. licheniformis* isolate synthesizes rhodanese that had a specific activity of 13.63 RU/mg, with a purification fold of 5.19, a percentage yield of 13.96% and an estimated native molecular weight of 34.44 kDa. The apparent K_m for KCN and Sodium thiosulphate ($Na_2S_2O_3$) were determined to be 32.9 mM and 21.0 mM respectively while their V_{max} were 5.6 RU /ml/min and 6.4 RU /ml/min respectively. The optimum pH and temperature of partially purified enzyme were 8.0 and 50°C respectively. The enzyme showed high stability at 50°C for 30 minutes. The enzyme showed specificity at 6.78 RU/ml/min and equally showed specificity for $Na_2S_2O_3$ while it was inhibited by other sulphur containing compounds such as 2-mercaptoethanol (2-MCPE), ammonium persulphate ($(NH_4)_2S_2O_8$), and sodium metabisulphite ($Na_2S_2O_5$). The enzyme activity was not inhibited by metal ions such as (K^+ , Mg^{2+} , Ba^{2+} , Ni^{2+} , Sn^{2+} and Na^+) at 1 mM and 10 mM and this was not significant ($p > 0.05$).

The high level of expression of rhodanese in *B. licheniformis* suggests that the enzyme possess functional cyanide detoxification mechanism, a bioremediation process for the survival of both plants and animals in the environment. Microbial enhancement of this kind is recommended to help in reduction of toxic materials from industrial effluents.

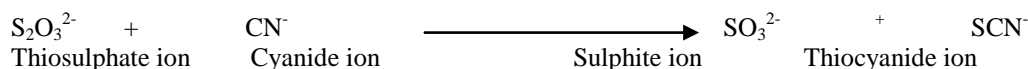
INTRODUCTION

Cyanide is commonly found as a contaminant in wastewater through release from metal finishing, electroplating and coal cooking which are the crucial factors that contribute to the bulk occurrence of cyanide in the environment (Dash *et al.*, 2009). In its gaseous form, it associates with a hydrogen ion to form hydrogen cyanide, an extremely toxic gas that smells slightly like almonds. Cyanide, in its solid form, is combined with various metal ions such as potassium and sodium to form white crystalline substances, which are stable. It was hypothesized that cyanide was the first organic compound on earth, from which chemical compounds are formed (Oparin, 1957; Rawls, 1997; Parmar *et al.*, 2012).

Cyanide is a natural compound produced by many organisms, including bacteria, algae, fungi, and plants, and is bio-produced in some cases as a defensive metabolite (Dubey and Holmes, 1995) or with invasive purposes (Pessi and Haas, 2004). Plants are the main source of cyanide in the biosphere because they co-generate cyanide with ethylene (Zagrobelyny *et al.*, 2008) in addition to generating cyanoglycosides and cyanolipids. Many microorganisms can use potassium or sodium cyanide as a sole source of carbon and nitrogen.

Rhodanese (thiosulphate cyanide sulphurtransferase: EC 2.8.1.1) is an ubiquitous enzyme that is *in vitro* catalyses the transfer of a sulphur atom from suitable donors to nucleophilic acceptors by way of a double displacement mechanism. During the catalytic process the enzyme cycles between a sulphur-free and a persulfide-containing form, via formation of a persulfide linkage to a catalytic cysteine residue (Domenico *et al.*, 2000). Several biological functions have been attributed to this ubiquitous enzyme which include cyanide detoxification

(Agboola and Okonji, 2004), construction of iron-sulphur centres in proteins (Ugulava *et al.*, 2000); selenium metabolism (Ray *et al.*, 2000); Thioredoxin metabolism (Nandi *et al.*, 2000); and also in sulphur supplier for key enzymes in sulphur energy metabolism (Aussignargues *et al.*, 2012).



MATERIALS AND METHODS

Potassium cyanide, sodium thiosulphate, peptone, yeast extract, boric acid, sodium borate, formaldehyde, ferric nitrite, nitric acid, citric acid, sodium citrate, ammonium sulphate (enzyme grade), potassium chloride and sodium chloride were obtained from BDH Chemical Limited, Poole, England. Glycerol, Coomassie Brilliant-Blue, Blue Dextran and Bovine Serum Albumin (BSA) were obtained from Sigma Chemical Company, St. Louis, Mo., USA. Sephadex CM-50 was obtained from Pharmacia Fine Chemical, Uppsala, Sweden. Disodium hydrogen phosphate and monosodium dihydrogen phosphate were products of Kermel Reagent Company Limited, Tianjin, China. and ammonium persulphate were products of Feinbiochemica, Heidelberg, Germany. Biogel P-100 was purchased from Bio-Rad Laboratories Inc., Benicia Ca., USA. Other chemicals, solvents and media ingredients used for the experimental purpose were of analytical grade and were procured from reputed chemical firms.

Sample Collection

Three (3) different 200ml sterile bottles were used in collection of the effluents samples from three (3) different sections of the steel rolling industry. The samples were taken straight to the laboratory for microbiological analysis.

Enzyme and protein assays

Rhodanese activity was measured according to the method of Lee *et al.*, (1995), as described by Agboola and Okonji (2004), on the principles of the colorimetric determination of thiocyanate formation. 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 Na₂S₂O₃, and 0.1 ml of the enzyme solution in a total volume of 1.0 ml. The mixture was incubated for 1 minute at room 37°C and the reaction was stopped by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5ml of Sorbo reagent. The absorbance was taken at 460nm. One Rhodanese Unit (RU) was defined as the amount of the enzyme that will convert one micro-mole (1µmol) of cyanide to thiocyanide in one minute at 37°C (Sorbo, 1951). The controls contained the reaction mixture as described above but does not have any enzyme. The enzyme was replaced with 0.1ml distilled water, the mixture was incubated for 1 minute at 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. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Enzyme isolation and purification

A millilitre of the water sample was taken from each of the sample bottle into a test tube containing 9ml of sterile distilled water. Serial dilution was carried out to thin out the load of microorganisms to be plated. Serial dilution up to 10⁻⁹ was carried out and dilution 7, 8 and 9 were pour plated. The plating was done by taking one millilitre of each of the dilution into separate petri dishes under aseptic condition and cooled but molten sterile nutrient agar was poured into each petri dishes, swirled to ensure proper mixing and distribution of the sample and the media. The media in the petri dishes were left to cool and set. The plates were incubated in an inverted position at 37 °C for 48 hours. All plates were labeled appropriately. After 48 hours the plates were observed and the ones with the most distinct colonies were aseptically picked using a sterile inoculating loop and then streaked on a different sterile petri dishes containing solid sterile nutrient agar. The streaking was done near a flame to prevent contamination of the sterile solidified nutrient agar plates. After streaking, the plates were covered, inverted, labeled and incubated at 37 °C for 24 hours. This process was repeated until pure colonies of each isolates were obtained.

The different isolates obtained were transferred into nutrient agar slants and then incubated for 24 hours to ensure growth of the organism. After 24 hours it was kept in a refrigerator to arrest growth and store the isolates.

Ammonium Sulphate Precipitation

The supernatant obtained from the centrifugation of the crude homogenate was brought to 85% ammonium sulphate saturation i.e 25.5 g of ammonium sulphate (analytical grade) was added and stirred slowly to 50 ml of crude enzyme. This was done for 1hour with occasional stirring until all the salt had dissolved completely in the supernatant. The mixture was left overnight in the fridge, followed by centrifugation at 12,000 rpm for 30 min at 10°C. The supernatant was discarded and precipitate was collected and suspended in 0.1 M Phosphate buffer pH 6.5. It was evaluated for rhodanese activity as described in section 3.11, the protein content was determined using the method of Bradford 1976.

Ion-Exchange Chromatography on CM-Sephadex C-50

CM-Sephadex C-50 cation exchanger was pre-treated by boiling twenty grams (30 g) of the resin in 300 ml distilled water for 1hr. This was followed by the addition of 100 ml 0.1 M HCl for 30 mins, after which the acid was decanted and the resin was washed with distilled water several times to ensure the total removal of the acid. Thereafter 100 ml of 0.1 M NaOH was added to the resin, which was decanted after 30 mins, followed by the thorough rinsing of the resin with distilled water to remove all traces of the base. The resin was then equilibrated with 0.1 M phosphate buffer (pH 6.5) before it was packed into a 2.5 x 40 cm column. Five millilitres (10 ml) of the enzyme solution from the preceding step was then applied on the column. The column was washed with 100 ml of 0.5M NaCl to remove unbound protein, followed by elution with 150 ml of 1.0 M NaCl in the same buffer. Fractions of 5 ml were collected from the column at a rate of 20 ml per hr. The fractions were assayed for rhodanese activity as described in section 3.11. The active fractions from the column were pooled and dialyzed against 50% glycerol in 0.1M phosphate buffer, pH 6.5. The yield and fold of purifications were calculated.

Gel Filtration Chromatography on Sephadex G-100

Gel filtration chromatography separate proteins and other molecules based on their molecular sizes. The Sephadex G-100 resin was swollen by dissolved 20g of the matrix in 200 ml of distilled water and boiled for 1 hour at 100 °C as described by Okonji *et al.* (2010a). The swollen gel was left overnight at 4 °C. The gel was then loaded and equilibrated on a 2.5 cm x 90 cm column. 7 ml of the dialysate from the previous step was loaded on the column and 2 ml of fractions were collected at 20 ml/hour. The rhodanese activity and protein concentration were monitored and the active fractions were pooled

Determination of Native Molecular Weight

The native molecular weight was determined on a Sephadex G-100 column (2.5 x 90cm). The standard proteins used to calibrate the column were bovine serum albumin (M_r 66000 Dalton; 10 mg/ml), ovalbumin (M_r 45000 Dalton; 10 mg/ml) and α -chymotrypsinogen (M_r 25,000 Dalton; 10mg/ml). The protein standards were prepared in 0.1M Phosphate buffer pH 6.5. Total sample volume of each of the protein markers applied to the column was 10ml. The proteins were eluted with phosphate buffer pH 6.5. Fractions of 5ml were collected and monitored by measuring absorbance at 280nm for the protein. The void volume (V_o) of the column was determined by the elution volume (V_e) of Blue dextran (5 mg/ml). A 10ml aliquot of the enzyme solution was then applied to the same column and the elution volume of the rhodanese was determined. The partition coefficient (K_{av}) of each of the standard proteins and the purified rhodanese was calculated. A plot of logarithm of the molecular weight of the marker proteins against K_{av} (partition coefficient) was made. The molecular weight of the enzyme was then extrapolated from the curve.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where;

V_e = elution volume
 V_o = void volume
 V_t = total volume

Determination of Subunit Molecular Weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Weber and Osborn (1975) to determine the subunit molecular weight of the enzyme on a 10% slab gel apparatus with a notched glass plate. Gels of 1.5 mm thickness were prepared by using perplex spacers of same size. Standard proteins were as contained in Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis. The standard molecular weight markers includes Bovine serum albumin (66.0 kDa), Ovalbumin (45.0 kDa), Chymotrypsin (25 kDa) and Lysozyme (14.3 kDa).

Determination of kinetic parameters

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentrations of 0.25M KCN between 0.005M and 0.05M at fixed concentration of 0.25M $Na_2S_2O_3$. Also, the concentration of 0.25M $Na_2S_2O_3$ was varied between 0.005M and 0.05M at fixed concentration of 0.25M 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 according to Lineweaver and Burk (1934).

Effect of pH on the Activity of Partially Purified Rhodanese

The effect of pH on the enzyme activity was performed according to the methods of Agboola and Okonji (2004). The enzyme was assayed using 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (6-8) and 50mM borate buffer (pH 3-11). The different buffers of different pH were substituted with the reaction buffer of the rhodanese assay.

Effect of Temperature on the Activity of Partially Purified Rhodanese

The enzyme was assayed at temperatures between 30°C and 80°C at an interval of 10°C to investigate the effect of temperature on the activity of the enzyme and to determine the optimum temperature of the enzyme. 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 control test contains distilled water instead of enzyme.

Effect of Heat at Different Temperatures on Partially Purified Rhodanese Activity

The heat stability of the enzyme was determined by incubating 2ml of the enzyme for 1h at 30°C, 40°C, 50°C and 60°C respectively. From the incubated solution, 0.1ml was withdrawn at 10min interval and assayed for residual activity. The activity at 40°C, 50°C and 60°C was expressed as a percentage of the activity of the enzyme incubated at 30°C which was the control. The residual activity of the enzyme was determined. The residual rhodanese activity was plotted against the different temperatures.

Effect of Metal Ions on Partially Purified Rhodanese Activity

The method of Lee *et al.*, (1995) was used to study the effect of various metal ions (cations) on the activity of partially purified rhodanese. The salts of the cations include $MgCl_2$, $CaCl_2$, $HgCl_2$, $NaCl$, $BaCl_2$ and KCl and at concentrations of 1.0 mM and 10 mM. A typical enzyme assay with 1ml of reaction mixture contained 0.5 ml 50 mM borate buffer pH 9.4, 0.2 ml of 250 mM KCN, 0.2 ml of 250 mM $Na_2S_2O_3$, 0.05ml of the respective salt solution. The substrate was mixed with the 0.05 ml of each metal concentration, followed by the addition of 0.05 ml enzyme. The mixture was incubated for one minute at room temperature, followed by the addition of 0.5 ml 15% formaldehyde and 1.5ml sorbo reagent. The control of each metal concentration did not contain metal.

Substrate specificity and kinetics

The substrate specificity of the enzyme will be determined by using different sulphur containing 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 as described in section 3.11. The activity will be expressed as a percentage activity of the enzyme using sodium thiosulphate as the control.

Results

Isolation of Bacteria and Screening of Isolates For Rhodanese Production

Ten bacteria were obtained from the water samples obtained from the steel rolling industry. The isolates were screened for their ability to produce rhodanese using the basal medium as described in section 3.8, the isolate with maximum rhodanese activity was selected for further studies. The isolate with the best activity was identified using microbiology procedure. Figure 1 shows the screening of the different isolates.

Identification of Rhodanese Producing Bacteria

The morphological characteristics of the isolate observed on nutrient agar plate after 24hr incubation at 35°C is presented in Table 1. Also, the Gram's reaction, spore staining test and other biochemical characteristics of the selected isolate was presented in Table 2 and the presumptive identity was based on comparison with Bergey's Manual of Determinative bacteriology (Buchanan and Gibbon, 1974) to be *Bacillus licheniformis*. The isolate appeared as flat, translucent colonies on agar plates. When Gram stained film was examined, the isolate appeared as gram positive rods.

Table 1. Morphological Characteristics of Isolates

Isolate	A5
Shape	Rod
Surface	Glistening
Opacity	Opaque
Elevation	Flat
Colour	Cream

Table 2. Gram's Staining, Spore staining, and Biochemical Characteristics of the Isolate

Isolate code	A5
Gram's Reaction	Positive
Shape	Rod
Catalase	Positive
Starch Hydrolysis	Positive
Spore Staining	Positive
NO ₃ ⁻ Reduction	Negative
Citrate	Negative
6.5% NaCl	Positive
MR/VP	Positive/Positive
Glucose	Positive
Size	0.6-1.0nm in length
Indole	Negative
Motility	Positive
Sulphur	Negative
Presumptive Identity	<i>Bacillus sp.</i>

Growth and Rhodanese Production

The result in Figure 1 showed the screening of the different bacterial isolates. The result as presented in figure 2 showed the time course of rhodanese production by *Bacillus licheniformis*. Maximum production was obtained at 39th hour with rhodanese activity of 3.09 RU/ml/min.

Effect of Carbon Sources on Production of Rhodanese by *Bacillus licheniformis*

The production of rhodanese was discovered to be highest with KCN as the sole carbon source with enzyme activity of 3.142 RU/ml/min as presented in figure 4 while maltose gave the least value of 0.618RU/ml/min.

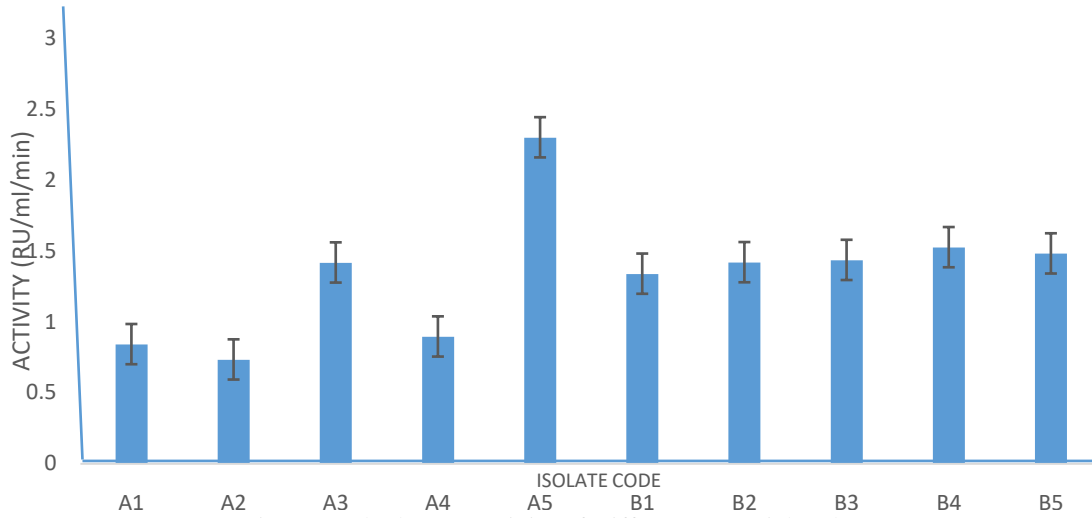


Figure 1. Rhodanese Activity of Different Bacterial Isolates

ISOLATE CODE

- A1 = *C. kutseri*
- A2 = *B. cereus*
- A3 = *B. macerans*
- A4 = *B. subtilis*
- A5 = *B. licheniformis*
- B1 = *B. macquariensis*
- B2 – B5 = *Aeromonas* spp

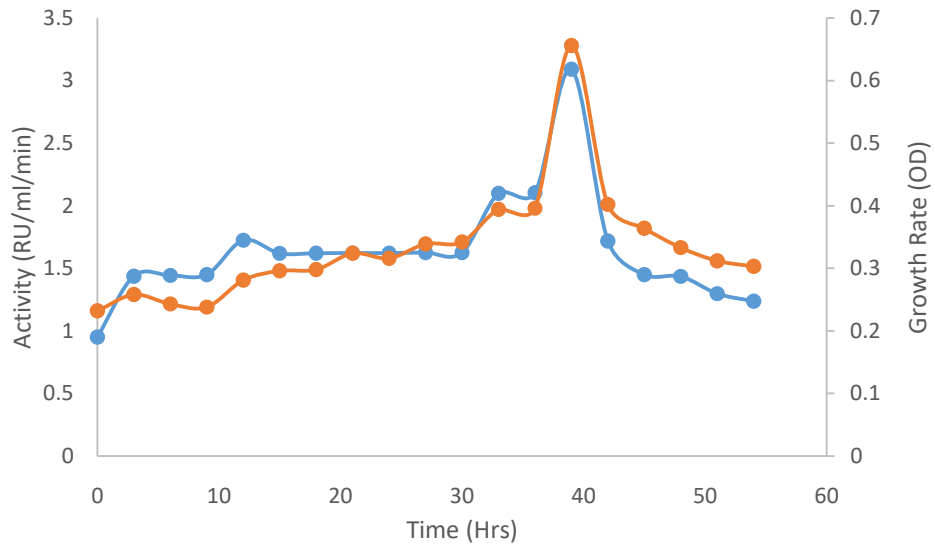
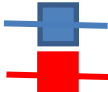


Figure 2. Incubation Time on Rhodanese Production and Cell Growth


Growth Rate (OD)
Rhodanese Activity

Different Concentrations of Potassium Cyanide on Rhodanese Production by *Bacillus licheniformis*

The effect of different concentrations of KCN showed that rhodanese production was highest at KCN concentration of 0.8g (w/v) and least with KCN concentration of 0.1g (w/v) with rhodanese activity of 3.344 RU/ml/min and 3.232 RU/ml/min respectively as shown in Figure 5.

Influence of Different Nitrogen Sources on Rhodanese Production by *Bacillus licheniformis*

The effect of different sources of nitrogen was studied by varying only the nitrogenous components of the media. Casein gave the highest activity with rhodanese activity of 3.674 RU/ml/min as shown in Figure 6 and 7 respectively.

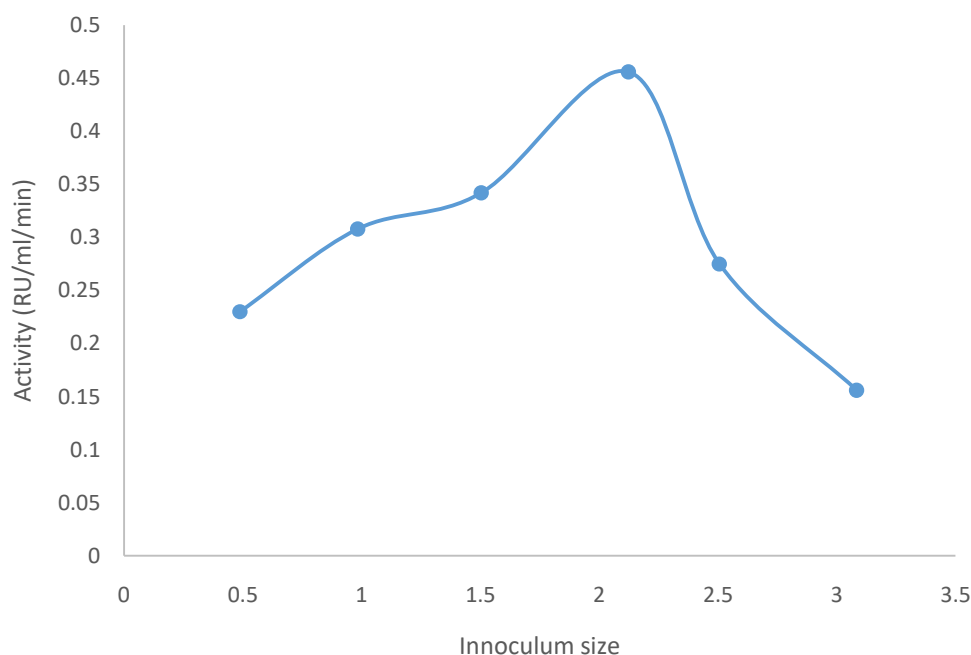


Figure 3. Inoculum size per 100ml of Basal Medium on Rhodanese Production by *Bacillus licheniformis*

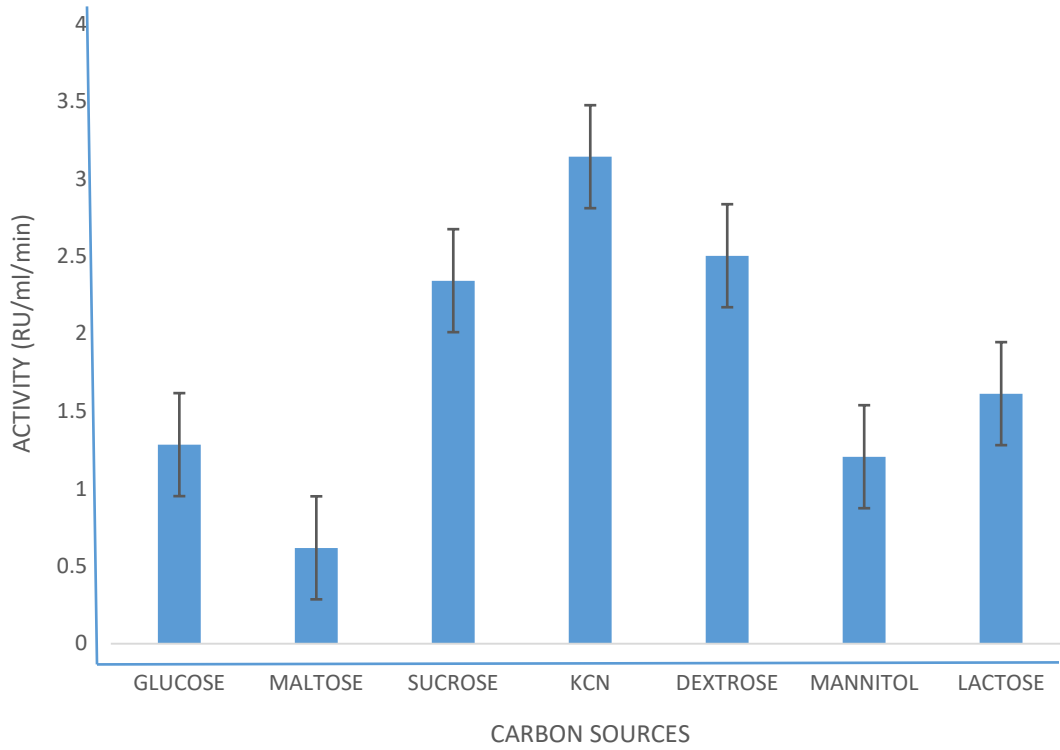


Figure 4. Effect of Different Carbon Sources on Rhodanese Production by *Bacillus licheniformis*

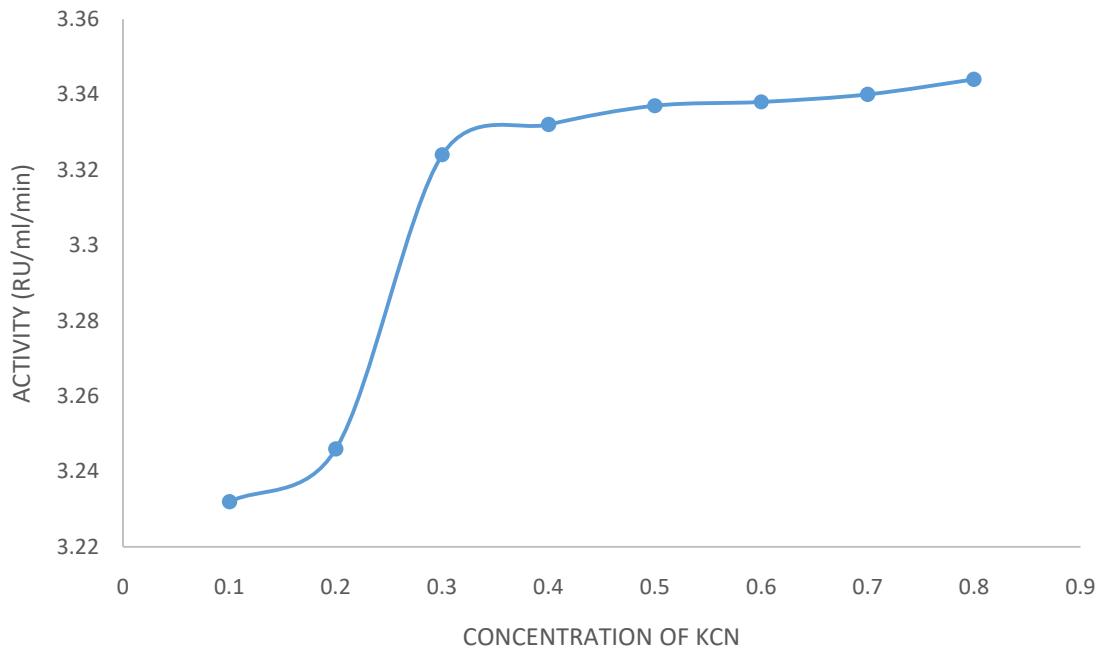


Figure 5. Effect of Different Concentrations of KCN on Rhodanese Production by *Bacillus licheniformis*

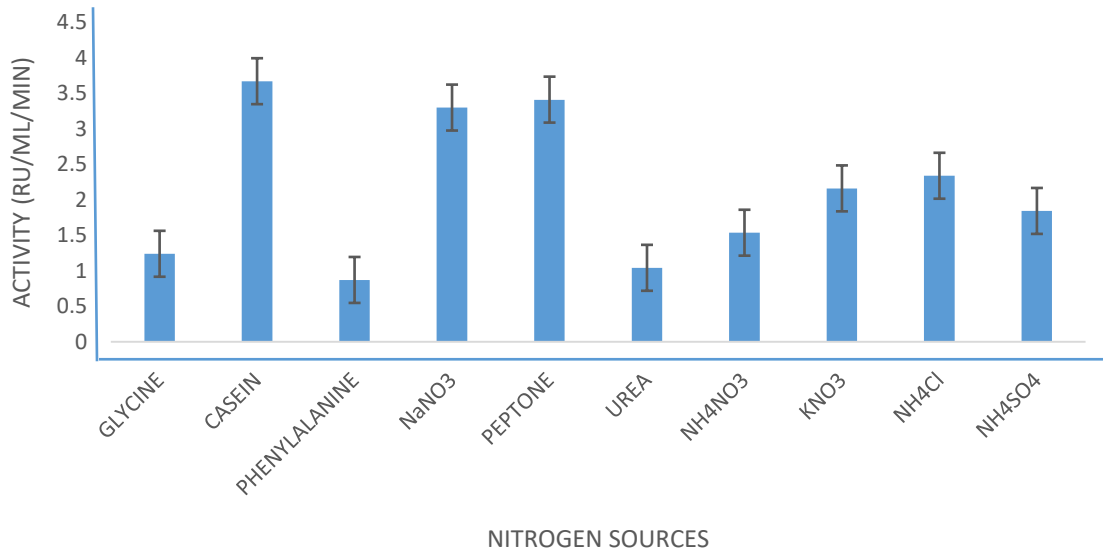


Figure 6. Different Nitrogen Sources on Rhodanese Production by *Bacillus licheniformis*

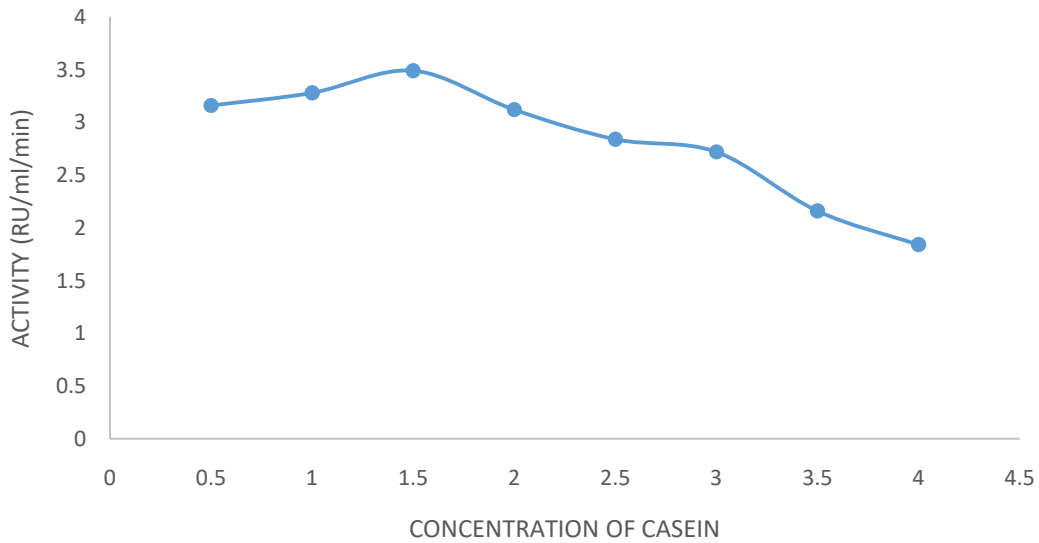


Figure 7. Effect of Different Concentrations of casein on Rhodanese Production by *Bacillus licheniformis*

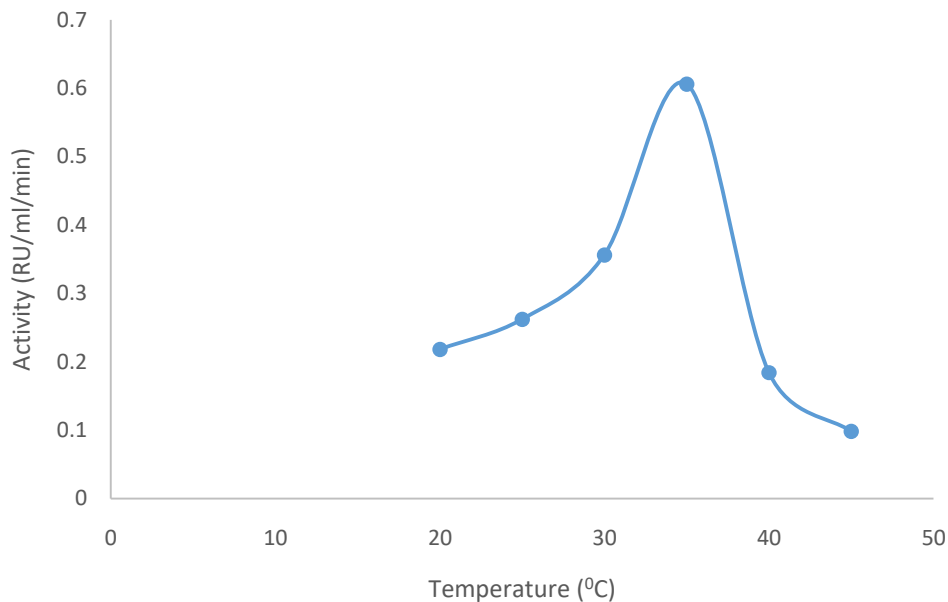


Figure 8. Effect of temperature on rhodanese production by *Bacillus licheniformis*

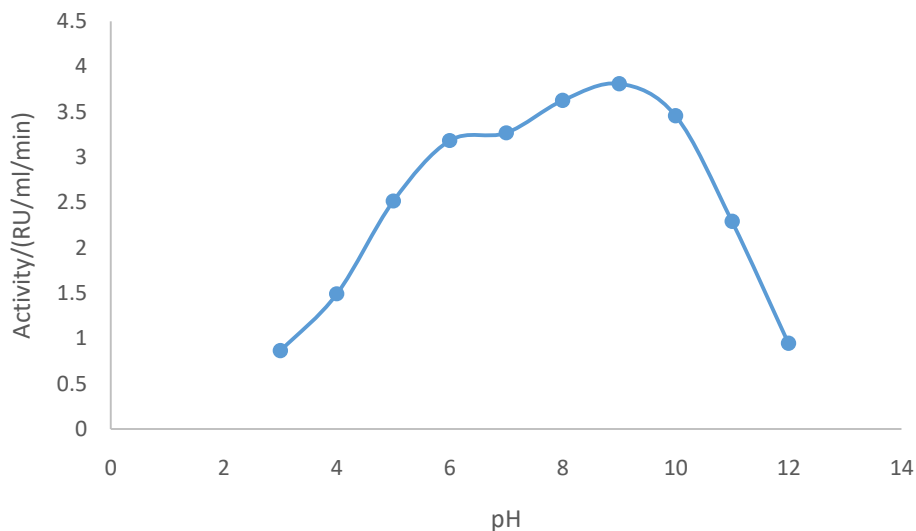


Figure 9. Effect of pH on rhodanese production by *B.licheniformis*

Summary of Partial Purification of Rhodanese Obtained From *Bacillus licheniformis*

The partial purification of rhodanese was carried out using ammonium sulphate precipitation, ion exchange chromatography and gel filtration. Results for the purification procedures are summarized in Table 3. The procedures resulted in 13.63 RU/mg specific activity with a purification fold 5.19 and a percentage yield of 13.69%.

The protein profile of partially purified rhodanese from *Bacillus licheniformis* by ion exchange is shown in Figure 10. The eluted protein was concentrated using concentrative dialysis in 50% glycerol and used for subsequent molecular weight determination on Sephadex G-100 gel filtration chromatography.

Table 3. Summary of Partial Purification Process of Crude Rhodanese Obtained From *Bacillus licheniformis*

Purification steps	Volume (ml)	Protein (mg/ml)	Activity (RU)	Total protein	Total activity	Specific activity	% yield	Purification fold
Crude enzyme	50.00	1.11	2.93	55.70	146.28	2.63	100.0	1.00
85% ammonium sulphate precipitation	20.00	1.01	2.96	20.25	59.08	2.92	40.39	1.11
CM sephadex C-50 iron exchange chromatography	10.00	0.04	0.48	0.39	4.77	12.23	3.26	4.66
Sephadex G-100 gel filtration	7.00	0.21	2.92	1.50	20.42	13.63	13.96	5.19

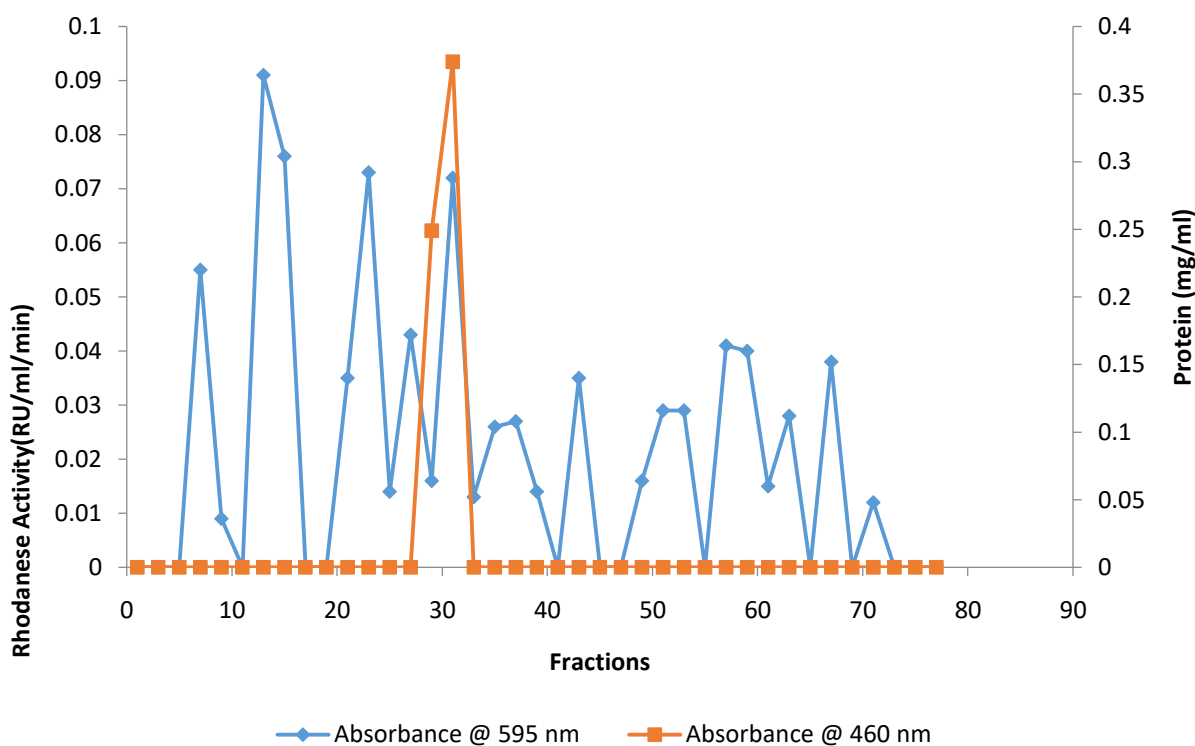
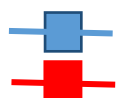


Figure 10. Separation by Ion-Exchange Chromatography on CM-Sephadex C-50 of Partially Purified Rhodanese from *Bacillus licheniformis*



Protein

Rhodanese Activity

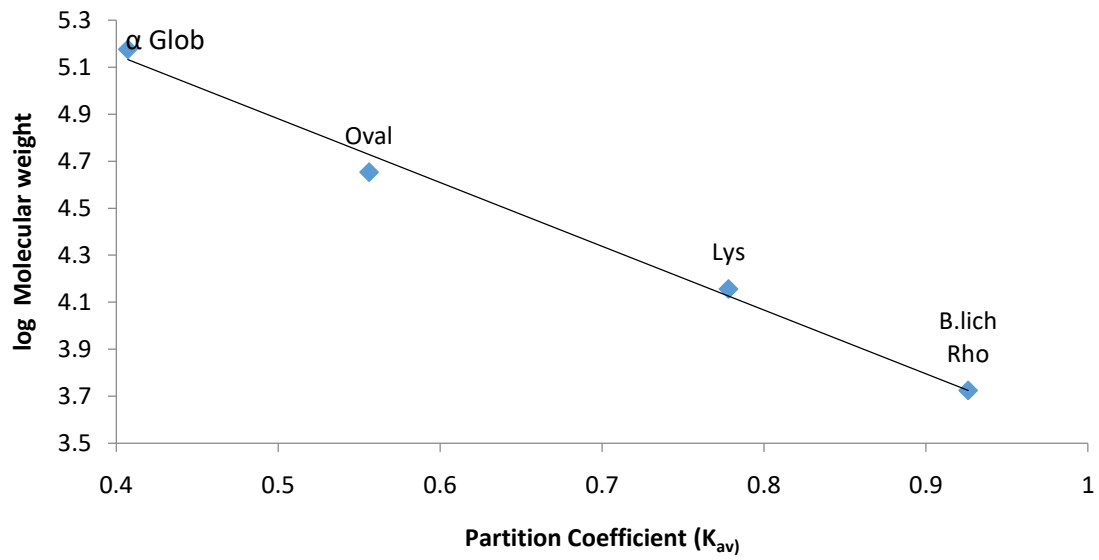


Figure 11a. Calibration Curve for the Determination of Native Molecular Weight of Partially Purified Rhodanese from *Bacillus licheniformis*

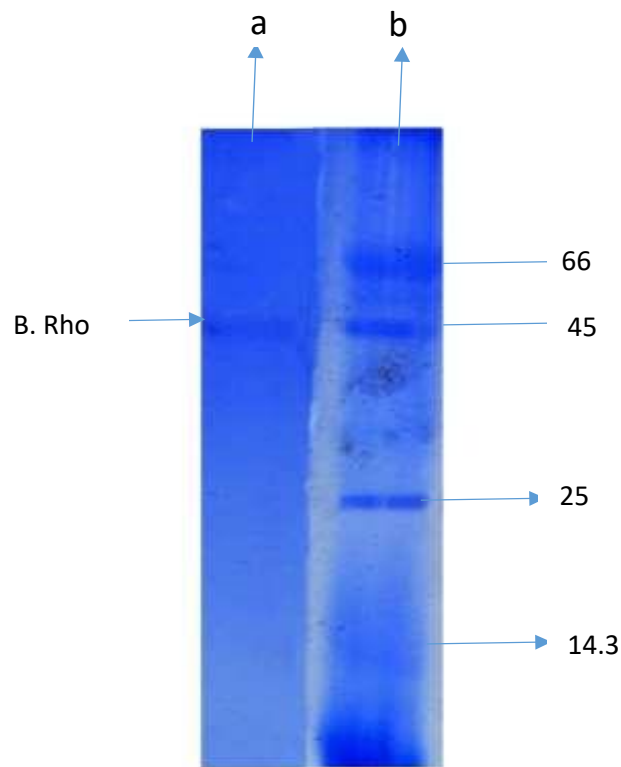


Figure 11b, SDS-PAGE for the Determination of Subunit Molecular Weight of Rhodanese from *Bacillus licheniformis*. Lane A = Purified rhodanese from the Microorganism *Bacillus licheniformis*. Lane B = Standard Proteins; The standard molecular weight markers include: i = BSA (66.0 kDa), ii = Ovalbumin (45.0 kDa), iii = Chymotrypsin (25 kDa), and iv = Lysozyme (14.3 kDa). B. RHO= *Bacillus licheniformis*. Rhodanese

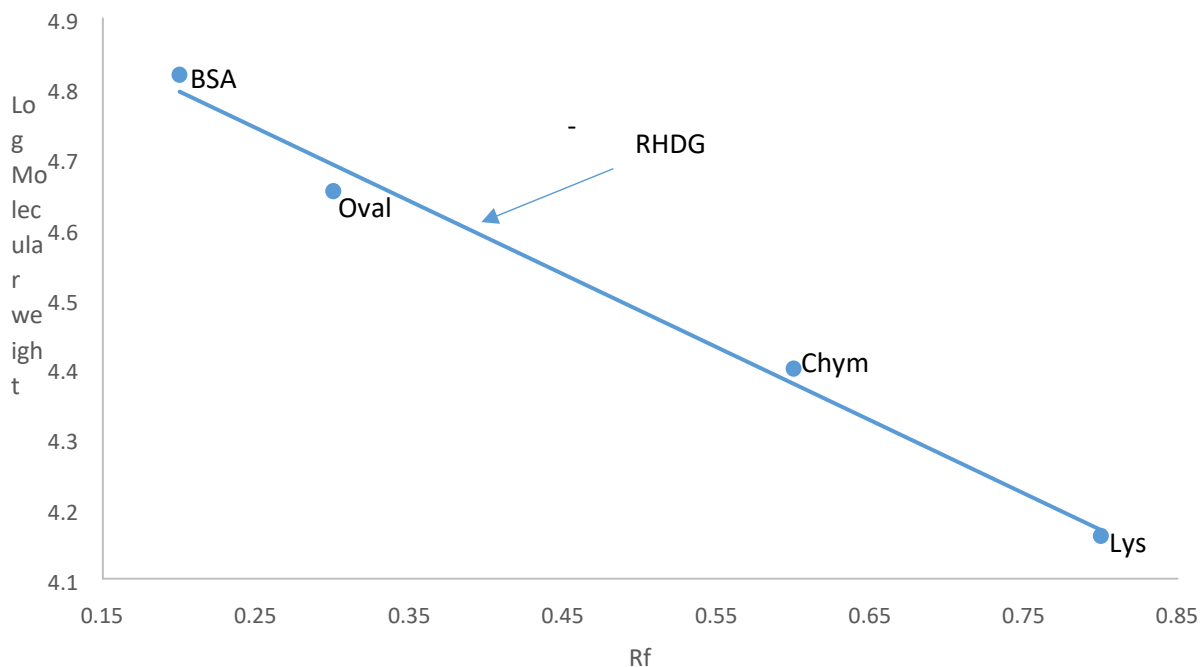


Figure 11c, Calibration Curve for the Determination of Subunit Molecular Weight of Rhodanese *Bacillus licheniformis*. The subunit molecular weight of the purified enzyme was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). BSA (bovine serum albumin; 66, 000 dalton) Oval (Ovine albumin; 45, 000 dalton) Chymo (Chymotrypsin; 25, 000 dalton) Lys (Lysozyme; 14, 500 dalton)

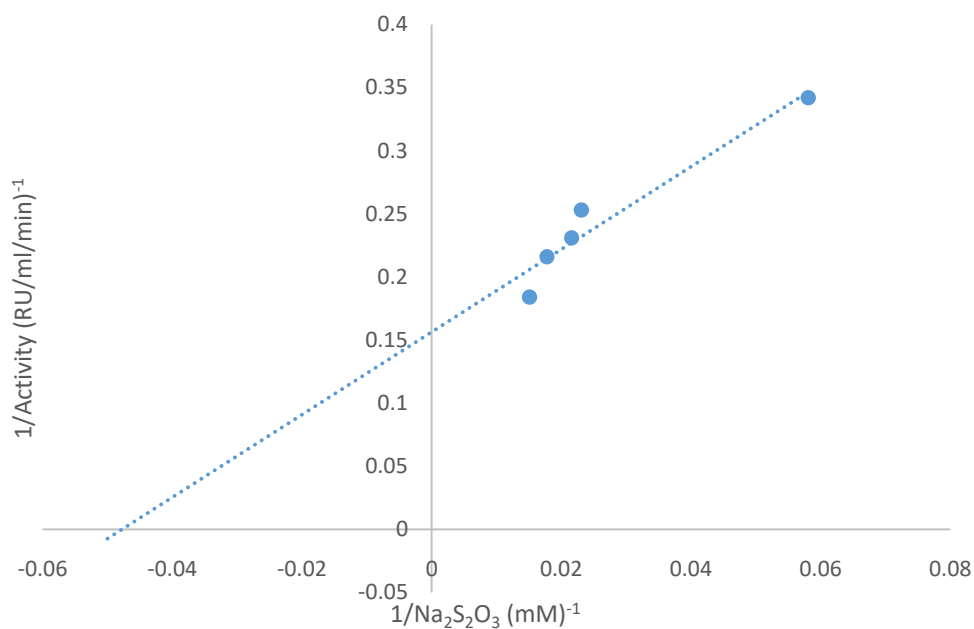


Figure 12. Lineweaver-Burk Plot for Varying Concentration of Sodium Thiosulphate Lineweaver-Burk plot of $1/V$ against $1/S$ at varying concentrations of $\text{Na}_2\text{S}_2\text{O}_3$ Between 5 mM and 50 Mm and a constant concentration of KCN at 25 mM

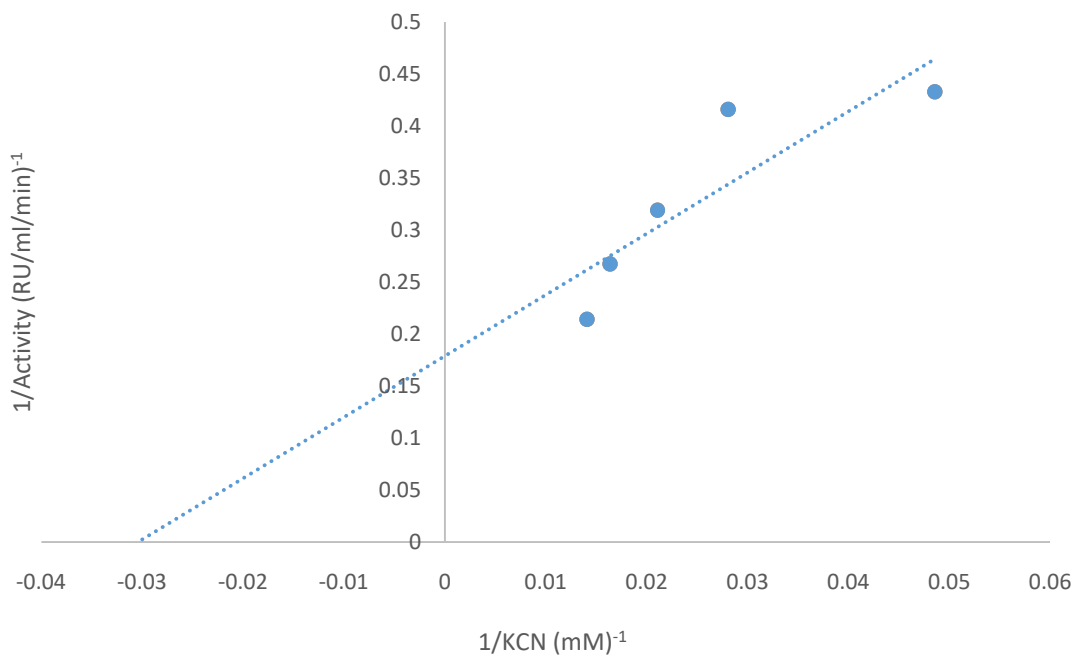


Figure 13. Lineweaver-Burk Plot for Varying Concentration of Potassium Cyanide Lineweaver-Burk plot of $1/V$ against $1/S$ at varying concentrations of KCN Between 5 mM and 50 Mm and a constant concentration of $\text{Na}_2\text{S}_2\text{O}_3$ at 25 mM

Table 4. Summary of Kinetic Parameters of Partially Purified Rhodanese from *Bacillus licheniformis* with KCN and Na₂S₂O₃ Substrates

Substrate	K _m (mM)	V _{max} (RU/ml/min)
KCN	32.90	5.60
Na ₂ S ₂ O ₃	21.00	6.40

Table 5. Result of Different substrates on Partially Purified Rhodanese From *Bacillus licheniformis*

Sulphur Compounds	% specificity
Sodium thiosulphate (Na ₂ S ₂ O ₃)	100.00
Sodium metabisulphite (Na ₂ S ₂ O ₅)	15.08
Ammonium persulphate ((NH ₄) ₂ S ₂ O ₈)	23.65
2-mercaptoethanol (CH ₂ (SH)CH ₂ (OH))	15.83
Sodium sulfite	22.09

Metal ions on rhodanese activity

The results of the effect of metal ions show that the activity of the enzyme was not inhibited by the investigated salts (Table 6).

Influence of Temperature on Partially Purified Rhodanese from *Bacillus licheniformis*

The assay was done to investigate the effect of temperature on activity of rhodanese from *Bacillus licheniformis* showed the enzyme to have its optimum temperature at 50°C, as shown in Figure 14

Heat Stability Study on Partially Purified Rhodanese from *Bacillus licheniformis*

The enzyme was found to be stable at 50°C for 30 minutes. Figure 15. showed the plot of percentage residual activity of the enzyme against time.

Table 6. Result of Metal Ions on Partially Purified Rhodanese from *Bacillus licheniformis*

METALS	ENZYME ACTIVITY (%)	
	10mM	1Mm
CONTROL	100.00	100.00
KCl	73.32 ± 3.04	74.41 ± 2.42
MgCl ₂	97.50 ± 3.04	99.81 ± 2.98
BaCl ₂	64.65 ± 0.72	80.44 ± 1.54
NiCl ₂	82.32 ± 2.77	86.59 ± 1.66
MnCl ₂	97.51 ± 2.01	83.98 ± 0.87
SnCl ₂	79.74 ± 0.12	93.66 ± 2.79
NaCl	100.00	89.32 ± 2.63

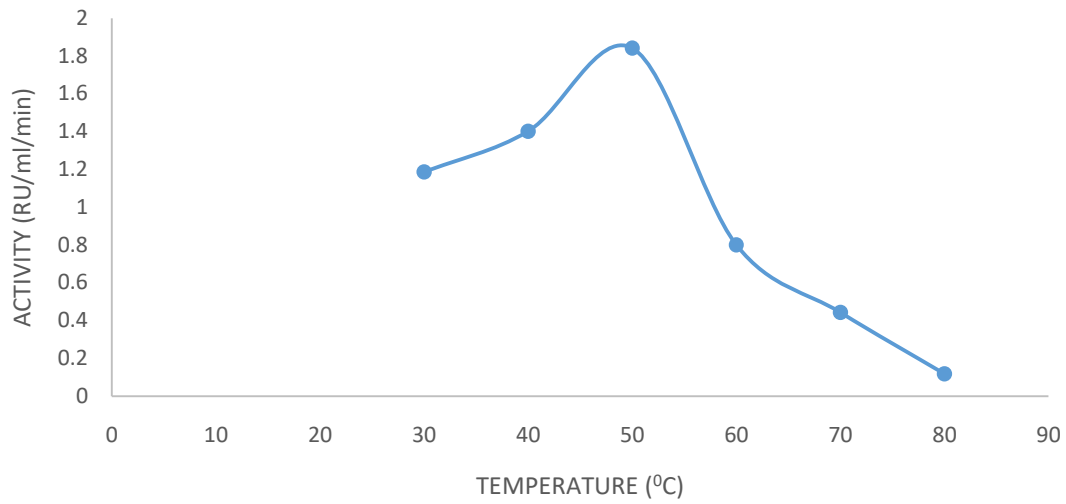


Figure 14. Effect of Temperature on Partially Purified Rhodanese Activity from *Bacillus licheniformis*

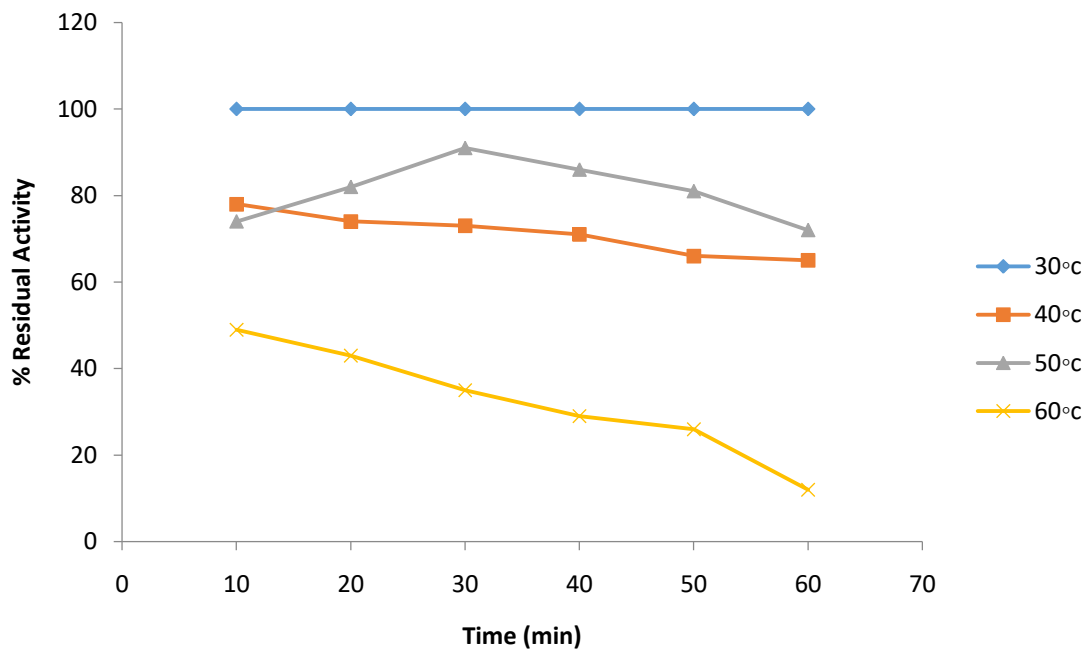


Figure 15. Heat Stability on the Activity of Partially Purified Rhodanese Obtained from *Bacillus licheniformis*

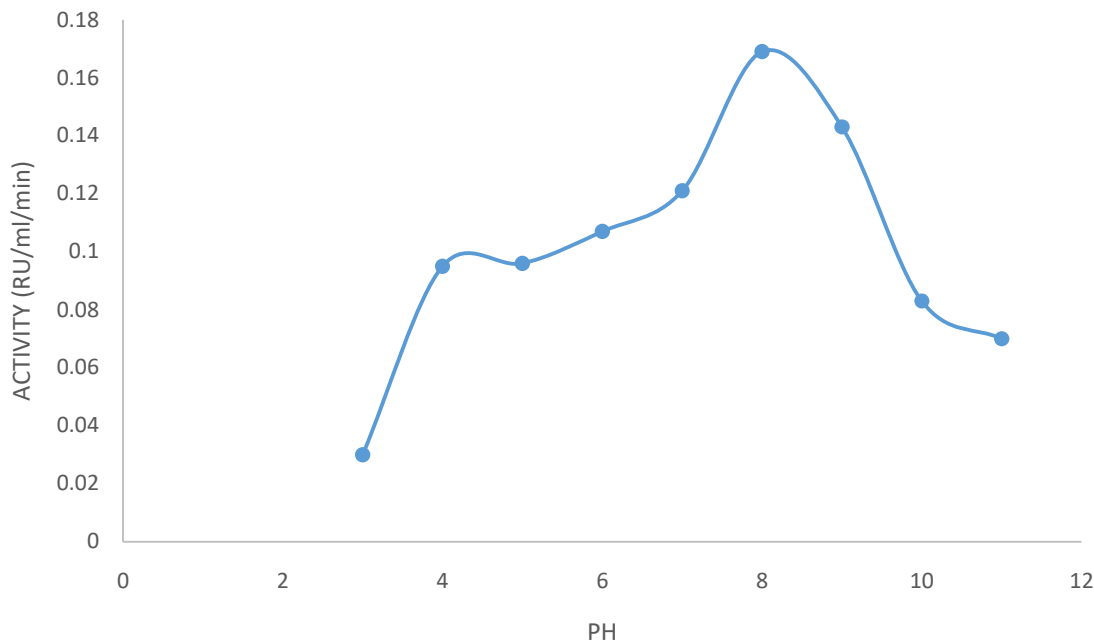


Figure 16. Effect of PH on the partially purified Rhodanese activity from *Bacillus licheniformis*

Discussion

The present study made successful attempt to isolate bacteria from the effluents of a steel rolling industry that is capable of producing the enzyme rhodanese. The isolate was gram positive, rod shaped, starch positive, spore forming and catalase positive. The bacterium with the highest activity among all the isolates was identified to be *Bacillus licheniformis* based on morphological and biochemical characteristics using Bergey's Manual of Determinative Bacteriology.

Production of enzyme such as rhodanese by *Bacillus licheniformis* is often depends on growth of the bacterium in the appropriate media composition. Rhodanese enzyme in this study is extracellular in nature, the bacteria will accumulate some cell components before the release of enzyme in the log phase, followed by decline in enzyme production during the stationary and death phase respectively. This was observed in *Bacillus licheniformis* as rhodanese production was maximum at 39 hour with gradual decline. The decline in rhodanese production may be due to exhaustion of the nutrients or accumulation of other products or metabolites which are both inhibitory to the growth of the bacterium and rhodanese production. Goyal *et al.* (2005) and Prakash *et al.* (2009) reported that inhibition of enzyme production may occur as a result of catabolic repression by metabolic monosaccharides such as glucose, increase in concentration of protease and rapid change in pH with the aim of conserving energy by bacteria. The bacterial growth pattern observed in this study was similar to that reported by Hoster *et al.* (2001) and Oyediji *et al.* (2013). Various researches have shown that enzyme synthesis is proportionate with the quality and concentration of carbon and nitrogen sources. These requirements differ from one organism to another (Pandey *et al.*, 2001; Schnurer and Jarvis 2010).

Naturally, 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 optimum nutritional requirement for rhodanese production was determined to obtain maximum enzyme production by *Bacillus licheniformis*. The effect of different nitrogen sources (1% and 0.5% w/v) on rhodanese production by *Bacillus licheniformis* was evaluated using submerged fermentation. The enzyme evaluation indicated that casein gave the maximum activity of the nitrogen sources utilized by *Bacillus*

licheniformis in the production of rhodanese with an activity of 3.49 RU/ml/min and when their concentrations were varied they gave optimum activity at 1.5g/dm³.

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this usually varies from one organism to another (Ehigie *et al.*, 2015). Bacterial rhodanases are produced at much wider range of temperature. In the result of Panos and Bellini it was reported that cyanide microbial activities increase as the temperature increases to 37°C (Panos *et al.*, 1999). However, in this study, the optimum temperature for the production of rhodanese was observed to be 35°C.

The source of carbon available for rhodanese producing bacteria is worthy of note owing to the fact that it is one of the basic requirements for growth and enzyme synthesis. The present study investigated the best carbon source for rhodanese production, potassium cyanide was found to have the highest activity of 3.14 RU/ml/min.

The result in this study showed that (0.8%) potassium cyanide composition of the enzyme production medium produced highest enzyme activity of 3.34 RU/ml/min which is very close to 3.21 RU/ml/min obtained with 0.25%. This is a clear indication that though different percentage of potassium cyanide composition may be used, 0.8% is the most suitable.

The optimum pH for the production of *Bacillus licheniformis* rhodanese is 9.0 with enzyme activity of 3.81 RU/ml. Schraft *et al.* (2006) reported *Bacillus licheniformis* as an organism with pH range of 4.3-9.3. This is in accordance with the result of Panos *et al.* (1999) who had reported 9.0 for optimum microbial cyanide degradation.

Rhodanese was produced under optimized conditions and harvested from the cultured broth for purification. Several methods have been used for the purification of rhodanese from various sources and the method used in this study for the purification of rhodanese from *Bacillus licheniformis* is similar to those of previous researchers (Cosby and Summer, 1945; Sorbo, 1953; Westley and Green, 1973). Extraction of enzyme was carried out by centrifugation of the cell broth at 12000 rpm for 15 min to obtain a cell free broth and was purified by a three-step procedure. The cell free broth was precipitated with 85% w/v ammonium sulphate precipitation. The rhodanese from *Bacillus licheniformis* was further purified by ion exchange chromatography on CM-Sephadex and finally by gel filtration on Sephadex G-100. The result showed a specific activity of 13.63 RU/mg with a purification fold of 5.19 and a percentage yield of 13.96%. Specific activity of different values from different sources has been reported for rhodanese. Agboola and Okonji (2004) also reported a value of 131 RU/mg for rhodanese from the fruit bat liver, while Fagbohunka *et al.* (2004) obtained a value of 20.1 RU/mg for Giant African snail. Akinsiku *et al.* (2010) obtained a value of 73 and 72 RU/mg for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDI) respectively. Okonji *et al.* (2011) obtained a value of 8.4 RU/mg for mudskipper liver rhodanese. Chew *et al.* (1972) obtained a yield of 7.8 and Ehigie *et al.* (2015) reported a percentage yield of 8.78%.

Native molecular weight of the enzyme was determined using gel filtration on Sephadex G-100. The molecular weight of the rhodanese was 34,440 Dalton. In general, a survey showed that the molecular weight of rhodanese falls between 31, 000 to 37, 000 Dalton (Lee *et al.*, 1995; Agboola and Okonji, 2004; Akinsiku *et al.*, 2010). An apparent molecular weight of 34, 500 Dalton and 36, 800 Dalton were reported for catfish rhodanese I (cRHD I) and catfish rhodanese II (cRHD II) respectively from the liver of African catfish (*Clarias gariepinus*) (Akinsiku *et al.*, 2010). Agboola and Okonji (2004) reported a molecular weight of 35,700 Dalton for rhodanese from the fruit bat liver. Sorbo (1953b) reported a molecular weight of 37,000 Dalton for bovine liver rhodanese. In 1995, Lee *et al.* (1995) reported a molecular weight of 34,800 Dalton for the mouse liver rhodanese and Nagahara and Nishino (1996) obtained a molecular weight of 34,000 Dalton for rat liver rhodanese.

The kinetic parameters (K_m and V_{max}) for potassium cyanide and sodium thiosulphate. Oyedeji *et al.* (2012) reported that, the K_m values for KCN and $Na_2S_2O_3$ as substrates for *Pseudomonas aeruginosa* rhodanese were 12.5 and 0.0066 mM, respectively, while the K_m values for the same substrates for *Bacillus brevis* rhodanese were 3.12 and 11.1 mM. Keith *et al.* (1987) reported the apparent K_m values of 78 mM and 17 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from *Escherichia coli*. Agboola and Okonji (2004) also reported K_m values of 13.5 mM and 19.15 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from fruit bat liver, while Hossein and Reza (2011) reported apparent K_m values of 36.81 mM and 19.84 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from rainbow trout liver. K_m values for KCN and $Na_2S_2O_3$ were 32.9 mM and 21.0 mM, and the V_{max} values for these substrates (KCN and $Na_2S_2O_3$) were 5.6mM and 6.4 mM, respectively for *Bacillus licheniformis*. The result from k_m indicates that the affinity of the *Bacillus licheniformis* enzyme for these substrates is higher which suggested a possible efficient cyanide catalytic

mechanism. However, it should be noted that cyanide detoxification is a secondary benefit of rhodanese, which has a number of major physiological roles, including the supply of sulphide for the formation of iron-sulphur centres for the electron transport chain.

Different optimum temperatures have been reported for rhodanese from different organisms (Ehigie *et al.*, 2013). Akinsiku *et al.* (2010) reported 40°C for the rhodanese from the liver of the African catfish from Asejire Lake. Himwich and Saunders (1948) also obtained an optimum temperature of between 38°C and 40°C for bovine liver rhodanese. Agboola *et al.* (2004) reported 35°C for the rhodanese in the cytosolic fraction of fruit bat liver. Ezzi *et al.* (2003) obtained a wide temperature optimum of 35 to 55°C for rhodanese of different strains of *Trichoderma*. The optimum temperature for *Bacillus licheniformis* rhodanese was 50°C which agree with the report of rhodanese from different sources (Emuebie *et al.*, 2011). Who obtained the optimum temperature of 50°C for mudskipper liver rhodanese, Sorbo (1953) reported an optimum temperature of 50°C for bovine liver rhodanese, while Okonji *et al.* (2011) obtained an optimum temperature of 50°C for mudskipper liver rhodanese. The heat stability showed that the enzyme is stable at 50°C for 30 minutes

As shown above, optimum pH for *Bacillus licheniformis* rhodanese was 8.0. This value is in line with the values found for rhodanese of bovine liver (between 8.0 and 9.0) (Sorbo, 1953), mouse liver (9.4) (Lee *et al.*, 1995), tapioca leaf (10.2–11) (Chew *et al.*, 1972), fruit bat liver (9.0) (Agboola *et al.*, 2004), and mudskipper liver (8) (Okonji *et al.*, 2011). Akinsiku working on the African catfish liver (6.5), obtained a high pH value of 6.5, (Akinsiku *et al.*, 2010) which is very close to the pH optimum of *Bacillus licheniformis* 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). These results are in agreement with results reported for rhodanases from different sources.

Bacillus licheniformis rhodanese was inactivated with 2-mercaptoethanol, ammonium persulphate, and sodium metabisulphite but activity was fully restored by addition of sodium thiosulphate. This studies shows that similar molecular properties between *Bacillus licheniformis* rhodanese and rhodanese from other sources (Westley 1973), and rhodanese enzyme was inactivated by 2-mercaptoethanol in tapioca leaf (Boey and Chew 1976). Okonji *et al.* (2011) reported the use of different sulphur compounds from a sulphane pool by rhodanese. Other researchers have also shown the specificity of rhodanese for sulphur from thiosulphate source (Lee *et al.*, 1995). Westley (1981) also reported the importance of sulphane sulphur pool in rhodanese cyanide detoxification mechanism.

The results of the inhibition study on the rhodanese from *Bacillus licheniformis* showed that the salts used did not have any significant effect on the activity of the enzyme. This could be as a result of consistent exposure to these metals due to their presence in the environment. Similar results have also been reported by Fagbohunka *et al.* (2004) and Okonji *et al.* (2011) on the rhodanese from the hepatopancrease of giant african snail and liver of mudskipper respectively. Other results showed little variations. Akinsiku *et al.* (2010) reported that land tortoise liver rhodanese was not affected by Mn^{2+} , Co^{2+} , Sn^{2+} , Ni^{2+} and NH_4^+ , while Ba^{2+} and Zn^{2+} inhibited the enzyme. Agboola and Okonji (2004) and Hossein and Reza (2012) also reported the effect of metals on the fruit bat liver and rainbow trout rhodanese respectively, stating that the inhibition of fruit bat liver and rainbow trout liver rhodanese by Hg^{2+} and Ba^{2+} was probably due to the interaction of these metal ions with sulphhydryl groups at the enzyme catalytic site or induction changes in the conformation of the enzyme (Ulmer and Vallee, 1972; Lee *et al.*, 1995; Nagahara and Nishino, 1996).

Conclusion

This study showed the presence of rhodanese activity in *Bacillus licheniformis* which has similar characteristics to rhodanases obtained from other sources. The high level of expression of rhodanese in *B. licheniformis* suggests that the enzyme may possess functional cyanide detoxification mechanism, a bioremediation process for the survival of both plants and animals in the environment. Microbial enhancement of this kind is recommended to help in reduction of toxic materials from industrial effluents. Enzymatic degradation of toxic waste in industrial effluents is highly recommended, before been discharged into the stream or river in order to prevent the ecosystem and its environment from being exposed to the toxicity of untreated effluents from metal processing industries

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