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Screening for rhodanese producing Bacterium in freshly pressed Cassava effluents of a Cassava processing industry channeled to Odo-Oba Stream in Ogbomoso-Nigeria

Adeola Folashade Ehigie*
Department of Biochemistry, College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria
Gbadebo Emmanuel Adeleke
Department of Biochemistry, College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria
Wasiu Ayinde Oladiran
Department of Biochemistry, College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria
Ona Leonard Ehigie*
Department of Biochemistry, College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria

*Corresponding author. E-mail: lehigie@lautech.edu.ng

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Abstract
Rhodanese is a key *enzyme* that plays an important role in cyanide detoxification. The enzyme was extracted, purified and physico-chemically characterised from *Bacillus licheniformis* which demonstrated the highest efficacy compared to the seven isolates of bacteria of the cassava processing industry effluent morpholologically and biochemically characterised. Statistical analysis was performed using one-way ANOVA and values were considered significant at $p<0.05$. This study showed that the optimum growth temperature was 35^0C at a pH 9.0. The highest duration time for the synthesis of rhodanese was at 40 hours. Potassium cyanide (KCN) and casein were the best carbon and nitrogen sources. The enzyme has a specific activity of 10.99 RU/mg, with a purification fold of 4.38, a percentage yield of 15.96%. The apparent K_m for KCN and Sodium thiosulphate ($Na_2S_2O_3$) were determined to be 30.24mM and 24.93mM respectively while their V_{max} were 5.40 RU /ml/min and 5.07 RU /ml/min respectively. The optimum pH and temperature were 8.0 and 50 ^0C respectively. The enzyme showed a high stability at 50^0C. The enzyme showed specificity at 6.78 RU/ml/min for $Na_2S_2O_3$ while it was inhibited by other sulphur containing substrates namely 2-mercaptoethanol, ammonium persulphate, and sodium metabisulphite The enzyme activity was not inhibited by metal ions such as (K^+, Mg^{2+}, Ba^{2+}, Ni^{2+}, Sn^{2+} and Na^+) at 1mM and 10mM and was not significant ($p>0.05$). Therefore, *B. licheniformis* have the potentials of reducing cyanide pollution thereby enhancing effective management of cassava mill effluent before eventual discharge into the environment and this may be developed into a more effective tool for bioremediation.

Keywords: *Bacillus licheniformis,* Cassava, Effluent, Rhodanase

INTRODUCTION

Cassava (*Manihot spp.*) is a root tuber crop widely grown in the tropics (Iyayi and Losel, 2001; Oboh and Akindahunsi, 2003). It is propagated by stem cutting and can grow to a height 6-8 ft. (O'Hair, 1995; Oboh, 2005). There are different varieties which may be classified on the basis of the amount of cyanogenic glycosides they contain. The glycosides are hydolysed endogenously to hydrogen cyanide. In Nigeria cassava processing mills are often cited near water bodies. This is so to allow easy cleaning of materials and less laborious disposal of wastes. Generally, wastes produced during processing include cassava peels and the liquid squeezed out of the mash (Oboh, 2005). This wastes are potential media for microbes which may either be harmful or beneficial. The waste could be turned to be beneficial at the instances when controlled fermentation are employed thus increasing the beneficial flora which can make the mash more nutritive by increasing the protein content of cassava products (Raimbault, 1998; Oboh *et al.*, 2002; Oboh and Akindahunsi, 2003), Nigeria is a major player in the production of cassava globally from which it derives a huge economic re-

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sources in the past (Daramola and Osanyinlusi, 2006). Processing of cassava tubers into a variety of products ensues the generation of liquid wastes which are hazardous to edaphic and aquatic factors. The indiscriminate discharge of this wastes poses serious environmental pollution. It is interesting to note that instead of leaving the waste as a pollutant, it could be recycled to animal feeds (Okafor, 1998; Oboh and Akindahunsi, 2003).

The occurrence of cyanide emerges from several processes other than cassava milling such as electroplating, coal cooking, mining (Dash *et al*., 2009, Aazam, 2014, Lovasoa 2017). Among all these, cyanide release is largely from cassava milling. As a gas, cyanide combines with hydrogen ion forming hydrocyanic acid which is extremely harmful with smell resembling that of almonds. The detoxification of cyanide is catalyzed by sulfurtransferases including Rhodanese. The enzyme also known as thiosulphate cyanide Sulphur transferase with Enzyme Commission number, EC 2.8.1.1, is an ubiquitous enzyme that, *in vitro,* catalyses the transfer of a sulphur atom from suitable donors to nucleophillic 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). The aim of this work was to isolate, purify and characterize Rhodanese in the cyanide-metabolising microbes isolated from stream contaminated with cassava wastes which could be further be employed in the agro industry for remediation of cyanide.

MATERIALS AND METHODS

Sampling area and water sample collections: Water samples were collected near a cassava processing factory behind a stream in Odo-Oba, Ogbomoso, in Oyo State, Southwestern - Nigeria. The village is made up of predominantly farmers. Odo-Oba lies within Latitude 7°35'0'' N and Longitude 4°17'0'' E. The stream, apart from being used for domestic purposes, is also used for washing and soaking peeled cassava before milling for processing to edible products

Analysis of cyanide content in water sample: Alkaline picrate was mixed with the sample filtrate for the testand incubated for 5 mins. Absorbance was read with a spectrophotometer at 490 nm. Cyanide content was extrapolated from a standard curve obtained using varied concentrations of KCN solution containing 5 to50 µg cyanide and 25ml of 1N HCl.

Isolation and inoculation procedures: Conventional dilution-plate procedure was employed. 1 ml of the sample was added to 9 ml sterile water. Tenfold dilutions were prepared up to 10^{-9}. Portions of 10^{-7}, 10^{-8}, 10^{-9} dilutions were spread on agar plates containing (1.5% w/v), peptone (1% w/v), yeast extract (0.5% w/v), sodium chloride (0.5% w/v), potassium cyanide (0.3% w/v).

The plates (in duplicates for each dilution) were incubated at 37°C for 48 h. Colonies were isolated from the plates for purification and identification. The colonies were streaked on the basal medium for purification and maintained on agar slants at 4° C. The different isolates were transferred to basal broth medium containing peptone (1% w/v), yeast extract (0.5% w/), sodium chloride (0.5% w/v), potassium cyanide (0.3% w/v) (dissolved in deionized water), pH 9.5.

For inoculation, MacFarland standards (0.5) of the isolates were prepared and the media were incubated at 37°C on a rotary shaker at 230 rpm for 48 h. Culture media were assayed for rhodanese production. Seven bacterial isolates which showed high enzyme activity was selected were screened for cyanide degrading potentiality. The best strain was further characterized based on cell morphology, cultural and biochemical characteristics (Holt *el al.,* 1994).

Enzyme production: The time course of the enzyme synthesis was determined and compared with microbial growth according to the method of Oluwatosin *et al.,* 2017. Optical density was determined at intervals of 3 h for 48 h to monitor microbial growth. Enzyme activity was determined at the same time course using the method of Lee *et al,* 1995. One Rhodanese Unit (RU) was defined as the amount of the enzyme that will convert one micro-mole (1 µmol) of cyanide to thiocyanide in one minute at 37°C Sorbo (1951).

Determination of protein concentration: Protein concentration was determined using Bovine Serum Albumin (BSA) (Bradford, (1976)) as protein standard. Absorbance was extrapolated from the standard curve. The absorbance was read at 595 nm.

Enzyme purification: Ammonium sulphate precipitation and ion exchange chromatography techniques were employed for partial purification of the enzyme following the methods of Agboola and Okonji, 2004, Oluwatosin *et al.,* 2017. The specific activity, yield and fold of purifications were calculated according to Agboola and Okonji (2004).

Characterization of the enzyme: The Kinetics of the enzymatic degradation of cyanide was studied using the partially purified enzyme preparation and modeled according to Lineweaver-Burk equation (Lineweaver and Burk (1934)). The enzyme was characterized with respect to pH, temperature, substrate specificity and metal ions. pH effect was determined using 50 mM of citrate buffer (pH 3-5); 50 mM phosphate buffer (6-8) and 50 mM borate buffer (pH 3-11) as substitute for the reaction buffer. The optimum temperature of the enzyme was investigated by assaying the enzyme

at temperatures between 30°C and 80°C at an interval of 10°C. The assay mixture was initially incubated at the specified temperature for 10 min before addition of an aliquot of the enzyme equilibrated at the same temperature. The substrate specificity study was carried out using different sulphur compounds namely sodium sulphite, 2-mercaptoethanol, ammonium persulphate, ammonium sulphate and sodium metabisulphite. Sodium thiosulphate was replaced with the sulphur compound. Percentage activity of the enzyme was determined using sodium thiosulphate as the control Agboola and Okonji (2004).

Effect of Metal Ions on Partially Purified Rhodanese Activity was studied using the method of Lee *et al.,* (1995). The compounds used were $MgCl_2$, $CaCl_2$, $HgCl_2$, NaCl, $BaCl_2$ and KCl 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. A volume of 0.05 ml of each metal concentration was mixed with the substrate, with addition of 0.05 ml enzyme and incubated for one minute at room temperature, followed by the addition of 0.5 ml 15% formaldehyde and 1.5 ml ferric nitrate.

RESULTS AND DISCUSSION

This study made successful attempt to isolate bacteria from the effluents of a cassava processing industry channeled to Odo-Oba Stream in Ogbomoso, 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* (Fig. 1) characterized based on cell morphology (Table 1) and biochemical characteristics (Table 2) (Holt *el al.,* 1994).

Enzyme synthesis such as that of rhodanese by *Bacillus licheniformis* is dependent on growth of the bacterium in an appropriate media. From the growth-activity study a correlation was found be-

Table 1. Morphological Characteristics of Isolate A 5.

Isolate	A 5
Shape	Rod
Surface	Glistering
Opacity	Opaque
Elevation	Flat
Colour	Cream

Table 2. Gram's Staining, Spore staining, and Biochemical Characteristics of the Isolate A5.

Isolate code	A 5
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	2.5-5.0 micrometer in length
Indole	Negative
Motility	Positive
Sulphur	Negative
Presumptive Identity	*Bacillus sp.*

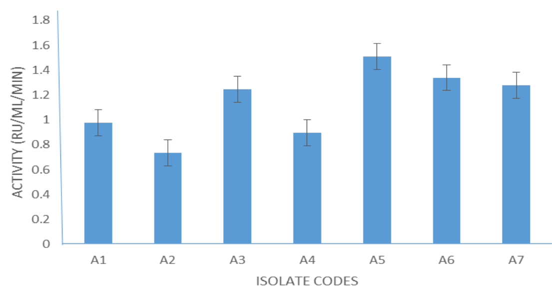

Fig. 1. *Rhodanese Activity of Different Bacterial Isolates (ISOLATE CODE). A1 = B. macerans;A2 = C. Kutsceri;A3 = B. cereus;A4 = B. macquariensis;A5 = B. licheniformis; A6 = B. subtilis;A7 = Aeromonas*

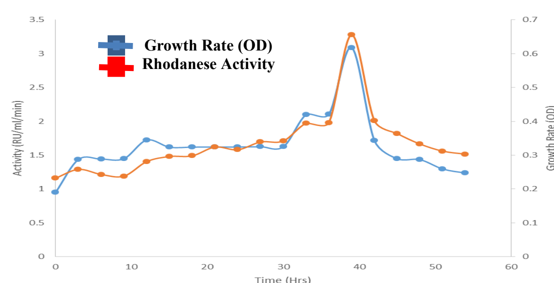

Fig. 2. *Incubation time on rhodanese production and cell Growth of B. licheniformis.*

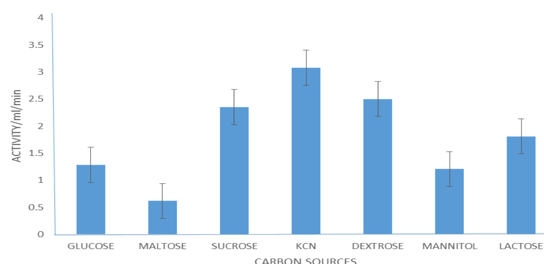

Fig. 3. *Influence of different carbon sources on rhodanese synthesis by B. licheniformis.*

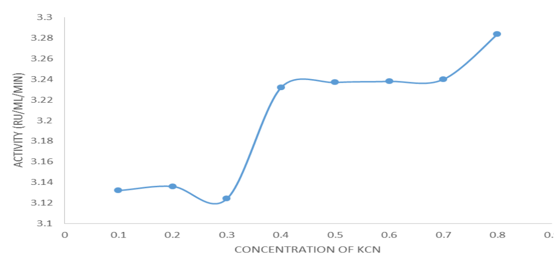

Fig. 4. *Effect of different concentrations of KCN on rhodanese synthesis by B. licheniformi.s*

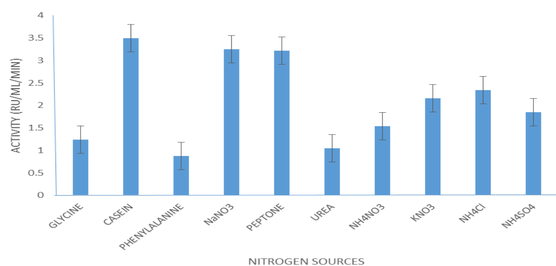

Fig. 5. *Effect of different nitrogen sources on rhodanese production by B. licheniformis.*

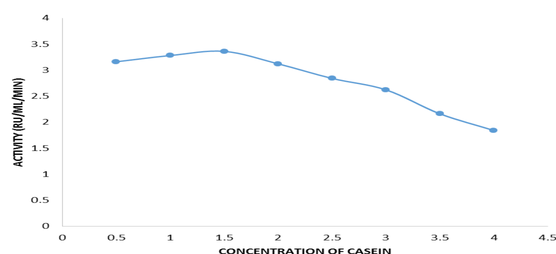

Fig. 6. Effect of different concentrations of casein on rhodanese production by *B. licheniformis.*

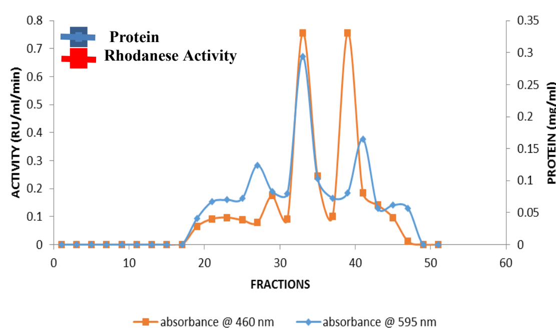

Fig. 7. Separation by Ion-exchange chromatography on Sephadex C-25 of partially purified rhodanese from *B. licheniformis.*

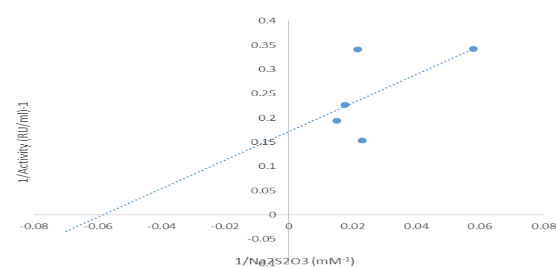

Fig. 8. *Lineweaver-Burk plot of 1/V against 1/S at varying concentrations of* $Na_2S_2O_3$ *(5 mM - 50 mM) and a constant concentration of KCN at 25 mM.*

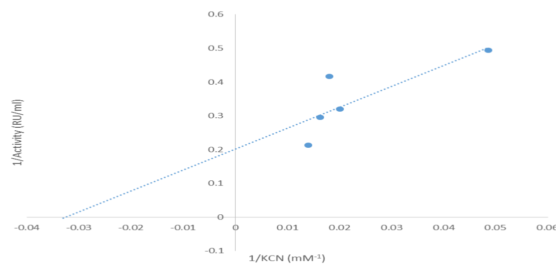

Fig. 9. *Lineweaver-Burk plot of 1/V against 1/S at varying concentrations of KCN (5 mM -50 mM) and a constant concentration of* $Na_2S_2O_3$ *at 25 mM.*

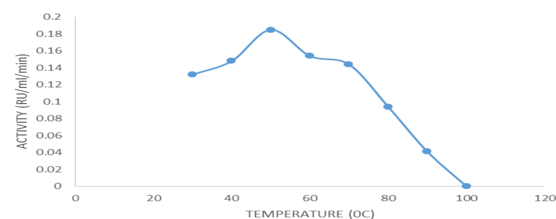

Fig. 10. *Influence of temperature on partially purified rhodanese activity from B. licheniformis.*

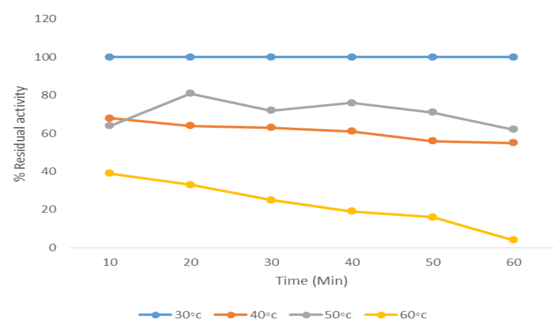

Fig.11. *Effect of heat stability on the activity of partially purified rhodanese obtained from B. licheniformis.*

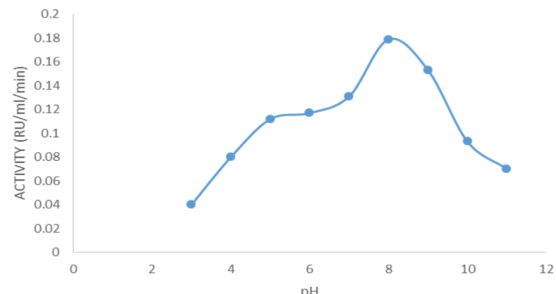

Fig. 12. *Effect of pH on the partially purified rhodanese activity from B. licheniformis.*

tween the enzyme activity and the rate of growth. The optimum incubation time for both maximum rhodanese activity and growth was at 39 h (Fig. 2) followed by gradual decline. The reduction in rhodanese production may be due to depletion of available 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)

The effect of different carbon and nitrogen sources was tested on the rhodanase enzyme synthesis of *B. licheniformis*. 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

Table 3. Partial purification procedure of crude rhodanese obtained from *B. licheniformis*.

Purification steps	Volume (ml)	Protein (mg/ ml)	Activity (RUnit)	Total protein (mg)	Total activity (Unit/mg)	Specific activity (Unit/mg)	% yield	Purification fold
Crude enzyme	50	1.097	2.756	54.85	137.8	2.512	100	1.0
85% ammonium sulphate precipitation	20	1.004	2.897	20.08	57.94	2.89	42.05	1.15
sephadex C-50 iron exchange chromatography	10	0.109	0.576	1.09	5.76	5.28	4.18	2.11

Table 4. Summary of Kinetic parameters of partially purified rhodanese from *B. licheniformis* with KCN and $Na_2S_2O_3$ substrates.

Substrate	K_m (mM)	V_{max} (RU/ml/min)
KCN	30.71	4.59
$Na_2S_2O_3$	17.11	5.83

Table 5. Effect of different substrates on partially purified rhodanese from *B. licheniformis.*

Sulphur Compounds	% specificity
Sodium thiosulphate ($Na_2S_2O_3$)	100
Sodium metabissulphite ($Na_2S_2O_5$)	13.76
Ammonium persulphate ($(NH_4)_2S_2O_8$)	13.63
2-mercaptoethanol ($CH_2(SH)CH_2(OH)$	14.15
Sodium sulfite	14.28

Table 6. Effect of metal ions on partially purified rhodanese from *B. licheniformis.*

Metals	Enzyme activity (%)	
	10mM	1mM
CONTROL	100	100
KCl	76.41 ± 2.93	73.31 ± 2.51
$MgCl_2$	95.51 ± 3.14	98.65 ± 2.78
$BaCl_2$	66.65 ± 0.81	83.12 ± 1.54
$NiCl_2$	82.32 ± 2.67	87.52 ± 1.66
$MnCl_2$	97.51 ± 2.01	91.08 ± 0.86
$SnCl_2$	77.54 ± 0.16	91.37 ± 2.39
NaCl	100.0	86.77 ± 2.51

carbon source for rhodanese production. When Potassium cyanide was used, the enzyme was found to show the highest activity of 3.081 RU/ml/min as shown in Fig. 4 with 0.8% potassium cyanide composition of the enzyme production medium producing the highest enzyme activity of 3.244 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 well tolerated.

The effect of different nitrogen sources (1% and 0.5% w/v) on rhodanese production by *B. licheniformis* was evaluated using submerged fermentation. The enzyme evaluation indicated that casein gave the maximum activity of the nitrogen sources utilized by *B. licheniformis* in the production of rhodanese with an activity of 3.454 RU/ml/min (Fig. 5).

Several methods have been used for the purification of rhodanese from various sources and the method used in this work for purification of rhodanese from *B. licheniformis* have been conventionally used by researchers (Cosby and Summer, 1945; Sorbo, 1953; Westley and Green, 1983; Ehigie *et al*., 2019; Itakorode *et al*., 2019). 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 enzyme from *B. licheniformis* was subjected to ion exchange chromatography on CM-Sephadex. A specific activity of 10.99 RU/mg, purification fold of 4.38 and 15.96% yield were obtained (Table 3). Different values of specific activity have been obtained from different sources. Rhodanese from the fruit bat liver had a value of 131 RU/ mg (Agboola and Okonji (2004)), while a value of 20.1 RU/mg was obtained for Giant African snail by Fagbohunka *et al*. (2004), 15.5 RU/ mg for some common plant tubers (Ehigie *et al.* (2013)), 73 and 72 RU/mg for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDII) respectively (Akinsiku *et al.* (2010)). Oluwatosin *et al* (2017) reported 16.48 RU/mg for rhodanese isolated from cyanide contaminated cassava site.

The kinetic parameters (K_m and V_{max}) for KCN and $Na_2S_2O_3$ are shown in Table 4. K_m values for KCN and $Na_2S_2O_3$ are 30.24 mM and 24.93 mM, and the V_{max} values for these substrates are 5.4mM and 5.07 mM, respectively for the *B. licheniformis* enzyme (Fig.s 8 and 9). K_m values for KCN and $Na_2S_2O_3$ as substrates for *Pseudomonas aeruginosa* rhodanese were 12.5 and 0.0066 mM, respectively (Oyedeji *et al*. (2013)), while the Km values for the same substrates for *B. brevis* rhodanese were 3.12 and 11.1 mM. The apparent K_m values of 78 mM and 17 mM for potassium cyanide and sodium thiosulphate were obtained respectively for rhodanese from *Escherichia coli* by Saidu (2005) and Chae *et al* (2006). The result of the kinetic analysis is an indication of high affinity of the *B. licheniformis* enzyme for these substrates is suggesting the catalytic efficiency of the enzyme.

Temperature is a crucial factor that regulates the growth and production of metabolites and enzymes by microbes (Banerjee, & Bhattacharyya 1992); Kumar and Takagi, 1999). In this study, the optimum temperature for the production of

rhodanese was observed to be 50 ºC (Fig. 10). Several similar or nearly similar observations have been reported for rhodanese from different organisms. In the result of Panos and Bellini it was reported that cyanide microbial activities increased as the temperature increased to 37ºC (Panos et al., 1999). Bovine liver rhodanese had optimum temperature between 38°C and 40°C (Himwich and Saunders (1948)). Agboola *et al*. (2004) reported 35°C for the rhodanese in the cytosolic fraction of fruit bat liver. Itakorode *et al*., (2019) and Sorbo (1953) obtained a temperature optimum of 50°C for *B. cereus* and bovine liver rhodanese respectively similar to the observation of Ehigie *et al.,* (2016) on mesocarp and capsule of Snake tomato, *Trichosanthes cucumerina*. However, the enzyme was observed to be stable up to 60 ºC (Fig. 11) which is similar to that reported by Oluwatosin *et al.* (2017) for *Klebsiella edwardsii*.

The pH optimum of 9.0 was obtained for the production of *B. licheniformis* rhodanese with enzyme activity of 3.792 RU/ml (Fig. 12) which is in tanderm with result from *B. cereus* (Itakorode *et al*., 2019). Oluwatosin *et al.* (2017) for *Klebsiella edwardsii* reported a pH of 6. Schraft *et al.* (2006) reported *B. licheniformis* as an organism with pH range of 4.3-9.3. This is in consonance with the result of Panos et al. (1999) who reported 9.0 for optimum microbial cyanide degradation. Rhodanese activity has been shown to be enhanced at alkaline pH level (Sorbo, 1953). Mouse liver rhodanese is active at pH of 9.4 (Lee *et al*., 1995), rhodanese from tapioca leaf at 10.2–11 (Chew and Boey, 1972), fruit bat liver rhodanese at 9.0 (Agboola *et al*., 2004).

Enzymes shows substrate specificity, rhodanese is no exception (Westley 1983, Okonji *et al.,* 2017). *B. licheniformis* rhodanese was inhibited by 2-mercaptoethanol, ammonium persulphate, and sodium metabisulphite but its activity was enhanced by addition of sodium thiosulphate (Table 5). In tapioca leaf, rhodanese was inactivated by 2 -mercaptoethanol (Boey and Chew 1976).

The inhibition study from metal ions revealed that the enzyme activity was not significantly inactivated by the metal ions tested (KCl, $MgCl_2$, $BaCl_2$, $NiCl_2$, $MnCl_2$, $SnCl_2$, NaCl) (Table 6). We inferred that these ions might be present in the environment of the organism making it to build tolerance or resistance to the ions. Fagbohunka *et al.* (2004) and Okonji *et al.* (2011) obtained similar results on the enzyme isolated from hepatopancrease of giant african snail and liver of mudskipper respectively. There have been discrepancies though. 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.

Conclusion

Microbial enzymatic degradation of toxic material in waste water effluent from cassava mill is highly recommended, before being channelled into Odo-Oba stream in Ogbomoso-Nigeria in order to safeguard the ecosystem from the dread of poisonous chemicals. The high level of expression of rhodanese in *B. licheniformis* suggests that the biodegradation capacity of the isolate might be a base study to develop the production of potential local bioremediation agents in toxic cassava mill effluent treatment technology. It is noteworthy that the cognate enzyme was not significantly inhibited by any of the metal ions tested. This could give an edge over other microbes which are affected by presence of heavy metals during the bioremediation process. We concluded that *Bacillus licheniformis* can be successfully applied for the removal of cyanide from aqueous solutions.

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