



## Purification and characterization of betacyanoalanine synthase from *Pseudomonas straminea*

R. E. OKONJI<sup>1\*</sup>, O. S. ADEDEJI<sup>2</sup>, B. ITAKORODE<sup>1</sup>, N. TORIMIRO<sup>3</sup> and C. ONWUDIEGWU<sup>4</sup>

<sup>1</sup> Department of Biochemistry and Molecular Biology, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

<sup>2</sup> Department of Biochemistry, Faculty of Science, Federal University Oye Ekiti, Ekiti state, Nigeria.

<sup>3</sup> Department of Microbiology, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.

<sup>4</sup> African Institute for Science Policy and Innovation, Faculty of Technology Obafemi Awolowo University, Ile-Ife, Nigeria.

\* Corresponding author; E-mail: [okonjire@yahoo.co.uk](mailto:okonjire@yahoo.co.uk); [reokonji@oauife.edu.ng](mailto:reokonji@oauife.edu.ng); Tel: +2348060164991

### ABSTRACT

Beta-cyanoalanine synthase (EC 4.4.1.9) is a cyanide detoxifying enzyme obtained from *Pseudomonas straminea* isolated from the effluents of Iron and Steel Smelting Company in Ile-Ife, Osun State. The effluent water samples were screened for their ability to detoxify cyanide and isolates obtained were then screened for b-cyanoalanine synthase production. The samples were screened for their ability to detoxify cyanide and isolates obtained were then screened for  $\beta$ -Cyanoalanine Synthase production. The enzyme was purified through Lyophilization, ion exchange chromatography on CM-Sephadex c-25 and Gel filtration on Biogel P-100. The enzyme had a specific activity of 273.75 U/mg with a purification fold of 2.41 and a yield of 28.90. The native molecular weight of the enzyme was estimated as 54.9 KDa and subunit molecular weight of 60.3 KDa. The  $K_m$  values of the enzyme for sodium cyanide and L-Cysteine were  $8.33 \pm 0.05$  mM and  $1.25 \pm 0.26$  mM respectively while their maximum reaction velocities were  $14.705 \pm 0.32$  MU/ml/min and  $0.1 \pm 2.48$  MU/ml/min respectively. The optimum temperature and pH for activity were determined to be 40 °C and 9.0. The metals tested at the different concentrations of 1 mM, 5 mM and 10 mM enhanced the activity of the enzyme. Inhibition studies on  $\beta$ -Cyanoalanine Synthase *Pseudomonas straminea* showed that the activity of the enzyme was affected by mercuric chloride, glycine, EDTA and iodoacetamide. The study concluded that  $\beta$ -Cyanoalanine Synthase from *Pseudomonas straminea* may possess potential bioremediation properties for detoxification of cyanide polluted wastewaters.

© 2018 International Formulae Group. All rights reserved.

**Keywords:** Beta-cyanoalanine synthase, Hydrogen cyanide, Bacterium, *Pseudomonas straminea*.

### INTRODUCTION

Beta-cyanoalanine synthase ( $\beta$ -CAS) (EC 4.4.1.9) is an enzyme which catalyses the reaction between L-cysteine and hydrogen cyanide to form  $\beta$ -cyanoalanine, by

transferring the sulfhydryl moiety from a cysteine residue to CN to form the  $\beta$ -cyanoalanine, a nonprotein amino acid according to the reaction (Bonner et al., 2005).  $\beta$ -cyanoalanine synthase has been

verified widespread in bacteria (Ezzi Mufada and Lynch, 2002), higher plants (Ezzi Mufada and Lynch, 2002) and insects (Ogunlabi and Agboola, 2007). L-cysteine is the natural primary substrate and cyanide as cosubstrate for cyanoalanine synthase (Claus et al., 2005).

Cyanide is a carbon–nitrogen radical, which may be found in a wide variety of organic and inorganic compounds. Cyanide is a very powerful and fast acting toxin (ATSDR, 2006). Cyanide is found in a wide range in the various life forms including photosynthetic bacteria, algae, fungi, plants such as cassava, tapioca, almonds, corn, lima beans, etc (Claus et al., 2005) and even in the animal kingdom such as beetles, butterflies and grasshoppers (Ogunlabi and Agboola, 2007). Humans are in close contact with cyanide in their daily life through food, drink, and medicines. Other routes of cyanide exposure include smoking tobacco (0.5mg/cigarette), respiring exhaust from vehicles, handling certain type of pesticides and insecticides (Akcil and Mudder, 2003). Cyanide is widely distributed in the environment and this causes severe environmental problems when produced in high amounts by anthropogenic activities, such as the electroplating industry (Parga et al., 2003), metal finishing or mining industries/processes (Mansfeldt et al., 2004), iron and steel mill, publicly owned wastewater treatment facilities and organic chemical industries. As cyanides are produced regularly by industries in large quantity in waste water streams, it is a potent health hazard for human and ecosystem (Rodrigo et al., 2005). To protect the environment and water bodies, these effluents containing cyanides must be treated before discharging into the environment. The United States Environmental protection Agency has proposed a limit for drinking and aquatic biota waters regarding total cyanide disposal in waters (USEPA, 2010).

Cyanides can be removed from industrial wastes by biodegradation, physical and chemical methods (Patil and Paknikar,

2000). Chemical and physical methods include chemical oxidation through alkaline chlorination, ozonization in presence of UV, hydrogen peroxide, Air/SO<sub>2</sub> process and chlorine dioxide gas (Lee et al., 2004; Parga et al., 2003), adsorption on granulated active carbon, ion exchange, membrane concentration, air stripping and evaporation. The biodegradation method of cyanides removal is better to physical and chemical methods. Biodegradation is more economical and faster, it is more efficient and has less capital and operative cost (Kao et al., 2006). Biological treatment involves the use of microorganisms and their enzymes which have the ability to degrade cyanide into less toxic compound like ammonia, formic acid and formamide. Microbes can utilize these compounds as a source of nitrogen and carbon for their growth under aerobic as well as anaerobic conditions (Ebbs, 2004). Here, we isolated, purified and characterized a bacterial- *Pseudomonas straminea* beta-cyanoalanine synthase from the effluents of Ife iron and steel smelting company with the aim of evaluating the potentials of this enzyme for possible bioremediation of cyanide-contaminated industrial effluents.

## MATERIALS AND METHODS

### Collection of sample

Effluents were collected from the study area, 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 labeled, put in ice packs, transported to the laboratory and analyzed within 30 minutes to 1 hour of collection

### Isolation of cyanide degrading microbes

Using a sterile inoculating loop, a loopful of the stock effluent was streaked on the modified Bushnell Hass agar and incubated inverted at 25 °C for 96 hrs to screen for cyanide degrading bacteria. All plates were labelled appropriately. The

distinct lines of the organism were picked, and subcultured on nutrient agar plates to obtain pure colonies. The bacterial isolates were subsequently transferred into nutrient agar slants, incubated at 25 °C for 24 hrs and stored in the refrigerator (-4°C) for further tests.

### **Characterization and identification of isolates**

Six bacterial isolates were screened for their ability to degrade free cyanide. The best strain was selected for further study and characterized. The best selected  $\beta$ -cyanoalanine synthase-producing strains were identified based on their cell morphology, cultural and biochemical characteristics using Bergey's manual of determinative bacteriology as a standard.

### **Preparation of Basal Medium for $\beta$ -Cyanoalanine Synthase Production**

The modified liquid basal medium for  $\beta$ -Cyanoalanine synthase production was prepared as described by Zlosnik and Williams (2004). The medium contained peptone (1% w/v), yeast extract (0.5% w/v), sodium chloride (0.5% w/v), sodium cyanide (0.3% w/v) at pH 9.5. One millilitre of the standardized pure bacteria isolate in normal saline was aseptically inoculated into each conical flask containing basal medium and incubated at 37 °C for 48 hrs on a rotary shaker (model) at 140 rpm.

### **Screening and Selection of Isolates for $\beta$ -Cyanoalanine Synthase Production**

Six isolates obtained were screened individually for  $\beta$ -cyanoalanine synthase activity using a modified basal medium containing peptone (1% w/v), yeast extract (0.5% w/v), sodium chloride (0.5% w/v), sodium cyanide (0.3% w/v), the pH was adjusted to 8.5 (Zlosnik and Williams, 2004). Each conical flask was then autoclaved at 121 °C for 15 mins and then allowed to cool. The screening was done in 100 ml of the basal medium containing 1 ml of inoculum of 0.5

McFarland standard. This was incubated at 37 °C for 48 hr with agitation at 170 rpm.

### **Enzyme Production by Submerged Fermentation**

This procedure was applied to the bacterial isolate with the highest activity obtained from the screening and selection procedure. The enzyme production for the selected isolate was determined by inoculating a 100 ml of the sterile basal medium with 1 ml standard inoculum of 0.5 McFarland standard in an Erlenmeyer flask. This was incubated at 37 °C for 48 hr with agitated at 170 rpm. At intervals of 3 hrs, 5 mls of samples were collected aseptically for a period of 48 hrs, the optical density of each sample was checked at 590 nm using a colorimeter and recorded. Each sample at the different time intervals was further centrifuged at 12,000 rpm for 30 mins to separate the cells from supernatant, the supernatant which served as the crude was checked for  $\beta$ -CAS activity at 650 nm and the protein at 595 nm.

### **$\beta$ -Cyanoalanine Synthase Assay**

Activity of  $\beta$ -Cyanoalanine synthase was carried out according to the method described by Ogunlabi and Agboola (2007). Assay was performed in a 10 ml serum bottle with the rubber cork. The assay mixture contained 1ml of the substrate solution (25 mM L-cysteine and 25 mM NaCN in 0.1 M Tris-HCl buffer, pH 8.5) and 1 ml of appropriately diluted enzyme solution. The mixture was incubated at 30 °C for 10 min and the reaction terminated by the addition of 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulphate (in 7.2 N HCl) and 0.5 ml of 30 mM FeCl<sub>3</sub> in (1.2 N HCl) through the rubber cork using a calibrated syringe and needle. The mixture was shaken vigorously and placed in the dark for 20 minutes. The solution was clarified by centrifugation and the absorbance was read at 650 nm. The absorbance at A<sub>650</sub> of 1.0 is

equivalent to 0.5  $\mu\text{mol}$  of sulphide produced under the assay condition. One unit of enzyme activity was defined as the amount of enzyme yielding 1.0 nmol of  $\text{H}_2\text{S}$ /min under the assay condition. Protein concentration was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard. The reaction mixture consisted of 100  $\mu\text{l}$  of the enzyme solution and 1.0 ml of Bradford reagent. The absorbance was read at 595 nm.

### **Lyophilization**

The crude enzyme was concentrated by freeze drying the enzyme solution using a lyophilizing machine. Earlier attempt using acetone or ammonium sulphate precipitation did not produce desired result. The freeze dried enzyme was later reconstituted by dissolving in appropriate buffer solution.

### **Ion-Exchange Chromatography on CM-Sephadex c-25**

The resuspended fraction was applied to CM-Sephadex c-25 column (2.5 x 40cm) pre-equilibrated with 0.1 M Tris HCl buffer (pH 8.5). The bound proteins were eluted with 1.0 M NaCl in the same buffer. Fractions of 3 mls were collected from the column at a flow rate of 36 ml per h. The fractions were assayed for  $\beta$ -cyanoalanine synthase activity as described and protein profile was monitored using Bradford (1976) method.

### **Gel Filtration Chromatography on Biogel P-100**

The swollen Biogel P-100 resin was then loaded and equilibrated on a 1.5 x 40 column. 3 ml of the post CM-sephadex c-25 sample was layered on the column. The column was eluted with 200 ml of 0.1 M Tris-HCl buffer, pH 8.5. Fractions of 3 ml were collected from the column at a flow rate of 12 ml/hour. The enzyme activity and protein concentration were monitored and the active fractions were pooled.

### **Determination of Native Molecular Weight**

The native molecular weight of the enzyme was determined under non-denaturing conditions by gel filtration on Sephacryl S-200. The standard proteins used include Lysozyme (14000 Da; 2 mg/ml), chymotrypsin (25000; 2 mg/ml), ovalbumin (45000; 2mg/ml), BSA (66000; 2 mg/ml). Total sample volume of each of the protein markers applied to the column was 5 ml. The proteins were eluted with 0.1 M Tris HCl buffer pH 8.5. Fractions of 5 ml were collected and monitored by measuring absorbance at 280 nm for the protein. The void volume ( $V_0$ ) of the column was determined by the elution volume ( $V_e$ ) of Blue dextran (2 mg/ml). A 5 ml aliquot of the enzyme solution was then applied to the same column and the elution volume of the enzyme was determined.

### **Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

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.

A mixture of the enzyme sample and sample buffer containing SDS (10%), bromophenol blue,  $\beta$ -mecaptoethanol and glycerol was prepared in a concentration of 1:1, and was then heated in boiling water bath for 3 minutes and then allowed to cool. Fifteen microlitre (15  $\mu\text{l}$ ) of the mixture along with the same volume of protein standards were applied to different wells on the slab and electrophoresed at a constant voltage of 70 volts to allow stacking of the proteins. The standard proteins that were used for the calibration of the gel were myosin (212 KDa),  $\beta$ -galactosidase (118 KDa), bovine serum albumin (66 KDa) and carbonic anhydrase (29

KDa), trypsin inhibitor (20 KDa) and lysozyme (14 KDa) which had also been denatured by mixing with SDS and heating for 3 minutes. After stacking was achieved, the voltage was increased to 100 volts to allow separation of the proteins in the resolving gel. After electrophoresis, the gels were stained in 0.04% Coomassie brilliant blue R-250 in 3.5% perchloric acid solution for 1 h, followed by destaining in a solution containing 7.5% acetic acid to increase the sensitivity of detection. After destaining, the lengths of the gels as well as the distance of migration by the different protein bands were measured.

#### Determination of Kinetic Parameters

The kinetic parameters  $K_m$  and  $V_{max}$  of the enzyme were determined by varying concentrations of NaCN between 0.5 mM and 5 mM at a fixed concentration of 10 mM L-cysteine. Also, the concentration of L-cysteine was varied between 0.2 mM and 2.0 mM at a fixed concentration of 10 mM NaCN. 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 Enzyme Activity

The activity of  $\beta$ -cyanoalanine synthase in different buffers at different pH values ranging between pH 5.0 -11 was investigated. All buffers were 0.2 M in concentration. The buffers used were sodium citrate (pH range 5.0 – 6.5), Tris HCl (pH range 7.0 – 9.0), sodium phosphate (pH range 7.0 –7.5) and borate buffer (pH 9.0-10.0). The substrate solution was prepared with the different buffer concentrations and assay was carried out accordingly.

#### Effect of Temperature on the Enzyme Activity

The activity of 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 reaction mixture containing 1 ml of the substrate solution (25 mM L-cysteine and 25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5) was incubated at the indicated temperature and initiated by the addition of an aliquot of the enzyme. The residual enzyme activity was plotted against the different temperatures. Also, the heat stability of the enzyme was determined by incubating 1 ml of the enzyme for 1 h at 30 °C, 40 °C, 50 °C, 60 °C and 70 °C respectively. From the incubated solution, 0.1 ml was withdrawn at 15 min intervals and assayed for residual activity

#### Effect of Metal Ions on the Enzyme Activity

The effect of various metal chlorides on the activity of purified  $\beta$ -CAS was also carried out. The salts of the cations include: NaCl, MnCl<sub>2</sub>, BaCl<sub>2</sub>, KCl, MgCl and NH<sub>4</sub>Cl at concentrations of 1 mM, 5 mM and 10 mM. The salt was incorporated into the substrate solution, containing 25 mM L-cysteine and 25 mM NaCN in 100 mM Tris-HCl buffer, pH 8.5. The effect of different compounds (EDTA, glycine, iodoacetamide, and mercury chloride) on  $\beta$ -CAS activity was investigated. The activity of  $\beta$ -cyanoalanine synthase was determined by assaying the enzyme in the presence of these compounds, at concentrations of 1 mM, 5 mM and 10 mM. The residual activity was also expressed in percentage (%).

#### RESULTS

A total of six bacterial strains were isolated from the effluents of iron and steel company out of which one was selected for further study based on its appreciable  $\beta$ -cyanoalanine synthase production. Cultural, morphological, biochemical and physiological characteristics of the isolate was examined and it was identified as a strain of *Pseudomonas straminea*. The isolate was subjected to growth and  $\beta$ -cyanoalanine activity studies and it was found that the enzyme activity correlates with the rate of growth (Figure 1). The optimum incubation time for maximum  $\beta$ -cyanoalanine activity by *Pseudomonas straminea* was at the 15<sup>th</sup> h.

### Purification of $\beta$ -CAS

The results for the purification of  $\beta$ -CAS are summarized in Table 1. The elution profiles after ion-exchange chromatography on CM- cephadex c-25 and gel filtration on Biogel P100 are shown in Figures 2 and 3 respectively. The enzyme had a specific activity of 273.75 U/mg and a yield of 28.90%. The enzyme was purified by lyophilization, ion exchange chromatography and gel filtration.

### Molecular Weights

The native molecular weight obtained from the plot of the logarithms of molecular weight of standard proteins against the partition coefficient was 54.9 KDa. The plot of the  $K_{av}$  values against the logarithm of the molecular weight is shown in Figure. 4. Figures 5 and 6 show the electrophoregram and calibration curve respectively obtained for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight estimated from SDS-PAGE was 60.3 KDa.

### Kinetic parameters

Lineweaver-Burk plots for varying concentration of sodium cyanide at fixed concentration of L-cysteine gave KM and  $V_{max}$  of  $8.33 \pm 0.05$  mM and  $14.705 \pm 0.32$  MU/ml/min respectively (Figure 7) while that of varying concentration of L-cysteine at fixed concentration of sodium cyanide are respectively  $1.25 \pm 0.26$  mM and  $0.1 \pm 2.48$  MU/ml/min (Figure 8). The values of the kinetic parameters of the Lineweaver-Burk plots are presented in Table 2.

### Effect of pH on the activity of purified *Pseudomonas straminea* $\beta$ -Cyanoalanine synthase

The effect of pH on purified *Pseudomonas straminea*  $\beta$ -CAS activity was determined at different pH ranging from 3.0–10.0. The highest enzyme activity of  $\beta$ -Cyanoalanine synthase from *Pseudomonas*

*straminea* was found to be optimum at pH 9.0. The result is shown in Figure 9.

### Effect of temperature on the activity of purified *Pseudomonas straminea* $\beta$ -Cyanoalanine Synthase

The effect of temperature on the enzyme activity of purified  $\beta$ -Cyanoalanine synthase from *Pseudomonas straminea* was determined by assaying for the enzyme at temperatures ranging from 30 – 90 °C. The activity increases gradually from 30 to the optimum temperature of 40 °C beyond which the enzyme activity decreased gradually. This result is shown in Figure 10.

### Heat Stability of Purified $\beta$ -Cyanoalanine Synthase from *Pseudomonas straminea*

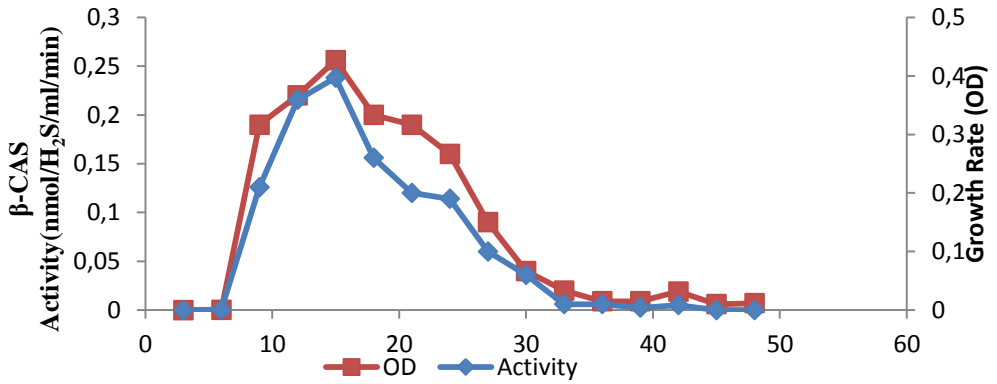
The stability of purified  $\beta$ -Cyanoalanine synthase from *Pseudomonas straminea* at various temperatures was studied by incubating an aliquot of the enzyme at temperatures between 30 – 60 °C.  $\beta$ -Cyanoalanine synthase from *Pseudomonas straminea* was stable at 30-40 °C as it retained about 50% or more of its activity after incubating for one hour at this temperature (Figure 11). At increased temperatures ranging from 50-60 °C, the enzyme lost more than 60% of its activity after incubating for one hour.

### Effect of Metal Ions on $\beta$ -Cyanoalanine Synthase from *Pseudomonas straminea*

All metal ions tested ( $\text{NaCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{NH}_4\text{Cl}_2$ ,  $\text{KCl}_2$ , and  $\text{MnCl}_2$ ) enhanced the activity of the enzyme at the different concentrations tested. The result is as shown in Table 3.

### Effect of Inhibitors on $\beta$ -Cyanoalanine Synthase from *Pseudomonas straminea*

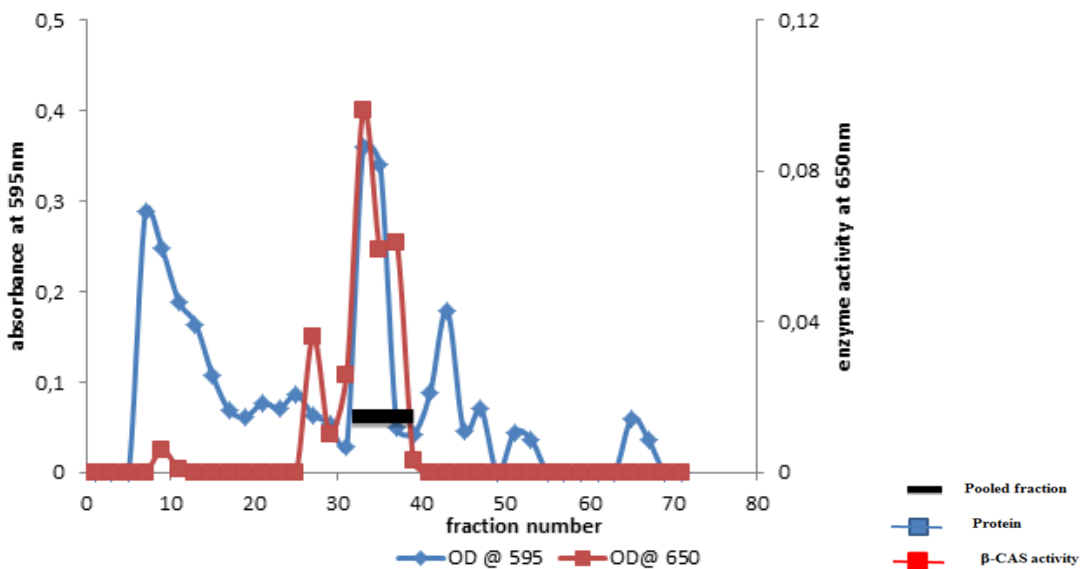
Activity of *Pseudomonas straminea*  $\beta$ -cyanoalanine synthase was greatly affected by mercuric chloride, EDTA, and iodoacetamide with more than 60% loss of activity at 10 mM concentrations of Iodoacetamide and EDTA. The result is as shown in Table 4.



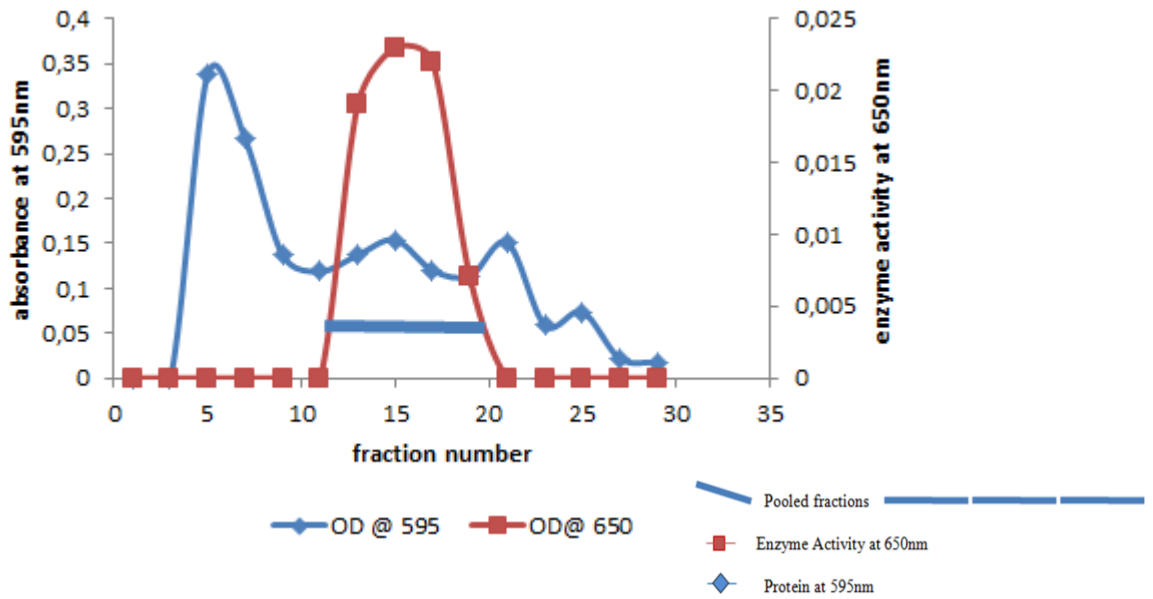
**Figure 1:** Effect of incubation time on  $\beta$ -CAS production and cell growth.

**Table 1:** Summary of purification procedure for *Pseudomonas straminea*  $\beta$ -Cyanoalanine synthase.

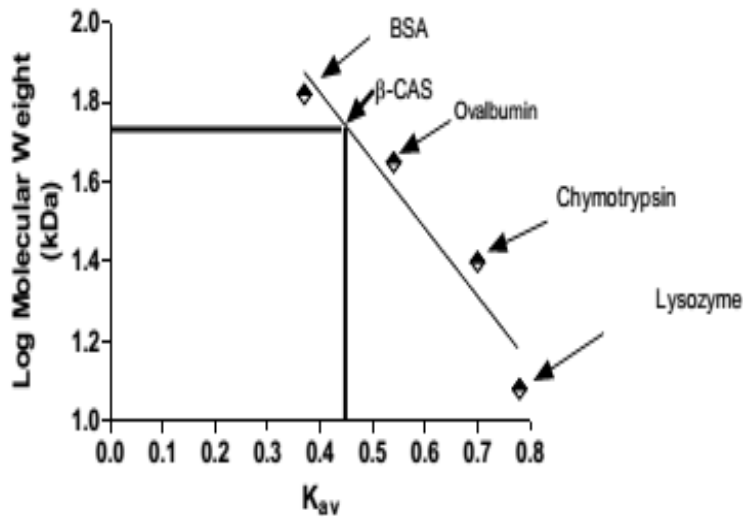
	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification fold
Lyophilization	2291.21	17.02	134.62	66.2	1.19
Ion-exchange on CM-Sephadex c-25	1986.02	13.00	152.75	57.4	1.34
Gel Filtration on Biogel P-100	1002.94	3.66	273.75	28.90	2.41



**Figure 2:** Ion-Exchange Chromatography of *Pseudomonas straminea*  $\beta$ -Cyanoalanine synthase on CM-Sephadex c-25.



**Figure 3:** Gel filtration chromatography on Biogel P-100 of  $\beta$ -Cyanoalanine synthase from *Pseudomonas straminea*.

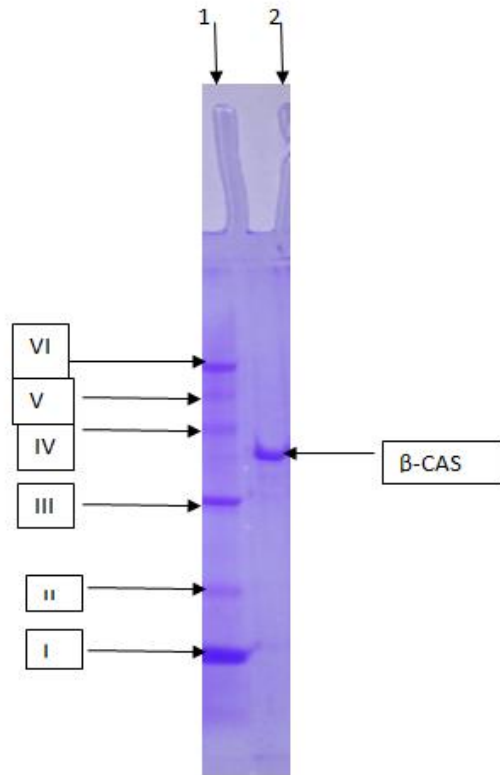


**Figure 4:** Plot of Logarithm of Molecular Weight against Partition Coefficient.

Marker proteins were applied to a Sephacryl S-200 column (1.5 x 40) and eluted using 0.1 M Tris-HCl buffer, pH 8.5 at a flow rate of 20 ml/hour. The void volume,  $V_0$  was determined by the elution of 2 mg/ml solution of Blue Dextran under the same condition.

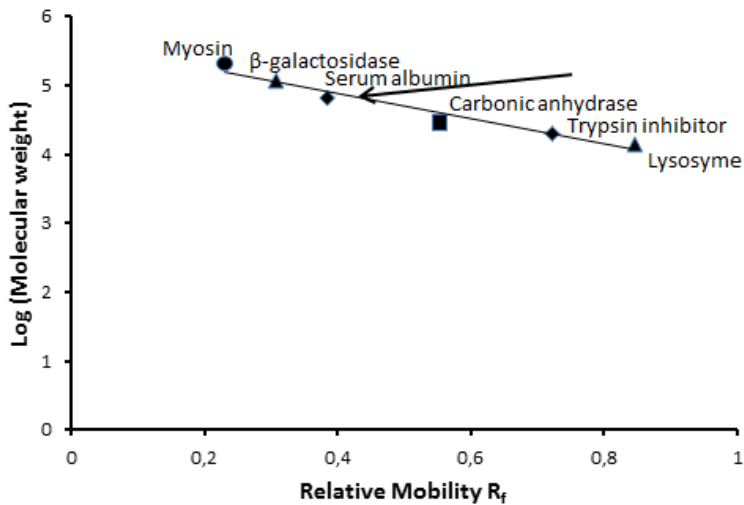
Marker proteins used include Lysozyme (14 kDa), Chymotrypsinogen A (25 kDa), Ovalbumin (44 kDa) and BSA (66 kDa). The position of *Pseudomonas straminea*  $\beta$ -Cyanoalanine Synthase is indicated as shown - $\beta$ -CAS



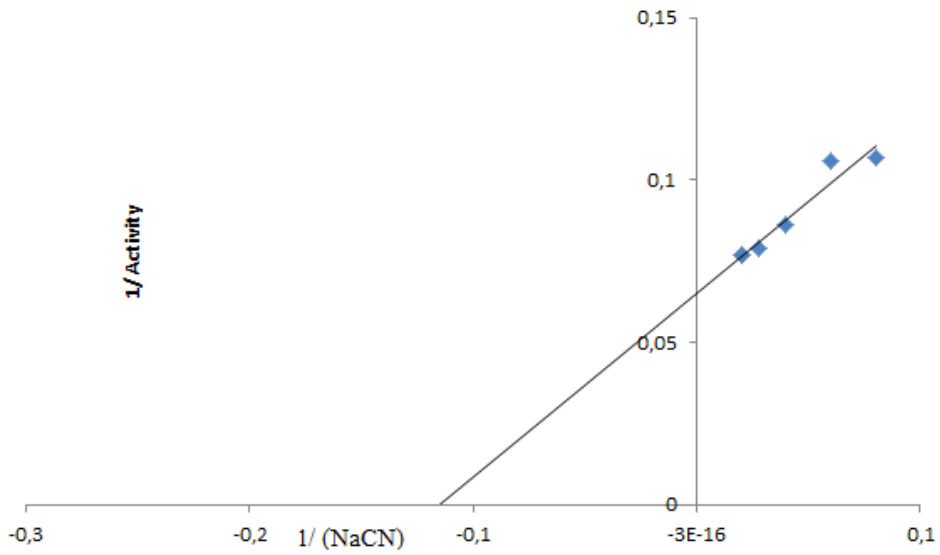


**Figure 5:** Electrophoregram of SDS-Polyacrylamide Gel Electrophoresis of *Pseudomonas straminea*  $\beta$ -Cyanoalanine Synthase.

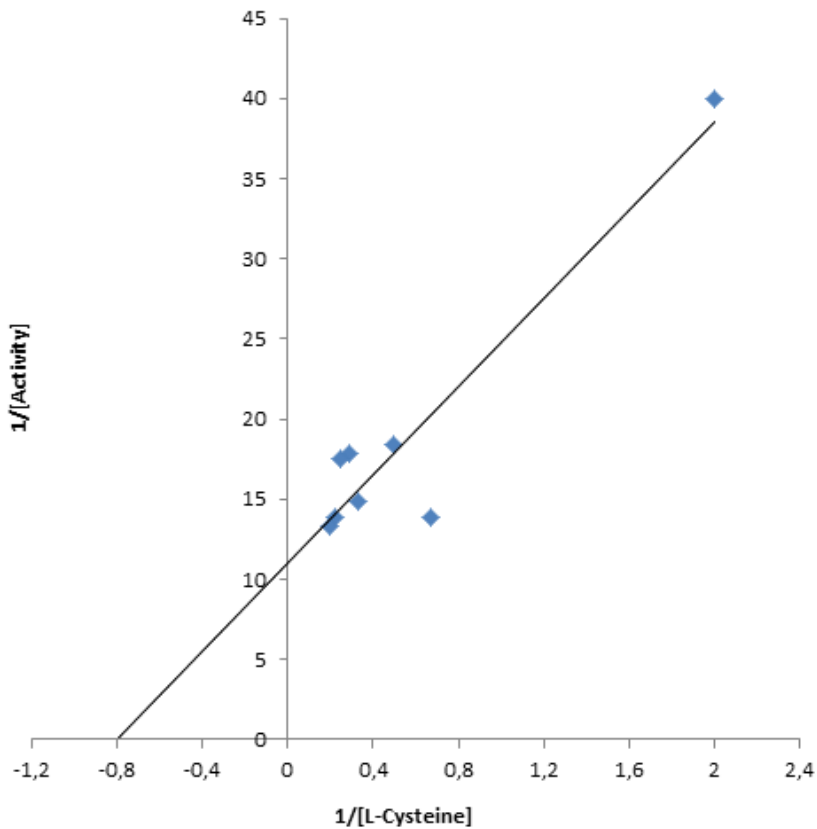
Lane 1 is the molecular weight ladder while Lane 2 is *Pseudomonas straminea*  $\beta$ -Cyanoalanine Synthase. The standard molecular weight markers include I= lysozyme (14 KDa), II= Trypsin Inhibitor (20 KDa), III= Carbonic anhydrase (29 KDa), IV= Serum albumin (66 KDa), V=  $\beta$ -galactosidase (118 KDa), VI= Myosin (212 KDa).



**Figure 6:** Plot of Logarithm of Molecular Weight of Protein Standards against the Relative Mobility ( $R_f$ ). The Relative Mobility of protein standards and  $\beta$ -CAS on the SDS-PAGE gel were determined. The molecular weight of *Pseudomonas straminea*  $\beta$ -CAS was interpolated from the plot of the logarithm of molecular weight of protein standards against the Relative Mobility.



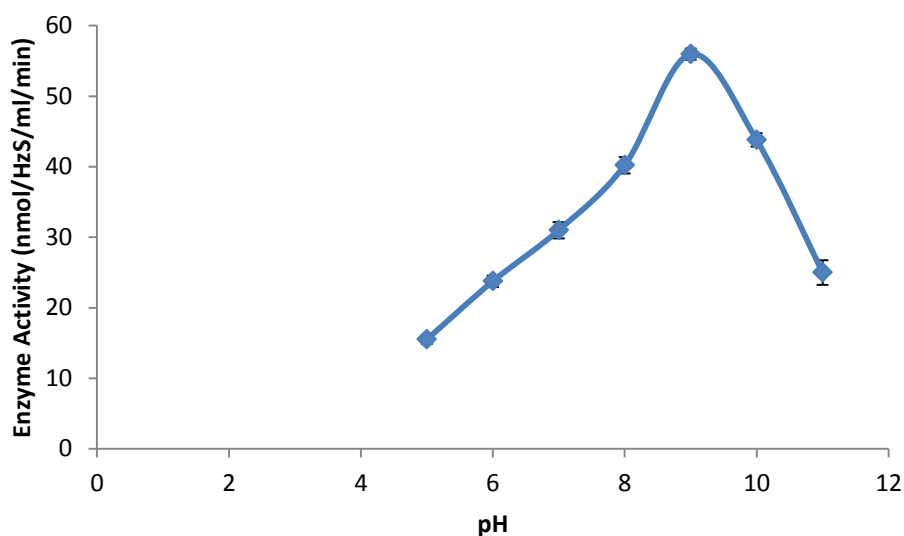
**Figure 7:** Lineweaver-Burk Plot for Varying Concentration of Sodium Cyanide. Lineweaver Burk plot of  $1/V$  against  $1/S$  at varying concentration of NaCN between 0.5 mM and 5 mM at a fixed concentration of 10 mM L-cysteine.



**Figure 8:** Lineweaver-Burk Plot for Varying Concentration of L-cysteine. Lineweaver Burk plot of  $1/V$  against  $1/S$  at varying concentration of L-cysteine between 0.2 mM and 2.0 mM at a fixed concentration of 10 mM NaCN.

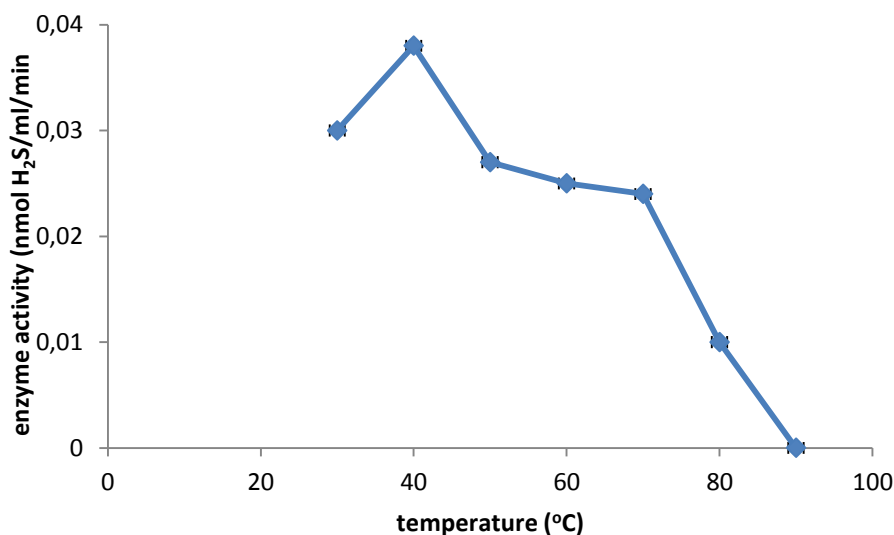
**Table 2:** Summary of the Kinetic Parameters for *Pseudomonas straminea*  $\beta$ -Cyanoalanine Synthase.

Substrate	$K_m$ (mM)	$V_{max}$ (MU/ml/min)
NaCN	$8.33 \pm 0.05$	$14.705 \pm 0.32$
L-cysteine	$1.25 \pm 0.26$	$0.1 \pm 2.48$



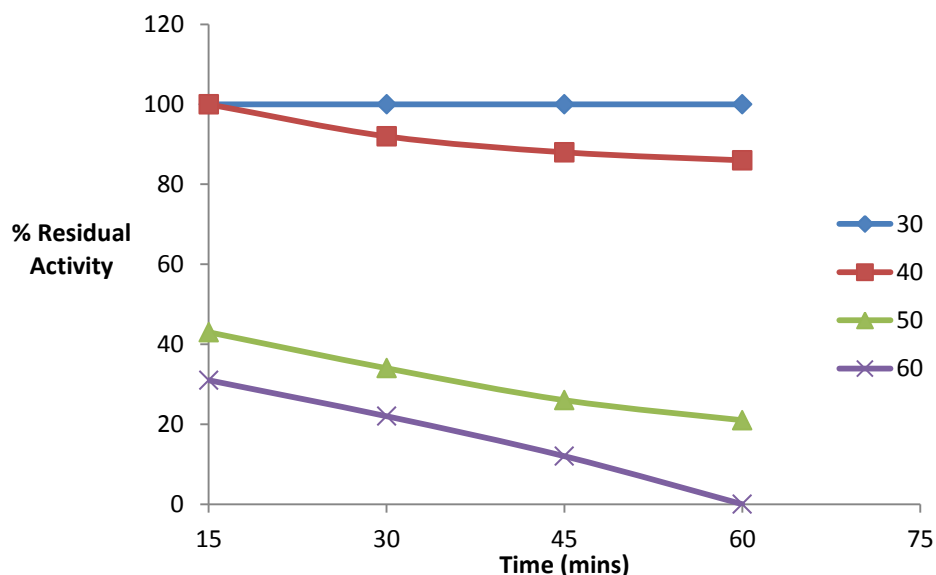
**Figure 9:** Effect of pH on  $\beta$ -CAS activity from *Pseudomonas straminea*.

Optimum pH was obtained using 0.2 M Sodium citrate buffer (pH 5.0-7.5), Sodium phosphate buffer (pH 7.0-7.5), Tris-HCl buffer (pH 7.0-9.0), and Borate buffer (pH 9.0-10.0).



**Figure 10:** Effect of Temperature on  $\beta$ -CAS activity from *Pseudomonas straminea*.

The effect of temperature on enzyme activity was determined by assaying for its activity at temperatures between 30 °C – 90 °C.



**Figure 11:** Heat Stability of  $\beta$ -CAS from *Pseudomonas straminea*.

Aliquots of  $\beta$ -CAS were incubated at different temperatures (30 °C-60 °C) for 1 h. An aliquot of the enzyme solution was taken at every 15 min interval and assayed for  $\beta$ -CAS activity and the residual activity was determined under the standard reaction conditions. The activity at 30 °C, 40 °C, 50 °C, 60 °C was expressed as a percentage of the enzyme incubated at 30 °C which was the control.

**Table 3:** Effect of Metal Salts on  $\beta$ -CAS from *Pseudomonas straminea*.

Salts	1mM	5mM	10mM
NaCl	143.75 ± 1.2	145.31 ± 0.6	112.5 ± 1.1
BaCl <sub>2</sub>	125.0 ± 0.87	117.0 ± 1.1	56.25 ± 1.2
MgCl <sub>2</sub>	135.93 ± 2.4	178.12 ± 1.9	179.68 ± 1.4
NH <sub>4</sub> Cl	131.25 ± 1.2	136.56 ± 0.4	126.81 ± 0.2
KCl	135.31 ± 3.2	173.43 ± 3.6	190.63 ± 2.8
MnCl <sub>2</sub>	120.31 ± 1.8	123.43 ± 0.7	139.06 ± 1.6

Assays were carried out in final concentrations of 1.0 to 10.0 mM of chloride salts of Sodium, Barium, Magnesium, Ammonium, Potassium and Manganese. The relative activity was determined by measuring  $\beta$ -CAS activity in the control that contained no chloride salt and taken as 100%.

**Table 4:** Effect of Inhibitors on the Activity of  $\beta$ -CAS from *Pseudomonas straminea*.

Inhibitors	Relative Activity (%)		
	1mM	5mM	10mM
Glycine	84.12 ± 1.2	56.14 ± 0.9	47.36 ± 2.9
Iodoacetamide	93.65 ± 1.5	32.46 ± 1.8	29.82 ± 0.8
EDTA	57.01 ± 2.4	50.87 ± 1.8	35.08 ± 1.7
HgCl	72.80 ± 3.7	58.77 ± 2.1	24.56 ± 1.4

Assays were carried out in final concentrations of 1.0 to 10.0 mM of inhibitors. The relative activity was determined by measuring  $\beta$ -CAS activity in the control that contained no inhibitor and taken as 100%.

## DISCUSSION

In this research work, bacterial isolates obtained from effluents of iron and steel smelting company were screened for the production of the enzyme  $\beta$ -cyanoalanine synthase, the isolate with the highest  $\beta$ -CAS activity was identified as a *Pseudomonas straminea*.

$\beta$ -CAS produced by *Pseudomonas straminea* was purified and the physicochemical properties investigated. The enzyme was purified using lyophilization, Ion-exchange chromatography on CM-sephadex c-25 and gel filtration on Biogel P-100. The results showed a specific activity of 273.75 U/mg with a percentage yield of 28.90% and a purification fold of 2.41. Specific activity of different values has been reported for  $\beta$ -CAS from different sources. Maruyama et al. (2001) purified  $\beta$ -CAS 140-fold from mitochondrial acetone powder of blue lupine seedlings using a combination of ammonium sulphate precipitation, acetone precipitation, fractionation on Sephadex G-100 column chromatography. Machingura et al. (2016) reported yields of 12%, 15%, 12% and specific activities of 13.8, 6.3 and 16.5 mmol/ml/mg starting with 83.2, 69.8 and 85.3 mg of protein of the leaf, rind and tuber of cassava, respectively.  $\beta$ -CAS was purified from the cytosolic fraction of the gut of grasshopper *Zonocerus variegatus* (L.) by ion-exchange chromatography on DEAE-Cellulose and gel filtration on Sephadex G-100 columns (Ogunlabi and Agboola, 2007). This gave a specific activity of 37.5 nmolH<sub>2</sub>S/min/mg and a yield of 25.6%. The specific activity obtained for  $\beta$ -CAS from *Pseudomonas straminea* may be as a result of difference in the nature of starting material, as against the mitochondrial fractions reported from other plant sources.

The native molecular weight of  $\beta$ -CAS from *Pseudomonas straminea* was determined using gel filtration on Sephacryl S-200. In this study, the molecular weight was estimated as 54.9 KDa. This compares well with the range of molecular weight estimated for  $\beta$ -CAS from other sources which varies from 50,000

to 62,000 Da. (Maruyama et al., 2001; Marrero et al., 2011; Machingura et al., 2016). In blue lupine,  $\beta$ -CAS is a monomeric enzyme, with a molecular weight of about 52 kDa, and contains one mole pyridoxal phosphate per mole of protein, which is essential for the catalytic activity (Nakamura et al., 2000). In spinach (*Spinacia oleracea*) and *Lathyrus latifolius*, the enzyme contains two identical subunits of 28 kDa to 30 kDa, each containing one molecule of pyridoxal phosphate, similar to the  $\beta$ -CAS of the cyanide-producing eubacterium *Chromatium violaceum* (Maruyama et al., 2000; Marrero et al., 2011). The fraction when subjected to SDS-PAGE revealed the presence of a protein band with a molecular weight of 60.3 KDa which shows that it is a monomeric enzyme.

Lineweaver-Burk plots gave apparent  $K_m$  values of  $1.25 \pm 0.26$  mM and  $8.33 \pm 0.05$  mM for L-cysteine and cyanide respectively. The kinetic parameters for  $\beta$ -CAS compared very well with reported values of  $\beta$ -CAS from other sources, showing that *Pseudomonas straminea*  $\beta$ -CAS has a high affinity for L-cysteine. Machingura et al. (2013) reported  $K_m$  values of 0.05 mM for *Vicia angustifolia*. The maximum velocity,  $V_{max}$  obtained for *Pseudomonas straminea*  $\beta$ -CAS was 0.1 nmol H<sub>2</sub>S/ml/min and 14.705 nmol H<sub>2</sub>S/ml/min for L-cysteine and sodium cyanide respectively. Ogunlabi and Agboola (2007) reported  $V_{max}$  values of 2.17 nmol H<sub>2</sub>S/ml/min and 20.0 nmol H<sub>2</sub>S/ml/min for L-cysteine and NaCN, respectively for  $\beta$ -CAS from *Z. variegatus*.  $K_m$  is equivalent to the substrate concentration at which the reaction rate is half maximal and is often used as an indicator of the affinity of an enzyme for its substrate (Nelson and Cox, 2004); a high  $K_m$  indicates weak binding, that is, low affinity of the enzyme for the substrate while a low  $K_m$  indicates strong binding that is high affinity of the enzyme for the substrate (Berg et al., 2002).

$\beta$ -CAS from *Pseudomonas straminea* showed an optimum pH at 9. The optimum pH values reported for  $\beta$ -CAS from different sources fall within the alkaline pH of 8.0 – 10.0. The optimum pH obtained for  $\beta$ -CAS from the cyanide-producing bacterium,

*Chromobacterium violaceum* was found to be pH 9.15, with diethanolamine-HCl as the preferred buffer (Maruyama et al., 2000). Similarly,  $\beta$ -CAS purified from the leaves of spinach (*S. oleracea*) exhibited a single pH optimum at around pH 9.0-9.5 with Tris-HCl buffer (Marrero et al., 2011) and pH optimum for  $\beta$ -CAS from immature seeds of *Vicia angustifolia* was found to be pH 9.4-9.5, also with Tris-HCl buffer (Machingura et al., 2013). Meier et al. (2001) reported an optimum pH of about 9.5 for  $\beta$ -CAS from 10-day-old etiolated blue lupine seedlings.  $\beta$ -CAS from potato tubers was reported to have an optimum pH of 8.0-9.0 (Maruyama et al., 2000). An alkaline pH of 9.0 for optimum activity was reported for  $\beta$ -CAS from grasshopper (*Z. variegatus*) (Ogunlabi and Agboola, 2007).

Optimum temperature of 40 °C was observed for  $\beta$ -CAS activity. Meier et al. (2001) reported a temperature of about 30 °C for optimal temperature of both immobilized and dissolved  $\beta$ -CAS. Studies of  $\beta$ -CAS from cassava over temperature range of 20-45 °C showed that the enzyme had a maximum activity at 30 °C with the activity decreasing significantly above 40°C (Machingura et al., 2016). The optimum temperature for  $\beta$ -CAS from the grasshopper *Z. variegatus* was 30 °C with activity reducing to near zero at 45 °C (Ogunlabi and Agboola, 2007).

Thermal stability studies on the purified enzyme showed that the enzyme was relatively stable at 30-40 °C as it retained about 50% or more of its activity after incubating for one hour at this temperature. At increased temperatures ranging from 50-60 °C, the enzyme lost nearly 60% of its activity after incubating for one hour. This is similar to the  $\beta$ -CAS from cassava where studies over a temperature range of 20–45 °C showed that the enzyme activity decreased significantly above 40 °C (Machingura et al., 2016). A sharp decline in enzyme activity for *Pseudomonas straminea*  $\beta$ -CAS at 40 °C could imply that this enzyme is sensitive to thermal inactivation, a process which apparently results from thermally induced transitions of the native structure which leads

to the exposure of hydrophobic surfaces and irreversible protein association. Consequently, an enzyme loses its compact three dimensional structure at extremely high temperature leading to loss of activity.

The effects of metallic ions on  $\beta$ -CAS were also determined. Salts of  $\text{NH}_4^+$  and metal ions such as  $\text{Na}^+$ ,  $\text{K}^+$  have been reported to relatively stimulate the activity of  $\beta$ -CAS purified from the cyanide producing bacteria, *Chromobacterium violaceum* with 1 mM  $\text{K}^+$  causing 15-20% stimulation of activity; divalent ions such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  also had little effect, with 10 mM  $\text{Ca}^{2+}$  causing 10% stimulation of activity (Maruyama et al., 2000). In the case of *Pseudomonas straminea*  $\beta$ -CAS all the salts tested ( $\text{NaCl}$ ,  $\text{BaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{KCl}$ , and  $\text{MnCl}_2$ ) enhanced the activity of the enzyme at concentrations of 1 mM, 5 mM, and 10 mM.

Activity of *Pseudomonas straminea*  $\beta$ -CAS was greatly affected by iodoacetamide and mercuric chloride which are known site-specific inhibitors. Iodoacetamide can inactivate an enzyme by reacting with a critical cysteine residue (Berg et al., 2002). The reaction catalyzed by  $\beta$ -CAS begins with binding of cysteine to the active site; pyridoxal-5-phosphate (PLP) co-factor in the  $\beta$ -CAS structure identifies the active site. In the first half reaction, the  $\alpha$ -amine of the cysteine reacts with the Cys-49 of the PLP-Lys-95 Schiff base to release the Lys residue. Formation of PLP-Cys allows Lys-95 to act as a general base in the  $\alpha$ ,  $\beta$ -elimination of sulphide resulting in the formation of the  $\alpha$ -aminoacrylate intermediate (Yi et al., 2012). Total inhibition of activity was observed for  $\beta$ -CAS from *Chromobacterium violaceum* at 380  $\mu\text{M}$ . The inhibition was 85% relieved by 2 mM  $\text{Mg}^{2+}$  suggesting that a divalent metal was required for activity (Maruyama et al., 2000).

## Conclusion

The presence of  $\beta$ -Cyanoalanine Synthase in *Pseudomonas straminea* suggests that the enzyme may possess potential cyanide detoxification mechanism. The presence of the enzyme may also be effective for

bioremediation of cyanide effluents from industrial wastewaters.

#### AUTHORS' CONTRIBUTIONS

REO designed the study, supervised the execution and revised the manuscript. OSA participated in the execution of the study (part of MSc thesis) and drafted part of the manuscript. BI also participated in the execution of the study, especially, the microbial work. NT was involved in the microbial study, the culturing and isolation of the organism and CO was involved in the location of the study area and participated in the drafting of the manuscript. All the authors read and approved the final manuscript.

#### COMPETING INTERESTS

The authors wish to state that they have no competing interest

#### ACKNOWLEDGEMENTS

The authors acknowledge Dr. K. Awojobi, Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria, for his contribution in the study. Mr. S. Chukwuejim, Department of Biochemistry, Federal University Oye-Ekiti, Nigeria, is also appreciated for his support during the collection of samples.

#### REFERENCES

AGENCY for TOXIC SUBSTANCES AND DISEASE REGISTRY (ATSDR) (2006). *Toxicological Profile for Cyanide (draft for public comment)* Atlanta, GA, US Department of Health and Human Services. 2-45 pp.

Akcil A, Mudder T. 2003. Microbial Destruction of Cyanide Wastes in Gold Mining: Process Review. *Biotechnology Letter*, **25**: 445-450.

Berg JM, Tymoczko JL, Stryer L. 2002. *Biochemistry* (5th edn). W. H. Freeman: New York.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**: 248-254.

Bonner ER, Cahoon RE, Knapke SM, Jez JM. 2005. Molecular basis of cysteine biosynthesis in plants: Structural and functional analysis of O-acetylserine sulfhydrylase from *Arabidopsis thaliana*. *Journal of Biological Chemistry*, **280**: 38803–38813.

Claus MT, Zocher GE, Maier TH, Schulz GE. 2005. Structure of the O-acetylserine sulfhydrylase isoenzyme CysM from *Escherichia coli*. *Biochemistry*, **44**: 8620–8626.

Ebbs S. 2004. Biological degradation of cyanide compounds. *Current Opinion in Biotechnology*, **15**: 231–236. DOI: 10.1016/j.copbio.2004.03.006

Ezzi-Mufadda I, Lynch, JM. 2002. Cyanide catabolizing enzymes in *Trichoderma* spp. *Enzyme and Microbial*, **31**: 1042-1047.

Kao CM, Chen KF, Liu JK, Chou SM, Chen SC. 2006. Enzymatic degradation of nitriles by *Klebsiella oxytoca*. *Applied Microbial Biotechnology*, **71**(2): 228-33. DOI:org/10/007/S00253-005-02290

Lee TY, Kwon YS, Kim DS. 2004. Oxidative treatment of cyanide in wastewater using hydrogen peroxide and homogeneous catalyst. *Journal of Environmental Science and Health*, **39**(3): 787-801

Lineweaver H, Burk D. 1934. The Determination of Enzyme Dissociation Constants. *Journal of American Chemical Society*, **56**(3): 658–666.

Machingura M, Sidibe A, Wood AJ, Ebbs SD. 2013. The  $\beta$ -cyanoalanine pathway is involved in the response to water deficit in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry*, **63**: 159–169.

Machingura M, Salomon E, Jez JM, Ebbs SD. 2016. The  $\beta$ -cyanoalanine synthase pathway: beyond cyanide detoxification. *Plant, Cell and Environment*, **39**: 2329-2341

Mansfeldt T, Heike L, Kurt B, Ruben K. 2004. Cyanide Leaching From Soil Developed From Coking Plant Purifier Waste As Influenced By Citrate. *Vadose Zone Journal*, **3**: 471-479.

- Marrero-Degro J, Marcano-Velázquez J, Siritunga D. 2011. Isolation and Characterization of Novel  $\beta$ -Cyanoalanine Synthase and Cysteine Synthase Genes from Cassava. *Plant Molecular Biology Rep.*, **29**: 514-524.
- Maruyama A, Ishizawa K, Takagi T. 2000. Purification and Characterization of  $\beta$ -Cyanoalanine Synthase and Cysteine Synthases from Potato Tubers: Are  $\beta$ -Cyanoalanine Synthase and Mitochondrial Cysteine Synthase Same Enzyme?. *Plant Cell Physio.*, **41**(2): 200-208.
- Maruyama A, Saito K, Ishizawa K. 2001. Beta-cyanoalanine synthase and cysteine synthase from potato: molecular cloning, biochemical characterization, and spatial and hormonal regulation. *Plant Molecular Biology*, **46**: 749–760.
- Meier M, Janosik M, Kery V, Kraus JP, Burkhard P. 2001. Structure of human cystathionine beta-synthase: A unique pyridoxal 59-phosphate-dependent heme protein. *EMBO Journal*, **20**: 3910–3916.
- Nakamura T, Yamaguchi Y, Sano H. 2000. Plant mercaptopyruvate sulfurtransferases: Molecular cloning, subcellular localization and enzymatic activities. *European Journal of Biochemistry*, **267**: 5621-5630.
- Nelson DL, Cox MM. 2004. *Lehninger Principles of Biochemistry* (4th edn). New York: W. H. Freeman.
- Ogunlabi OO, Agboola FK. 2007. A soluble beta-cyanoalanine synthase from the gut of the variegated grasshopper *Zonocerus variegatus* (L.) *Insect Biochemistry and Molecular Biology*, **37**: 72-79. DOI: 10.1016/j.ibmb.2006.10.003
- Parga JR, Shukla SS, Carrillo-Pedroza FR. 2003. Destruction of cyanide waste solutions using chlorine dioxide, ozone and titania sol. *Waste Management* **23**(2): 183-191.
- Patil Y, Paknikar K. 2000. Biodegradation of silver–cyanide from electroplating industry wastewater. *Letter of Applied Microbiology*, **30**(1): 33-37.
- Rodrigo A, Almudena O, Jorge L, Fernando P. 2005. State Of The Art of Passive Treatment Technologies for The Abatement Of Cyanides From The Mining Industry: Experiences In Asturias (Spain). 9th International Mine Water Congress. Oviedo- Spain.
- UNITED STATES ENVIRONMENTAL PROTECTION AGENCY USEPA 2010. Hydrogen Cyanide and Cyanide Salts; CASRN Various. Integrated Risk Information System (IRIS), U.S. Environmental Protection Agency Chemical Assessment Summary, National Center for Environmental Assessment.
- Weber K, Osborn M. 1975. Protein and sodium dodecyl sulphate. Molecular weight determination, polyacrylamide gel and related procedures. In *The Proteins*, Newrath H, Hill RL (eds). Academic Press: New York. 1(3); 179-223.
- Zlosnik JEA, Williams HD. 2004. Methods for Assaying Cyanide in Bacterial Culture Supernatant. *Letters in Applied Microbiology*, **38**: 360–365