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Enzymatic cyanide detoxification by partially purified cyanide dihydratase obtained from *Serratia marcescens* strain AQ07

Detoxificación enzimática de cianuro mediante el uso de una dihidratasa parcialmente purificada de la cepa *Serratia marcescens* AQ07

K.I. Karamba[1,2], M.K. Sabullah[3], A. Zulkharnain[4], C. Gomez-Fuentes[5] and S.A. Ahmad[1*]

[1]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*
[2]*Department of Microbiology, Faculty of Science, Bauchi State University, Gadau Main Campus, Bauchi State, Nigeria.*
[3]*Faculty of Science and Natural Resources, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.*
[4]*Department of Bioscience and Engineering, College of Systems Engineering and Science, Shibaura Institute Technology, 307 Fukasaku, Minuma-ku, Saitama, 337-8570, Japan.*
[5]*Department of Chemical Engineering, Universidad de Magallanes, Avda. Bulnes 01855, Punta Arenas, Regiòn de Magallanes y Antàrtica Chilena, Chile.*

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Abstract

The partially purified enzyme of indigenously isolated *Serratia marcescens* strain AQ07 was utilised to develop the best form of cyanide detoxification method that is eco-friendly and cost effective. The present study evaluates the feasibility of the enzyme to degrade high cyanide concentrations and the possible metabolic pathways involved, for which the protein concentration and cyanide detoxification activity were quantified. Bacterial cells grown in cyanide incorporated medium were disrupted by sonication and the resultant cell free extract were tested for metabolic pathway. The cell free extract was precipitated by ammonium sulphate precipitation and partially purified by ion exchange chromatography using DEAE cellulose. The maximum enzyme activity achieved was 2125 μM/min. The partially purified enzyme was found to be able to detoxify 82% of 2 mM KCN in 10 min of incubation and cyanide degradation (or depletion) rate showing a linear increase with increasing enzyme concentration. The effective accruing of ammonia as metabolite illustrated that the detoxification was ensued via the function of cyanide dihydratase. Additional confirmation through SDS-Page showed that the molecular weight of enzyme was assessed to be ~38 kDa, which is tandem with the reported cyanide dihydratases. Hence, the use of enzyme as a substitute to live bacterial cells in detoxification of cyanide illustrates various advantages such as the capacity to withstand and detoxify higher cyanide concentration and total reduction in the total cost of process since nutrient provision is immaterial.

Keywords: *Serratia marcescens*, detoxification, enzyme, cyanide dihydratase.

Resumen

Una enzima parcialmente purificada aislada de la cepa nativa *Serratia marcescens* AQ07 se utilizó para desarrollar un método de detoxificación de cianuro ecológico y rentable. El presente estudio evalúa la factibilidad de la enzima para degradar altas concentraciones de cianuro y las posibles vías metabolicas implicadas, para lo cual se cuantifica la concentración de proteínas y la actividad de detoxificación de cianuro. Las células bacterianas cultivadas en medio con cianuro fueron lisadas por sonicación y el extracto libre de células resultante se analizó para determinar la vía metabólica de detoxificación. El extracto libre de células se precipitó mediante sulfato de amonio y se purificó parcialmente mediante cromatografía de intercambio iónico utilizando dietanolamina de celulosa. La máxima actividad enzimática alcanzada fue de 2125 μM/min. Se encontró que la enzima parcialmente purificada era capaz de detoxificar el 82% de KCN 2 mM en 10 minutos de incubación, mostrando la tasa de degradación del cianuro un incremento lineal con el aumento de la concentración de la enzima. La acumulación de amoníaco como metabolito ilustra que la detoxificación se produjo a través de la acción de la cianuro dihidratasa. Adicionalmente, a través de SDS-Page se determinó que el peso molecular de la enzima es alrededor ~38 kDa, valor que está en el rango de las dihidratasas de cianuro conocidas. Finalmente, el uso de enzimas como catalizador, en vez de las células bacterianas para la desintoxicación de cianuro muestra varias ventajas, como la capacidad de resistir y detoxificar una mayor concentración de cianuro y la reducción en el costo total del proceso, ya que en este caso la provisión de nutrientes es irrelevante.

Palabras clave: *Serratia marcescens*, destoxificación, enzima, cianuro dihidratasa.

* *Corresponding author. E-mail*: aqlima@upm.edu.my
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1 Introduction

Over the last few decades, studies on the remediation of xenobiotic compounds using microorganisms have been conducted and have proven to be one of the best alternatives (Interiano-López *et al*., 2019; Martínez-Sánchez *et al*., 2018; Esquivel-Viveros *et al*., 2009). In this technology, microorganisms or their constituents especially enzymes are used to degrade or transform the wastes (Balagurusamy, 2005; Bertrand *et al*., 2013). As a result of the disadvantages of conventional methods used in cyanide detoxification in industrial processes, biological methods have been generating a lot of interest (Dursun and Aksu, 2000). Cyanide known as HCN or CN^-, represented inclusively as CN. It is volatile and reactive leading to a short half-life ($t_{1/2}$). Cyanide occurs as ordinary metabolite in wide variety of plants, fungi and animals despite its renowned toxicity. Its toxicity can be quickly absorbed, through inhalation, ingestion or dermis (Bhandari *et al*., 2014). Two metabolic reactions have been suggested for cyanide degradation in bacteria, which includes hydrolytic and oxidative reactions in which the oxidative reactions are being catalysed by either cyanide monooxygenase or cyanide dioxygenase and the hydrolytic pathway is being catalysed by cyanide dihydratase (Ebbs, 2004). *Bacillus pumilus* C1, *Pseudomonas fluorescens* NCIMB 11764 and *Pseudomonas paucimobilis* were all reported to degrade cyanide, resulting in the formation of ammonia and carbon dioxide as by product, which indicates oxidative reactive reactions (Harris and Knowles, 1983; Whitlock and Mudder, 1986). Meanwhile, *Pseudomonas stutzeri* AK61, *Pseudomonas fluorescens* NCIMB 11764 and *Rhodococcus* sp. UKMP-5M were discovered to degrade cyanide via hydrolytic reactions, converting it to ammonia and formate with molecular weight of enzyme being ~38 kDa, which indicates the catalytic activity of cyanide dihydratase (Kunz *et al*., 1994; Watanabe *et al*., 1998; Maniyam *et al*., 2013). Furthermore, *Pseudomonas fluorescens* NCIMB 11764 isolated from activated sludge of a coke plant have been revealed to remove cyanide, forming ammonia and formate under aerobic and anaerobic conditions (Kunz *et al*., 1992).

Various manufacturing practices comprising the production of artificial fibres, chemical intermediates, pharmaceuticals and rubber along with coal processing, ore leaching, and metal plating engulf millions litres of cyanide annually (Ebbs *et al*., 2010). In addition, cyanide hydrolysis of some compounds by microbes, plants and insects could lead to the release of hydrogen cyanide, which adds to the natural manifestation of cyanide in the environment (Dash *et al*., 2009). Moreover, discharges of food and feedstuff production also contain a considerable amount of cyanide emanating from cyanogenic glycosides (Bolarinwa *et al*., 2006). Since cyanide is a powerful metabolic inhibitor that could be fatal to all living organisms, it is consequently vital to remove it from seepages before discharging it to the environment.

Considering these observations, various methods have been proposed for the management of cyanide-harbouring effluents so as to bring down the cyanide concentrations to regulatory limits. Alkaline chlorination oxidation process is the most commonly adopted technique for cyanide adulterated waste treatment. Nevertheless, applying such treatment is frequently subjected to recurrent disappointments, owing to several shortfalls such as high working cost and payments of royalty, frequently ineffectual in managing cyanide complexes as a result of slow reaction level, production of sludge, license is required before disposal, produce detrimental by products and are commonly appropriate only for the treatment of aqueous centred effluents (Dash *et al*., 2009). Thus, a better substitute designed to attain high removal efficiency at a cheap rate is preferred for the management of cyanide-bearing effluents (Maniyam *et al*., 2015).

Employing biological techniques bids economic benefits as well evolving as a better environmentally friendly method in cyanide-harbouring effluent detoxification (Dash *et al*., 2009; Karamba *et al*., 2015b; Perumal *et al*., 2013). Furthermore, biological treatment method has the capacity to achieve cyanide detoxification efficacy equal to those acquired via chemical or physical methods under optimum conditions (Akcil *et al*., 2003). Fascinatingly, the utilisation of enzymes isolated from microorganism could suggest a new benefit to eventually augment cyanide-removal capability in contrast to the use of whole microorganism having the enzyme (Maniyam *et al*., 2015). The use of enzymes offer substantial advantages like the ability to bear and remove higher cyanide concentrations, disregard the requisite for nutrient provision and the consequence of other noxious chemicals as well as providing the stage for momentously augmented kinetics (Adams *et al*., 2001). Considering these advantages, Novo Industries has effectively commercialised the application of

cyanide dihydratase from *Alcaligens denitrificans* with optimal cyanide detoxification capacity and steadiness with respect to ionic strength, pH and existence of heavy metal ions in treating of cyanide bearing waste waters (Maniyam *et al*., 2015).

In this research, the capacity of partially purified enzyme of *Serratia marcescens* strain AQ07 (Karamba *et al*., 2018) in detoxifying cyanide was assessed. The likely pathway for the detoxification of cyanide by the bacteria was clarified. This bacterium was studied for enzymatic cyanide degradation and the end product was detected after enzymatic assay. Favourable conditions such as pH, temperature were optimised for the enzymes. Kinetics studies of cyanide degradation were carried out and the possible pathway utilised by the enzyme was detected. Several literature studies indicated that the microbial cells or their enzymes are utilised in the treatment of sewages releases from industries (Parmar *et al*., 2013). Dubey and Holmes (1995) suggested that cyanase converts cyanate into ammonia and carbon dioxide. To date, there are no reports published on the role of cyanide dihydratase in cyanide detoxification by *Serratia marcescens* enzyme. Accordingly, this study is designed to explore the enzyme properties responsible for the biodegradation of cyanide to consider the mercantile relevance for the removal of cyanide in waste waters. In this paper, the purification and identification of this enzyme were reported which was established to be cyanide dihydratase catalysing cyanide conversion to ammonia.

2 Materials and methods

2.1 Cyanide degrading assay

The cyanide-degrading activity assay for the bacteria was carried out in a buffer medium (Potivichayanon and Kitleartpornpairoat, 2010) containing 200 mg/L filter sterilised potassium cyanide. Resting cells of the bacteria were diluted in the buffer medium to obtain a solution of O.D 600 of 1.0 using Shimadzu U.V. Mini 1240 spectrophotometer, which was then added into the medium. The mixture was incubated for 72 hrs on an orbital shaker at 150 rpm. Samples were taken at stipulated intervals and centrifuged at 10,000 xg. Concentrations of ammonia and residual cyanide were tested using phenate and γ-picoline barbituric acid method and bacterial growth was established using CFU/mL technique, respectively.

2.2 γ-Picoline barbituric acid method

This method was carried out based on the protocols described by Nagashima (1977). Sample (500 μL) was collected and placed in a centrifuge tube (1.5 mL). Buffer solution (250 μL) pH 5.2 was added. Chloramine T trihydrate salt (13 μL) was added. It was stirred vigorously with vortex mixture machine and let to stand for five min. γ-Picoline barbituric acid reagent (150 μL) was lastly added and let to stand for final 10 min. The presence of blue coloration illustrates the concentration of cyanide residue. Absorbance was taken at 605 nm by the use of spectrophotometer against a blank reagent. The real concentration of residual cyanide was read by use of a calibration curve ranging from of 0 to 3.5 mg/L KCN.

2.3 Enzyme activity and protein concentration assay

The activity of enzyme was established based on ammonia production detected according to phenate method as described by Rand *et al*. (1976). While, protein concentration was determined according to Lowry method as described by Lowry *et al*. (1951).

2.4 Preparation of cell free extract

The isolated strain of the bacteria was cultivated in 3 L of buffer medium containing 200 mg/L potassium cyanide. The bacteria was allowed to grow for a period of 48 hours and harvested at early stationery phase by centrifugation at 10,000 xg for 15 min. The pellet was washed two folds using 50 mM phosphate buffer (pH 7.0) and was re-suspended in the same buffer. The suspension of the washed cells was disrupted by sonication for 10 min on ice and centrifuged at 10,000 g for 15 min at 4 °C. The pellets were discarded and the supernatant was utilised as the cells free extract for cyanide degrading enzyme purification. All steps were carried out in the purification process at 4 °C.

2.5 Enzyme assays for oxidative reactions

Experiments for various cyanide degrading enzymes were conducted to ascertain the specific enzyme and biodegradation pathway responsible for the biodegradation of cyanide. For each enzyme activity conducted, samples were collected at 10, 20 and 30 min time intervals to check the time for maximum activity. Each experiment was conducted in triplicate and distilled water was used as a control sample. In order to detect oxidative reactions, three enzymes

were assayed, namely cyanide dioxygenase, cyanide monooxygenase and cyanase.

2.6 Cyanide dioxygenase assay

This process was conducted according to the protocol of Kunz *et al*. (1998). The mixture of reactions was prepared using 200 μL of crude extract, 5 μL of 2.0 M KCN, 10 μL of 4 mM NADH and 35 μL of 50 mM sodium potassium buffer pH 7.0. The reaction mixture final quantity is 250 μL. Reaction was allowed to occur at 27 °C. The final products of the reaction catalysed by cyanide dioxygenase are ammonia and carbon dioxide. The detection of ammonia illustrates enzyme activity (Rand *et al*. 1976).

2.7 Cyanide monooxygenase assay

The assay for the detection of this enzyme showed no difference with cyanide dioxygenase, but produced a variety of final product. Cyanate was formed in this reaction and is very difficult to estimate. Cyanase was being tested as an alternative method to prove the action of this enzyme due to the potential it has in converting the cyanate to ammonia (Parmar *et al*., 2013).

2.8 Cyanase assay

This method was carried out using the procedure described by Anderson (1980). 5 mL of the reaction mixture in cyanide dioxygenase protocol was taken and 400 μL of sodium bicarbonate as well as 0.04 μL of 50 mM potassium phosphate buffer pH 7.6 were added. The final volume of reaction mixture measured as 5.08 mL. Reaction mixture was allowed take place at 27 °C. It is assumed that cyanate formation by the activity of cyanide monooxygenase will trigger another reaction catalysed by cyanase. The end result is ammonia which indicates activity of enzyme illustrating degradation of cyanide by the enzyme.

2.9 Enzyme assays for hydrolytic pathway

In order to detect hydrolytic pathway, cyanide dihydratase assay was conducted according to the method described by Parmar *et al*. (2013). 2 mL of crude extract was taken and 1 mL of 2.0 mM KCN was added, followed by the addition of 50 mM phosphate buffer pH 8.0 and the final volume observed is 5.0 mL. Reaction was allowed to take place at 27 °C. Phenate method was conducted to test for the ammonia to confirm the activity of cyanide dehydratase (Rand *et al*., 1976).

2.10 Purification of cyanide-degrading enzymes

First purification method was conducted base on 35, 45, 55 and 65% of ammonium sulphate precipitation of the crude extract. Dialysis of the saturated sample was carried out using snake skin dialysis tubing 10K MWCO, which is a selective permeable membrane. Second purification method was Ion exchange chromatography using DEAE cellulose. An ion exchange column with 5 cm diameter and 80 cm height harbouring DEAE-Cellulose was provided. 5 mL of ammonium sulphate saturated solution was applied into the column with 50 mM potassium phosphate buffer pH 7.0, by using HPLC pump through the column set at a flow rate of 1 mL min^{-1}. The fraction that has high enzyme activity was concentrated using vivaspin 20 at 5000 g, 4 °C for 10 min. The active fraction was referred to as purified enzymes. The purified enzyme was kept at -20 °C. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% polyacrilamide gels to see the efficiency of cyanide dihydratase as described by Laemmli (1970).

2.11 Effect of temperature on cyanide dihydratase activity

The assay for the detection of temperature was carried out according to the protocol explained by Ahmad *et al*. (2017). The reaction mixture in the following order; 100 μL enzyme, 50 μL of 2 mM KCN and 100 μL of 50 mM phosphate buffer pH 8, was separately incubated at temperatures of 15, 18, 21, 24, 27, 30 33 and 36 °C (± 1 °C) for 25 min. The selection of temperatures was to determine the capability of mesophilic enzyme to perform in low, intermediate and high temperatures. Cyanide dihydratase was assayed as described previously.

2.12 Effect of pH on cyanide dihydratase activity

This study was conducted to examine the ideal pH for the activity of enzyme. A buffer system was utilized to determine the pH profiles for optimizing enzyme assay by the use of purified enzymes. The buffers involved include citrate buffer with the pH of 5 and 6, phosphate buffer with the pH of 7 and tris-HCL buffer with the

pH of 8 and 9 Ahmad *et al*. (2017). 100 μL of purified enzyme was incubated at different pHs for 25 min at room temperature. Cyanide dihydratase activity was examined as described previously.

2.13 *Kinetic study of cyanide-degrading enzyme*

The periodic loss of cyanide was determined at intervals in closed vials containing 100 μL enzyme and 50 μL of KCN in various concentrations. Therefore, to evaluate the influence of enzyme concentration in cyanide detoxification, increasing amount of enzyme was added to the reaction mixture containing 5 mM KCN and the rate of cyanide removal was assessed after 25 min of incubation. The cyanide detoxification activity was further examined at varying cyanide concentrations (1 to 6 mM) after 25 min of incubation. Appropriate samples of aliquot were periodically taken and diluted as required for the assessment of remaining cyanide. Discrete preparation without enzyme was carried out to act as the control and assessment of cyanide loss due to abiotic activity. The K_m and V_{max} values were analysed by Michelis-Menten and Lineweaver-Burk using Grap pad prismTM version 5.0 software and Microsoft excel.

3 Results and discussion

Several cyanide degrading bacteria were isolated from the soil of Universiti Putra Malaysia and were characterised for the best cyanide degradation (Karamba *et al*., 2015a; 2017; 2018). One of the bacteria designated as *S. marcescens* strain AQ07 exhibited the best cyanide degrading capacity. It was identified through gram staining as a gram negative, rod shaped bacterium, and possess the ability to proliferate under aerobic conditions.

3.1 *Cyanide degradation by S. marcescens strain AQ07*

Fig. 1 illustrates the overtime cultivation of *S. marcescens* strain AQ07 in a buffer medium containing 200 mg/L KCN. An initial lag phase of about 12 hours was observed during the degradation of cyanide. Growth of the bacteria started after then. Within 72 hours, 95.3% of the cyanide was removed from the bacteria. The final O.D 600 after 72 hours incubation was 0.52, equivalent to 1.37 x 1016 CFU/mL. In the process of cyanide degradation, continuous accrual of ammonia was detected in the reaction mixtures, though the accumulation was less within the first 24 hours after which the build-up ensued.

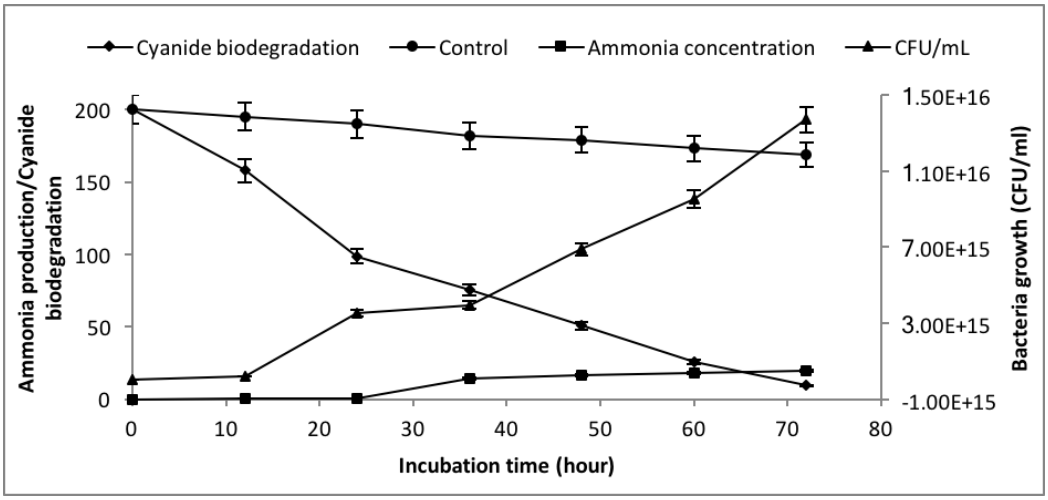

Fig. 1. Cyanide degrading activity, bacteria growth and ammonia production by *S. marcescens* strain AQ07. The control experiment contains no bacteria. Data represent mean ± STDEV, n=3.

The total ammonia accumulated after 72 hours was 19.6 μg. A lot of bacteria able to degrade cyanide and release ammonia as by product have been reported. *Pseudomonas fluorescens* NCIMB 11764 was reported to produce ammonia as end product after cyanide biodegradation (Dorr and Knowles, 1989). *Pseudomonas* sp., *Alcaligens xylosoxidans* subsp. denitrificans DF3, *Pseudomonas stutzeri* AK61 and *Bacillus pumilus* C1 were reported to degrade cyanide producing ammonia and formate as their end products (Ingvorsen *et al*., 1991; Meyers *et al*., 1991; Watanabe *et al*., 1998; White *et al*., 1988). *S. marcescens* strain AQ07 has the potential of removing 200 mg/L KCN with high growth yield and could withstand the effect of most heavy metals in the production medium (Karamba *et al*., 2016). This is a very good commercial property of the bacteria, which can be applied for biological treatment of cyanide. Furthermore, its enzyme can also be prepared for cyanide degradation.

3.2 Preparation of cell free extract

Gram negative bacteria possess an isolated rigid layer of additional materials roughly characterised as polysaccharide, protein and lipid beside murrain (Roberts and Cabib, 1982). The cell wall of *S. marcescens* is a total rigid layer composing murein with covalently linked lipoprotein molecules (Braun *et al*., 1970). In this study, after the disruption of cell wall using a sonication machine, the harvested cells of the bacteria (supernatant) was regarded as cell free extract and was used to determine the extent of cells disruption by measuring the total protein concentration. The highest amount of protein obtained was 498.8 mg BSA/L in 30 min of incubation and the enzymatic activity of the enzymes stands at 0.799 μg (Fig. 2).

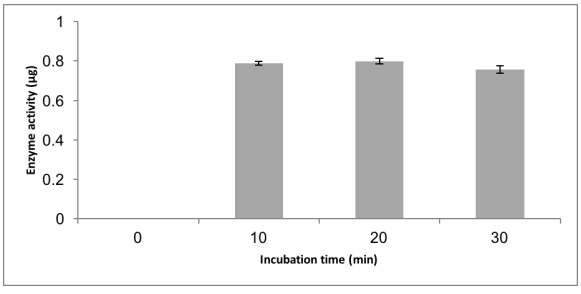

Fig. 2. Enzyme activity of cyanide dihydratase via hydrolytic reaction. Data represent mean ± STDEV, n=3.

Table 1. Various enzyme activities obtained in the course of different reactions.

Enzyme assay	Enzyme activity (Min)		
	10	20	30
Cyanide dioxygenase	-	-	-
Cyanide monooxygenase	-	-	-
Cyanase	-	-	-
Cyanide dihydratase (cyanidase)	0.778	0.799	0.756

3.3 Discovering cyanide degradation pathway

Enzymatic assays on three various enzymes catalysed two essential reactions; oxidative and hydrolytic, which were conducted as shown in Table 1. The results indicated that no activity was recorded for cyanide dioxygenase, cyanase and cyanide monooxygenase. Though in cyanide monooxygenase assay cyanate is being formed in the reaction which is very difficult to estimate, cyanase was being assayed as an alternative technique to prove the action of this enzyme because it has the potential of converting cyanate to ammonia (Parmar *et al*., 2013). Cyanide dihydratase activity of 0.799 μg was recorded after 20 min of incubation (Table 1 and Fig. 2). This result suggests that strain AQ07 might use hydrolytic pathway by converting KCN to ammonia and formate. Conversion of cyanide to ammonia has proven the metabolism of cyanide via different metabolic reactions. *Rhodococcus* sp. UKMP-5M has been reported to demonstrate hydrolytic pathway, leading to the formation of ammonia and formate (Maniyam *et al*., 2013). *Pseudomonas* sp. was reported to demonstrate oxidative pathway catalysed by cyanide dioxygenase responsible for the formation of ammonia and carbon dioxide (Parmar *et al*., 2013). *Bacillus pumilus* was reported to degrade cyanide using cyanide dihydratase enzyme, indicating hydrolytic reaction (Jandhyala *et al*., 2003). *Fusarium lateritium* has been reported to remediate cyanide, taking hydrolytic pathway catalysed by cyanide hydratase (Nolan *et al*., 2003). It is well established that there are several metabolic pathways that operates with the help of catalytic enzymes, since the degradation of cyanide illustrates that *S. marcescens* strain AQ07 possesses the essential enzymatic metabolic action to remove cyanide. This prompts the investigation of specific enzymes resulting to the metabolism. Several enzymatic assays were conducted to ascertain the type

of enzymes by testing the end product of the reaction product. Several literatures on different pathways indicate that the majority of the final products from reaction products formed are ammonia with or without intermediates (Parmar *et al*., 2013). It was also confirmed that ammonia have been detected in this study, demonstrating that cell free extract obtained from *S. marcescens* strain AQ07 have the capacity to metabolise KCN added in the medium as substrate. Though the test for formate and formamide was not conducted in this research, the lack of enzyme activity in the oxidative reactions carried out and the affirmation of activity observed in hydrolytic reaction conducted illustrates the possible formation of formate or formamide as end product in the reaction mixture.

3.4 Purification of cyanide dihydratase

The results (Fig. 3) attained 55% saturation levels demonstrates the paramount fraction for advance purification step as the enzyme activity attained is 0.261 μg trailed by 65% with 0.202 μg. The fraction with 35% saturation shows the lowermost activity with enzyme activity of 0.15 μg. Fraction 45% saturation illustrated low enzyme activity of 0.16 μg. The precipitated enzyme, which is the active fraction of 55% and dialysed, was preceded to ion exchange chromatography after concentration with viva spin 20. It has been reported that cyanide dihydratase enzyme has a molecular weight of ~38 kDa (Watanabe *et al*., 1998; Maniyam *et al*., 2013), based on SDS page electrophoresis (Fig. 4), which illustrates that a band was produced at ~38 kDa after the electrophoresis.

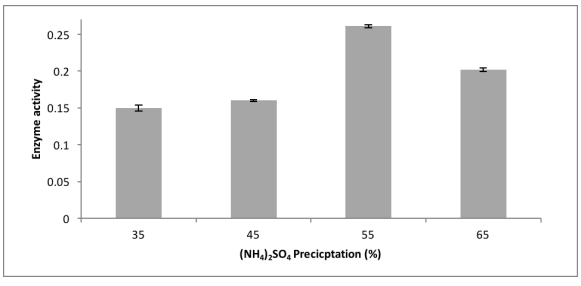

Fig. 3. Protein concentration and enzyme activity after ammonium sulphate precipitation. Data represent mean ± STDEV, n=3.

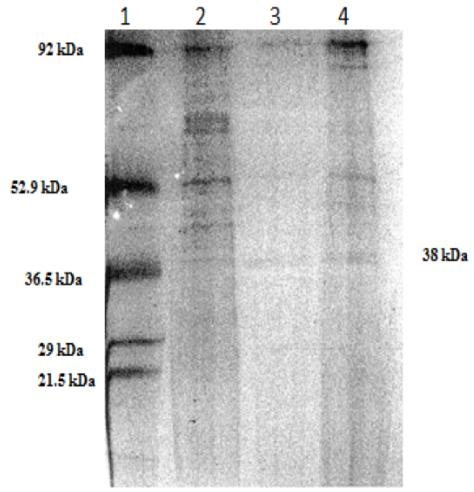

Fig. 4. SDS Page for purified cyanide dihydratase. Lane 1 Molecular mass marker in kDa (Prestained SDS page marker low range); Lane 2: Cell free extract of cyanide dihydratase after high speed centrifugation; Lane 3: Purified cyanide dihydratase after ion exchange chromatography (DEAE Cellulose). 4: Precipitated cyanide dihydratase after ammonium sulphate precipitation.

This result may suggest that the functional enzyme responsible for the degradation is a protein aggregate that consisted ~38 kDa polypeptide subunit and goes in tandem with the pathway determination in which activity was obtained at cyanide dihydratase method of enzyme assays. The results of the purification steps are illustrated in the cyanide dihydratase purification table (Table 2). It demonstrates the total activity for crude extract to be 79.9 and total protein of 49, 880 mg BSA/L given a specific activity of 1.60184E-05. After ammonium sulphate precipitation, the total activity reduces 2.61 and total protein reduces to 4813 mg BSA/L with specific activity of 5.42281E-05, this may perhaps be due to precipitation of the protein by ammonium sulphate, since it reveals the hydrophobic interior of the environment and make the protein less soluble. Moreover, after the purification process by DEAE cellulose, the total activity reduces to 0.165 and total protein to 17.4 mg BSA/L with specific activity of 0.00105364. The yield for ammonium sulphate precipitated sample is 3.27% and has a purification fold of 3.385 because protein is accrued in this step thereby illustrating lesser purification fold while the yield for DEAE cellulose is 0.207% with purification fold of 65.78 illustrating very good purification fold.

Table 2. Cyanide dihydratase purification table.

Step	Volume (mL)	Activity (U)	Total Activity (units)	Protein (mg BSA/ L)	Total Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude	100	0.799	79.9	498.8	49,880	1.60184E-05	100	1
Ammonium sulphate precipitation	10	0.261	2.61	481.3	4,813	5.42281E-05	3.266	3.385
DEAE cellulose	3	0.055	0.165	17.4	52	0.00105364	0.206	65.777

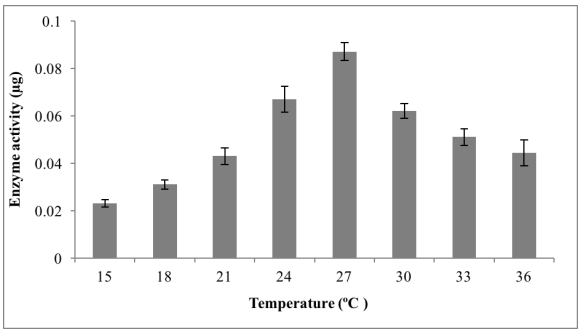

Fig. 5. Effect of temperature on cyanide dihydratase activity. The error bars represent mean ±STDE. n=3.

3.5 Effect of various temperatures on cyanide dihydratase activity

The temperature effects on the activity of cyanide dihydratase were determined in an array of 15 to 36 °C (Fig. 5). This range was considered to be the most active range for cyanide biodetoxification (Parmar *et al*., 2013). It is observed from the graph shows that cyanide dihydratase is active in these ranges and shows it is a mesophilic enzyme because it is most active within the intermediate temperatures. The augmentation of temperatures from 15 to 27 ºC shows increase in the potential energy of the molecules in the system. These energies may include translational energy, vibrational energy, and rotational energy of the molecules with the maximum activity at 27 ºC. Nonetheless additional increase in temperatures from 27 to 36 ºC decreases the degree of enzyme activity. This may perhaps be as a result of denaturation of the enzyme due to higher temperature and thus the enzyme becomes sedentary (Ahmad *et al*., 2017). Preceding reports demonstrates ideal temperature for cyanide dihydratase activity from *Pseudomonas* species to be 27 ºC (Parmar *et al*., 2013), which is the same with this project report. Cyanide dihydratase acquired from *B. pumilus* was reported to be active at normal room temperature, which might be approximately 27 ºC (Jandhyala *et al*., 2003). Cyanide dihydratase purified from *B. pumilus* C1 was described to be most active at 26 ºC (Meyers *et al*., 1993), which is very close to result acquired in this research.

3.6 Effect of pH levels on cyanide dihydratase activity

The effect of pH was studied using five different pHs with 3 different buffers; citrate buffer (pH 5 and 6), phosphate buffer (pH 7) and tris-HCL buffer (pH 8 and 9), each at 50 mM. Fig. 6 shows the outline and peak level of cyanide dihydratase activity for the enzyme on pH. It illustrates that the enzyme is active in all ranges studied, but most active at pH 8. Enzyme activity was found to be low at pHs 5, 6, 7 and 9. Previous reports have testified that cyanide hydratase purified from *Pseudomonas* species have maximum activity at pH 8.5 (Parmar *et al*., 2013). Jandhyala *et al*. (2003) reports that the optimal pH for cyanide dihydratase obtained from *B. pumilus* is 8, while Meyers *et al*. (1993) reports that pH of 7.8 to 8 are the optimum pH for *B. pumilus* C1 cyanide dihydratase.

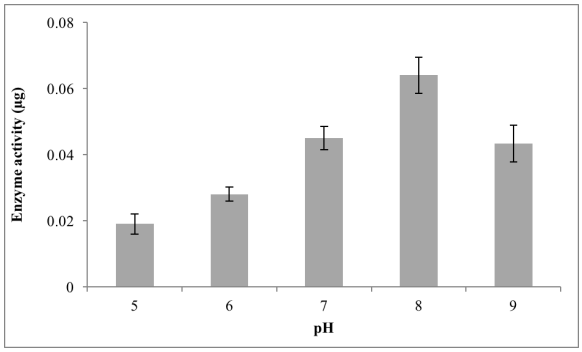

Fig. 6. Effect of pH on cyanide dihydratase activity.

Error bars represent ± mean STDVE. n=3.

Varying pHs could change the ionic state of amino acid side chains in the active sites, there by resulting to an optimum activity concurring to the formation of strongest bond and deactivation as a result of suboptimal non-covalent formation with substrates. In general, hydrogen and ionic bonds are commonly affected by pH and they are vital bonds in affixing substrates to enzymes (Sabullah *et al*., 2017; Halmi *et al*., 2014).

3.7 Determination of cyanide dihydratase temperature stability

Purified cyanide dihydratase acquired from *S. marcescens* isolate AQ07 was found to be very stable at -20 ºC for 70 days. At 4 ºC and room temperature, cyanide dihydratase activity was reduced to 4 and 1 days respectively, illustrating the instability of the enzyme (Fig. 7). Enzyme instability as such observed to be due to certain factors like tertiary and quaternary protein denaturation by thermal vibration, which results to a loss of co-factors, contaminating protease due to handling and improved activity at elevated temperatures, enhanced oxidation of sulfhydryl groups at elevated temperatures (Scopes, 2013). Purified cyanide dihydratase from *Rhodococcus* sp. UKMP 5M has been reported to be stable for 40 days at -80 ºC and 3 days at room temperature.

3.8 Recovery of cyanide detoxification activity in partially purified enzyme of S. marcescens strain AQ07

Rates of cyanide utilisation when applied at concentration of 1 to 5 mM KCN, respectively was examined with partially purified enzyme collected from the resting cells of *S. marcescens* strain AQ07 cultured with 200 mg/L KCN (Fig. 8). It was obviously evident that the addition of lesser cyanide concentration abetted cyanide detoxification significantly related to that of applying 5 mM KCN. The enzyme succeeded in reducing cyanide concentration from 1 to 0.17 mM within 10 min of incubation, which stands at 83% reduction. Instead, the addition of 5 mM observed detoxification of cyanide to 1.61 mM after 10 min of incubation stands at 67.8% reduction. No degradation was further noticed until 25 min. Fascinatingly, the enzyme of this bacteria can detoxify 1 to 2 mM of KCN within 10 min of incubation, whereas free and immobilised cells of the bacteria degrade 200 mg/L in 72 and 24 hrs, respectively (Karamba *et al*., 2017b) illustrating the outstanding capacity of the bacteria to be employed in detoxifying real cyanide containing effluents. No cyanide loss was obtained in the control experiment that contains no enzyme. The influence of enzyme concentration on cyanide detoxification activity determined at an initial concentration of 5 mM KCN was studied.

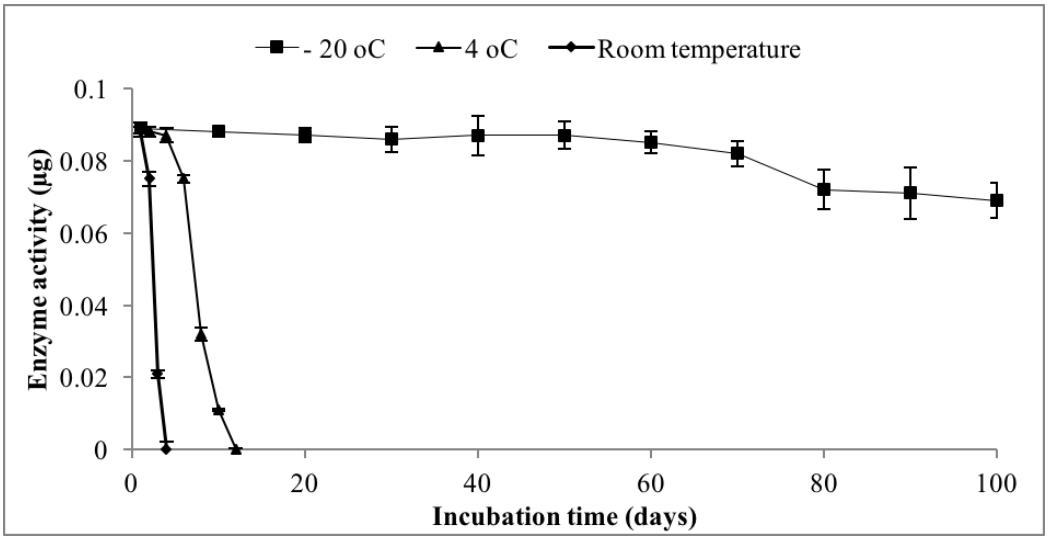

Fig. 7. Effect of prolong pre-incubation temperatures on cyanide dihydratase. Error bars means ± mean STDE. n=3.

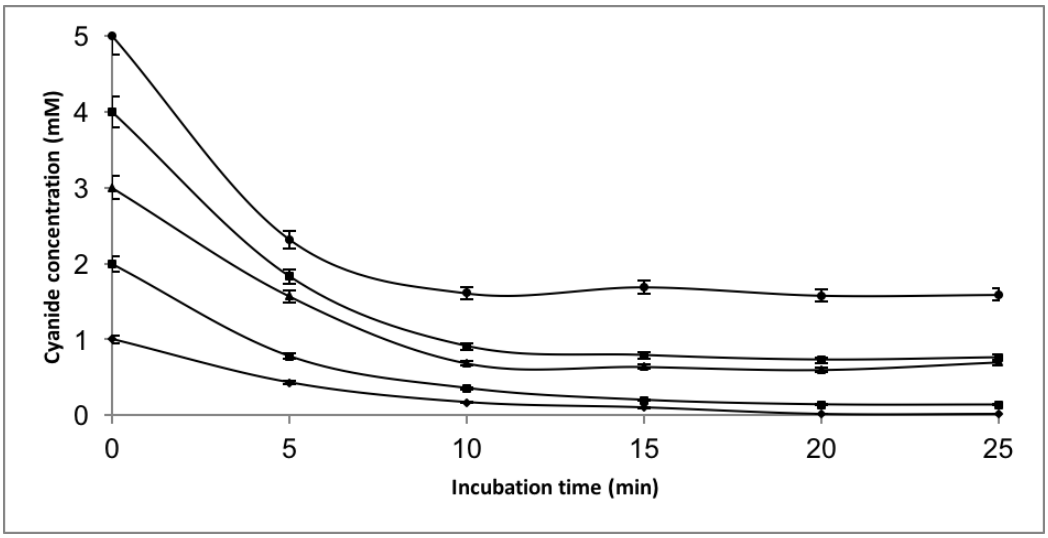

Fig. 8. Phase progression detoxification of 1 to 5 mM KCN by partially purified enzyme of *Serratia marcescens* strain AQ07.

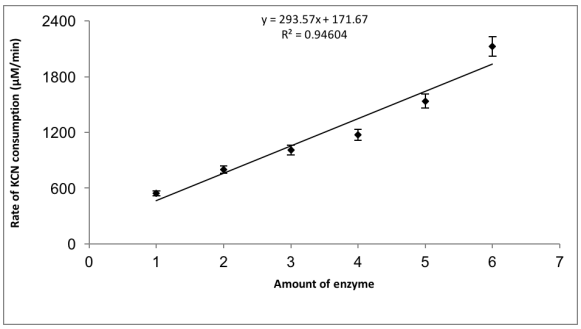

Fig. 9. Rate of cyanide disappearance measured as a function of enzyme concentration. Data represent mean ± STDEV, n=3.

It was pretty clear in Fig. 9 that the cyanide detoxification activity was reliant on the enzyme concentration under selected reaction conditions. A linear connection was experimental among the rate of cyanide utilisation and concentration of enzyme. When the enzyme concentration was amplified for five times, the cyanide consumption rate amplified from 546 μM/min to 2125 μM/min, recording a significant raise of cyanide detoxification activity. It was pretty clear from these results that the cyanide degradation rate accelerates as the quantity of enzyme is augmented. Increasing enzyme concentration led to more quantity of active enzyme in the continuous reaction system, which results in greater total activity that eventually improved the degrading activity of cyanide by the *S. marcescens* strain AQ07 enzyme. Furthermore, the cyanide detoxification enzyme does not require the addition of any cofactor, which demonstrates economic advantage (Maniyam *et al*., 2015).

3.9 Kinetics study of cyanide degradation by partially purified enzyme of S. marcescens strain AQ07

The initial velocity of enzymatic detoxification of cyanide by partially purified enzyme of *S. marcescens* strain AQ07 was studied over concentration of cyanide in the array of 0.1 to 5 mM (Fig. 10). The initial rate of substrate depletion improved as a task of cyanide concentration under the planned experimental design. Maximum rate of cyanide consumption was obtained with utmost activity of 0.181 μg/min experiential at 5 and 6 mM cyanide. Hydrolysis of cyanide by partially purified enzyme fits to simple Michaelis-Menten inundation kinetics when examined over cyanide concentration of 5 mM KCN (Fig. 11). Lineweaver-Burk plot (Fig. 12) revealed a linear response at 5 mM KCN and less. Michaelis-Menten constant (K_m) for finest fit standards of 26.52 and V_{max} value of 1.13 were determined.

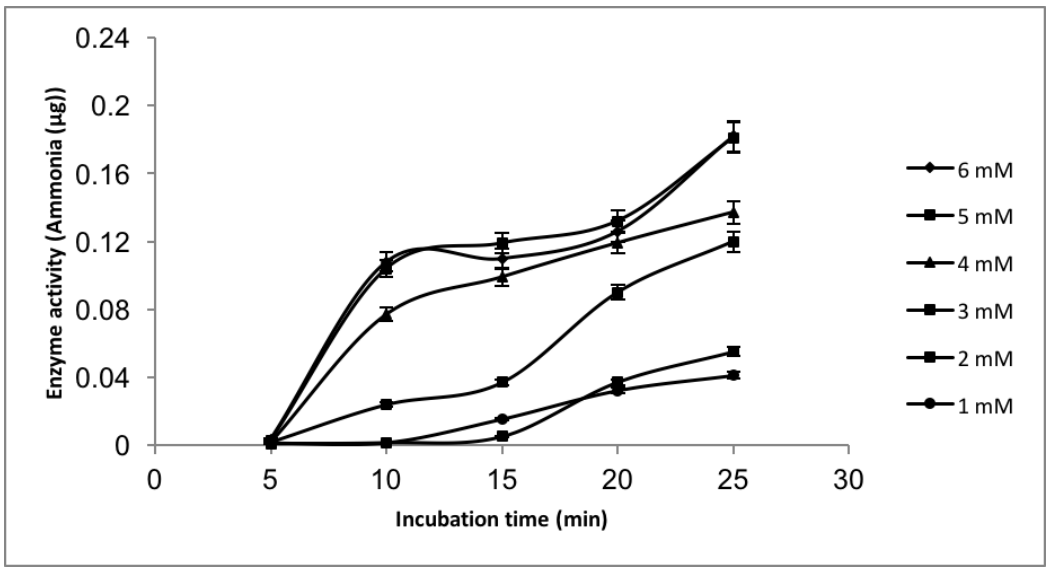

Fig. 10. Enzyme activity as a result of cyanide detoxification of 1 to 6 mM KCN by partially purified enzyme of *Serratia marcescens* strain AQ07. Data represent mean ± STDEV, n=3.

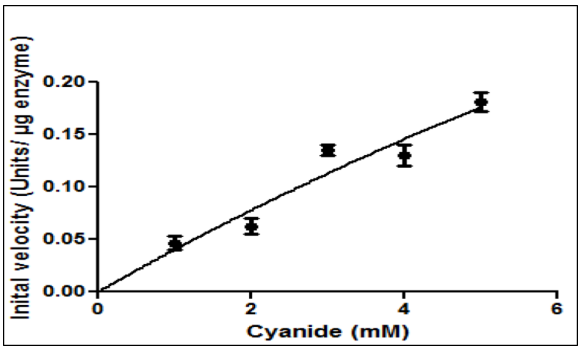

Fig. 11. Michaelis-Menten Plot with cyanide substrate. Data represent mean ± STDEV, n=3.

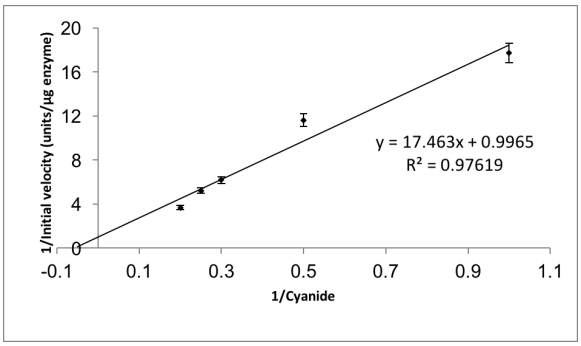

Fig. 12. Lineweaver-Burk Plot with cyanide as substrate. Data represent mean ± STDEV, n=3.

Conclusions

The present study deals with the deployment of partially purified enzyme of *S. marcescens* strain AQ07, which indicates encouraging result in the detoxification of cyanide to harmless end products via the function of cyanide dihydratase. Optimisation of the enzymatic detoxification of cyanide was provided by manipulating the parameters such as enzyme and cyanide concentrations. In this study the maximum protein obtained using Lowry method was 498.8 mg BSA/L within 30 min incubation with a maximum enzymatic activity of 0.799 μg. These results illustrate that the strain follows hydrolytic pathway in detoxifying cyanide. The maximum temperature effect obtained on cyanide dihydratase activity was 27 °C and the maximum effect of pH was 8. The stability of the enzyme was tested which illustrates that it is very stable at -20 °C for 70 days and completely unstable at 4 °C. Detoxification by enzyme offers various discrete advantages compared to live bacterial cells methods like the employment of a better straightforward process, supporting the utilisation of enzymes, vibrant activity under serious reaction circumstances and the capacity to eliminate environmental distresses. The enzyme of these bacteria succeeded in reducing the 83% of 1 mM cyanide within 10 min and 67.8% of 5 mM enzyme in 10 min. The maximum rate of 6 mM cyanide

consumption was 0.181 μg/min which fits Michaelis-Menten inhibition kinetics and Lineweaver-Burk plot. Due to these confident qualities, the use of enzymes offers a more achievable substitute to the existing general procedures applied for the management of industrial wastes harbouring cyanide. Furthermore, no obligation for co-factor is required throughout the enzymatic reaction that additionally backs the probability of bringing out an effective enzymatic system for applied management of effluents bearing cyanide in industrial scale. Moreover, it was found that it is only suitable to immobilise the enzyme using a suitable encapsulation matrix to be utilised for hands-on employment in managing real effluents bearing cyanide. Henceforward, more intricate investigations need to be conducted by considering the prolonged existence and reusability of the immobilised enzymes to realise capable cyanide detoxification technology.

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