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Isolation, screening and optimization of *Bacillus cereus* for a thiosuphate sulphur transferase production

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ABSTRACT

This research was carried out to isolate rhodanese, a thiosuphate sulphurtransferase producing bacterium from effluents of Ife Iron and Steel Smelting Company with the aim to screen the isolates for rhodanese production, to select the best producer of the enzyme and to optimize the production conditions. The isolates were presumptively identified using Bergey's manual of determinative bacteriology. The best rhodanese-producing strain was further identified by 16S rRNA gene sequencing. Optimal conditions for rhodanese production were determined using standard methods. Twelve bacteria strains were isolated from the effluent and one active strain was selected for further study. The organism was identified as *Bacillus cereus* by sequencing of its 16S rRNA gene. The optimum production was obtained at the 39th hr at 30ºC and optimum pH of 8.0. Best nitrogen and carbon source for production were found to be casein and potassium cyanide (KCN) with rhodanese activity of 0.930 ± 0.04 and 0.346 ± 0.002 RU/ml respectively. The production of rhodanese by *B. cereus* suggests the involvement of the enzyme in cyanide detoxification mechanism and this may be employed in the treatment of cyanide-containing effluents.

KEY WORDS: *Bacillus cereus*, Effluents, Isolate, Optimization, Rhodanese, Cyanide.

1. INTRODUCTION

Cyanide is an important chemical produced on a large scale for use by several industries such as mining (gold extraction) and electroplating industry. These industries release large quantities of untreated liquid waste which contains mostly heavy metals (White, 2000). The high metabolic inhibition potential of free cyanide classified it as the most toxic form (Knowles, 1986). Exposure to cyanide through water surfaces is the major exposure route for most animals affected by cyanide poisoning. High cyanide lipid solubility enhance its absorption through inhalation and skin. High concentration of cyanide can permanently damage some living species in sea and rivers. Industrial activities such as ore leaching, organic synthesis, and metal plating produce significant quantities of cyanide. Cyanide obstructs the normal metabolism in two ways. It binds with the ferric form of heamoglobulin and methemoglobin in the blood to form cyanomethemoglobin which cannot transport oxygen and secondly, it inhibits the last enzyme complex (cytochrome c oxidase) of the electron transport chain in cell mitochondria (Chin, 2000). The continuous discharges of untreated effluents to water bodies pose a danger to the environment. Chemical processes applied in most cases for cyanide degradation are toxic when discharge to the environment. Biological methods of removing cyanide from industrial wastewaters have been reported to be cost-effective (Watanabe, 1998; Akcil, 2003; Siriantapiboon, 2007). Some microorganism such as *Bacillus* and *Klebsiella* have been reported to survive in cyanide solution due to their ability to metabolize cyanide to non-toxic end products such as thiocyanate (Kao, 2003; Rao, 2010; Ebbs, 2004). The enzyme rhodanese (EC 2.8.1.1) has been reported to be involved in cyanide detoxification in the microorganism (Colnaghi, 1996). Enzymatic bioremediation may provide efficient means of escape for an environment under cyanide stress because enzymes are not affected by inhibitors of microbial metabolism. Rhodanese has been known to be one of the enzymes responsible for cyanide detoxification to a less toxic compound (thiocyanate) using thiosulphate as substrate (Itakorode, 2019). Recently, the presence of rhodanese was reported in *Bacillus cereus* (Itakorode, 2019). However, the optimization conditions for production were not investigated. Cyanide-containing effluent must be properly treated before been discharged into the environment. This study, therefore, aimed to isolate, screen and optimized rhodanese producing bacterium from industrial effluents that may be employed in the treatment of cyanide-containing effluents.

2. MATERIALS AND METHODS

Collection of samples: Effluents were collected from Iron and Steel Smelting Company located at Fashina, Ile-Ife, Osun State, Nigeria 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 of collection

Screening and Isolation of bacteria: One milliliter of the effluent sample was taken into a test tube containing 9 ml of sterile water and was serially diluted. A dilution of 10^{-9} was plated on modified Bushnell Hass agar medium containing potassium cyanide (KCN) for primary isolation of the bacteria. The plates were incubated at 37°C for 96 h to select for cyanide degrading bacteria. To indicate the rhodanese producing potential of the organism, isolates

were grown separately in 100ml of a production medium containing 0.3% KCN, 1% bacteriological peptone, 0.5 % yeast extract, 0.5 % NaCl at pH 9.5. Standard solutions of the isolates were used to inoculate the media, followed by incubation at 30°C for 48 h on a rotary at 170 rpm. The media were then checked for enzyme activity.

Isolates Characterization and Identification: The bacteria strains were presumptively identified using Bergey's manual of determinative bacteriology. The best rhodanese-producing strain was further identified by sequencing its 16S rRNA gene. The molecular analyses were carried out using molecular techniques and equipment available at the Bioscience Centre of the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. Extraction of bacteria DNA was carried out using modified method of Trindade (2007).

Polymerase Chain Reaction (PCR) Amplifications: PCR amplification consists of 0.4 µl of 10mM dNTPs, 2 µl of 25 mM $MgCl_2$, 0.24 µl of Taq polymerase (1 U/µl) (Promega USA), 5 µl of 5× PCR buffer, 4 µl of the DNA solution and 1 µl of 10 pmol each of primer (Forward 5'- CCAGCAGCCGCGGTAATACG -3' and Reverse 5'- ATCGGCTACCTTGTTACGACTTC -3'). The solution was made up to 25µl with DNase free water. The PCR amplicons were visualized using 1.5% agarose gel electrophoresis. Sequence similarity search of the GenBank data was done using the National Centre for Biotechnology and Information (NCBI) Basic Alignment Search Tool (BLAST) program.

Enzyme production: The production medium contained 0.3% KCN, 1% bacteriological peptone, 0.5 % yeast extract and 0.5 % NaCl in 100 ml conical flask. The flasks were autoclave at 121ºC for 15 min. The One millilitre of the standardized pure bacterium isolate in normal saline was inoculated into a conical flask. This was incubated at 37ºC for 39 h with agitation at 170 rpm (De Souza, 2001). The optical density was checked, and it was centrifuged at 12000 g for 15 min. The supernatant was used as the crude enzyme.

Assay for Enzyme Activity: Rhodanese activity was determined using sodium thiosulphate and potassium cyanide as substrates. The assay mixture consisted of borate buffer (50 mM, pH 9.4), 0.25 M KCN (0.2 ml), 0.25 M (0.2 ml) $Na_2S_2O_3$ and 0.1 ml of the enzyme. The mixtures were incubated at room temperature and terminated by the addition of 15% formaldehyde (0.5 ml). 1.5 ml of Sorbo reagent (consisted of 10g Fe $(NO3)_2$. $9H_2O$, 20 ml HNO3 and 80 ml of distilled water) was then added (Sorbo, 1953). Absorbance was read using spectrophotometer at 460 nm. One unit of rhodanese activity (RU) is defined as the amount of the enzyme that catalyzed one micromole of the substrate to product in one minute (Sorbo, 1953). The protein concentration was determined by the Braford method, bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

Optimization process for maximum rhodanese production: The optimal conditions for the enzyme production were determined by subjecting enzyme production to various physiological conditions such as incubation time, inoculum size, carbon sources, nitrogen sources, temperature and hydrogen ion concentration (pH).

Effect of Incubation Time and Inoculum Volume on Enzyme Production: Incubation time was checked by incubating the production medium at 37ºC for 48 h with agitation at 170 rpm. 5 ml of the growth medium were withdrawn at regular intervals of 3 h, centrifuged and were analyzed for rhodanese activity. The effect of inoculum was checked by varying the volume from 0.5 ml to 3.0 ml at an interval of 0.5.

Effect of Carbon and Nitrogen Sources on Rhodanese Production: Different carbon sources (glucose, sucrose, maltose, mannitol, dextrose, and lactose) were investigated as alternative replacements for potassium cyanide in the production medium. Likewise, different nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, ammonium chloride, sodium nitrate, casein, and peptone) were investigated for their effect on rhodanese production by replacing the peptone in the production medium for rhodanese. The nitrogen sources were added at 1% concentration.

Effect of Temperature on Rhodanese Production: Conical flasks (250 ml) each containing 100 ml of production medium was inoculated with standard bacteria suspension. The flasks were incubated at a temperature ranging from 25-45ºC at an interval of 5ºC for each temperature.

Effect of Hydrogen Ion Concentration (pH) on Rhodanese Production: The broth medium was prepared by varying pH values ranging from 4-10 at 1.0 interval. The effect of pH production was studied in 250 ml conical flasks containing 100 ml of the production medium. The sterile medium was adjusted to different pH and inoculated with standard bacteria suspension.

3. RESULTS

Enzyme production and identification: Twelve rhodanese-producing bacteria were isolated from the effluents and the strain with high production potential was selected. The selected isolate was identified as a strain of *Bacillus cereus*. It shared 99% homology with *B. cereus* LN831988 and 100% homology with *B. cereus* KT381022. The percentage identities of the isolate nucleotide sequence with corresponding sequences of *Bacillus cereus* from the Genbank is shown in Table.1. The isolate stain was subjected to rhodanese activity studies and maximum *B. cereus* rhodanese activity was obtained at the 39th hour of incubation. (Figure.1).

Table 1. Percentage identities of the isolate nucleotide sequence with corresponding sequences of *Bacillus cereus* from the Genbank

Bacillus cereus							
ACCESSION	KT381022	KX65992	KT633716	LN831988	KM658260	KF973315	KY777586
% IDENTITY	100%	98%	100%	99%	99%	97%	99%

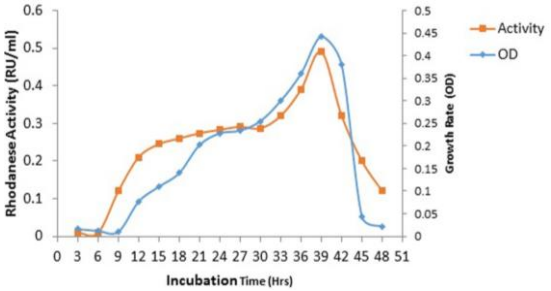

Figure.1. Growth and rhodanese activity of *B. cereus*.

Effect of Inoculum Size and Carbon Sources: The optimal inoculum size was observed to be 2 ml with rhodanese activity of 0.47 RU/ml. (Figure 2). Potassium cyanide (KCN) was found to be the best carbon source with rhodanese activity of 0.346 ± 0.002 RU/ml (Figure.3).

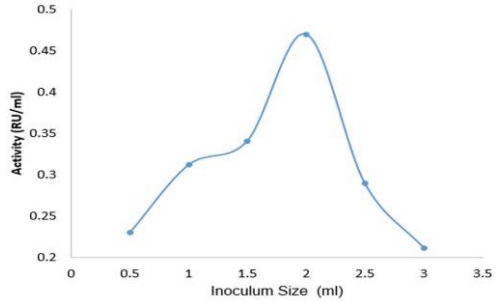

Figure.2. Effect of inoculum size on rhodanese production by *B. cereus*

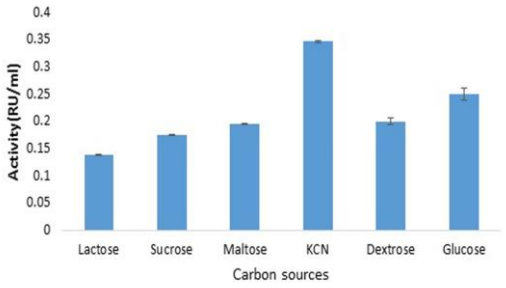

Figure.3. Effects of Carbon sources in replacement of KCN on rhodanese production by *B. cereus*

Effect of Nitrogen Source, pH and Temperature: The enzyme evaluation indicated casein (0.9 ± 0.04 RU/ml/) to be the best nitrogen source followed by peptone (0.6 ± 0.024 RU/ml) for optimum rhodanese production while potassium nitrate gave the least activity (Figure.5). Rhodanese production was optimum at pH 8.0 and 30ºC with enzyme activity of 3.9 RU/ml and 0.648 RU/ml respectively (Figure.4, 5 and 6).

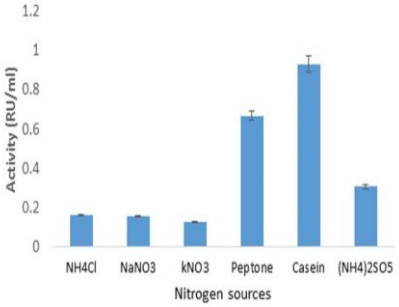

Figure.4. Effects of Nitrogen sources in replacement of peptone on rhodanese production by *B. cereus.*

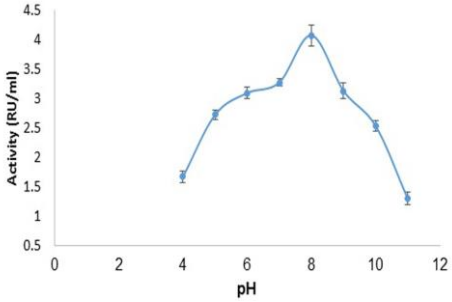

Figure.5. Effect of pH (4-11) on rhodanese Production by *B. cereus*

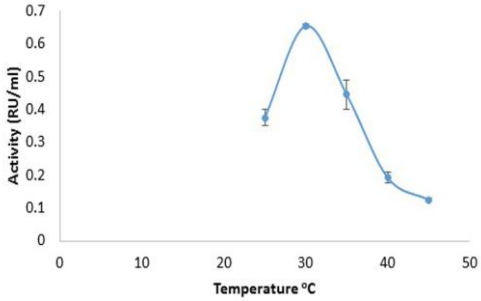

Figure.6. Effect of temperature on rhodanese production by *B. cereus*

DISCUSSION

The present study made a successful attempt to isolate bacteria from an industrial effluent for the purpose of producing industrially important enzyme. Bacteria strains were isolated from the Company effluents and the strain with appreciable rhodanese production was selected. The selected isolate was gram-positive, rod-shaped, spore-forming, and catalase positive and it was identified as *Bacillus cereus* with a 100% homology to other *Bacillus cereus spp.* on NCBI website. Rhodanese activity has been reported in different bacteria species such as *Azotobacter vinelandii*. *Bacillus brevis*, *Escherichia coli*, and *P. aeruginosa* (Chiu, 2009). Production of an enzyme such as rhodanese by the microorganism is often dependent on the growth of the bacterium in the appropriate media composition. The bacterium accumulates some cell components before the release of the enzyme in the log phase, followed by a decline in enzyme production during the stationary and death phase respectively. Production of rhodanese by *B. cereus* was maximum at 39 h with a gradual decline (Figure.1). This decline in production may be due to exhaustion of the nutrients or accumulation of other products or metabolites which are both inhibitory to the growth of the bacterium and rhodanese production. Also, the inhibition of enzyme production might have resulted from catabolic repression, increase in protease and hydrogen ion concentration of the growth medium (Goyal, 2005; Prakash, 2009). The growth pattern observed in this study is similar to the one reported by Hoster (2001) and Oyedeji (2013). Research has shown that enzyme synthesis is correlated with the quality and concentration of carbon and nitrogen sources and these requirements differ from one organism to another (Pandey, 2001; Schnurer, 2010).

The optimization of culture media and environmental conditions is essential for effective production as it tends to reduce the cost of production (George-Okafor, 2012; Mohammad, 2019). In this study, the observed optimal inoculum size was 2 ml with rhodanese activity of 0.47 RU/ml (Figure.2). The source of carbon available to rhodanese producing bacterium is worthy of note owing to the fact that it is one of the basic requirements for growth and enzyme synthesis. Potassium cyanide (KCN) as a carbon source, produced high rhodanese activity (0.346 ± 0.002 RU/ml) and the fact that the organism was isolated from cyanide contaminated effluents might be the reason for the observed enzyme activity. Also, literature has shown that *B. cereus* grow well in a cyanide solution (Sujatha, 2015). The ability of the *B. cereus* to make use of potassium cyanide as a carbon source suggest the use of the organism in the bioremediation of cyanide-contaminated effluents.

Impact of different nitrogen sources (1%) on rhodanese production by *B. cereus* was evaluated using both organic and inorganic nitrogen sources. Casein produced the highest rhodanese activity and this may be due to the ability of the organism to hydrolyse casein during growth and enzyme production as opposed to other nitrogen sources. The enzyme evaluation indicated that casein and peptone gave the maximum rhodanese activity of the nitrogen sources utilized by *B. cereus* (Figure 4).

The optimum pH for enzyme production was 8.0 (Figure.5). Schraft (2006), reported *B. cereus* as an organism with a pH range of 4.3-9.3. Also, Panos (1999), reported pH 9.0 for optimum microbial cyanide degradation. Kumar and Takagi (1999), reported the influence of temperature on the growth and production of metabolites by microorganism. Panon (1999), reported that microbial activities increase as the temperature increases to 37ºC. In this study, the optimum temperature was observed at 30ºC (Figure.6). Luo (2007) reported *B. cereus* to grow optimally at a temperature range of 20-40ºC.

4. CONCLUSION

In this study, we optimized various conditions for maximum rhodanese production by *B. cereus*. The production of rhodanese by this bacterium suggests the cyanide detoxification potential of the enzyme and this explained the reason for the survival of the bacterium in the environment. However, further studies are needed to increase the rhodanese production through genetic engineering techniques.

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