Research Article

Bioremediation of Inorganic Cyanide Pollutants using Recombinant Bacterial Rhodanese

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Abstract | Cyanide; a highly toxic compound, is a significant environmental pollutant generated by metallurgical industries. The high cost and environmental drawbacks of chemical treatments have driven research towards exploring biological methods as a more sustainable approach for cyanide detoxification. In particular, enzymatic degradation has been investigated for its effectiveness in removing cyanide from wastewater. In this study, we aimed to focus on cloning, expression, and immobilizing the rhodanese gene (*rhdA*) obtained from *Bacillus licheniformis* to test the role of immobilized recombinant rhodanase in the bioremediation of cyanide-polluted envirnonments. We engineered a genetic system to express recombinant *Bacillus licheniformis* rhodanese (r-RhdA) in *Escherichia coli* strain M15. A 954 bp DNA fragment encoding the *rhdA* gene was amplified and successfully expressed in *E. coli* M15 [pREP4] as a His-tagged fusion protein. The sodium dodecyl sulphate– polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the expression of a ~35 kDa RhdA protein, further validated using an anti-His antibody *via* western blot. Phylogenetic analysis revealed conserved regions among rhodanese proteins belonging to the *Bacillus* genus. The recombinant rhodanese showed a 4.08-fold increase in specific rhodanese activity compared to the native enzyme. Furthermore, r-RhdA was successfully immobilized in calcium alginate-gelatin composites, achieving a cyanide degradation efficiency of 71.6%. These obtained findings suggest the potential application of immobilized recombinant rhodanese as a useful bioremediation tool for cyanide-contaminated ecosystems.

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Introduction

Cyanides are compounds with the cyano (-C≡N) group that exist in nature in various forms due to their unique chemical characteristics. According to pH, cyanide can be detected as the ion cyanide (CN^-) in dissolution at great pH or vaporized as hydrogen cyanide at neutral or acid pH (pKa 9.2). The affinity of cyanide to metals is high, so complexes of cyanide and transition metals which are found in nature are highly stable (Akcil and Mudder, 2003; Baxter and Cummings, 2006). Cyanide compounds are involved in the manufacture of acrylic, rubber, and plastic; for industrial operations including electroplating,

production of steel, and metal extraction from ores such as gold and silver ores; and for fumigation, as well as insecticides (ATSDR, 2006; MacLennan and Moiemen, 2015).

Cellular respiration abruptly stops when cyanide binds to cytochrome oxidase inside the mitochondria. Cyanide has a strong affinity for the ferric ion of cytochrome a_3 portion of the enzyme, and as a result, the electron transport is effectively stopped and the oxidative phosphorylation is inhibited. In the end, this inhibition stops the adenosine triphosphate (ATP) production. Even when blood oxygen levels are adequate, cellular hypoxia occurs because cyanide inhibits the cell's ability to use oxygen. This caused a shift from aerobic to anaerobic metabolism, leading to increased lactic acid production, resulting in a great anion gap metabolic acidosis due to lactic acid accumulation. The brain, heart, and other organs with high oxygen demand are more susceptible to cyanide poisoning (Gracia and Shepherd, 2004; Shepherd and Velez, 2008). Additionally, convulsions, unconsciousness, and death are caused by high doses of cyanide inhalation, oral, or skin exposure. Lower cyanide exposures can cause headaches or lightheadedness. Nonlethal inhalation exposure to cyanide might result in dyspnea and upper respiratory irritation (ATSDR, 2006).

There are five main treatment methods that are commonly used to remove cyanide from wastewater. These methods are based on various principles: physicochemical, electrochemical, photochemical, chemical and biological procedures (Vaca-Escobar *et al*., 2024). Alkaline chlorination, ozonation, wet-air oxidation, and sulfur-based technologies are some of the chemical processes now used to treat wastewater that contains cyanide. Despite their usefulness, these technologies are relatively expensive and pose environmental risks due to the chemical agent(s) they release, which might cause secondary contamination. Similar to alkaline chlorination, a major disadvantage is the formation of toxic by-products, which is a common limitation of chemical treatment methods (Akcil and Mudder, 2003; Malmir *et al*., 2022). Recently, biological treatment has emerged as a favored approach for treating cyanide wastewater over other treatment approaches, including physical or chemical treatments. Biological approaches can lower the content of cyanide in wastewater to environmentally acceptable levels and produce fewer toxic by-products (Botz *et al*., 2016; Vaca-Escobar *et al*., 2024). For instance, the biodegradation of cyanide by *Rhodococcus* UKMP-5M may have involved the bacterial enzyme cyanidase through a hydrolytic pathway (Nallapan Maniyam *et al*., 2013). Similarly, the cyanide-degrading bacterium *Pseudomonas pseudoalcaligenes* CECT 5344 is capable of utilizing cyanide and various metal-cyanide complexes as its sole nitrogen source (Biełło *et al*., 2023). In addition, *Alcaligenes faecalis* is reported for its capability of degrading cyanide (Li *et al*., 2025). Microorganisms have developed several resistance and/or assimilation techniques for dealing with the toxicity of both endogenous and exogenous cyanide (Cipollone *et al*., 2006). Microbes employ diverse metabolic pathways to face the toxic substances in their environment, often producing specialized enzymes to degrade these chemicals. One crucial enzyme that is widely employed in the biodegradation of cyanide is rhodanese. Rhodaneses (thiosulfate: cyanide sulfurtransferases; EC 2.8.1.1), are widely distributed and highly conserved enzymes, that are thought to represent a crucial evolutionary mechanism for the detoxification of cyanides (Raybuck, 1992). *In vitro*, rhodanase catalyzes the irreversible transfer of a sulfur atom from a suitable donor (e.g., thiosulfate) to cyanide, resulting in the formation of less toxic sulfite and thiocyanate. Kinetic studies suggest a two-step catalytic mechanism, where the enzyme cycles between sulfur-free and persulfurated forms, with the catalytic cysteine residue undergoing a (de) persulfuration cycle (Alexander and Volini, 1987; Bordo *et al*., 2001; Cipollone *et al*., 2004). Rhodaneses have been discovered in a wide range of microorganisms including Aspergillus welwitschiae LOT1 isolated from battery-effluent contaminated soil (Lawal *et al*., 2023). Also, it is produced by the wild strain (KOJCM1665) and two selected mutant strains (KOJCM1665c and KOJCM1665d) of *Klebsiella oxytoca* JCM 1665 (Itakorode *et al*., 2024a). Immobilized enzymes exhibit superior catalytic activity, operational stability, reusability, and improved recovery and storage, compared to their free counterparts. Enzyme immobilization also enhances thermal stability and efficiency. These benefits make immobilized enzymes essential for industrial applications, leading to more effective catalysts and reduced manufacturing costs (Yaashikaa *et al*., 2022; Ishak *et al*., 2025).

For instance, rhodanese-producing fungus cell

have been successfully immobilized using sodium alginate matrics (Rishi *et al*., 2023). The use of alginate–gelatin–calcium hybrid carriers to enhance mechanical stability and minimize enzyme leakage, thereby enabling efficient encapsulation (Shen *et al*., 2011). In this study, rhodanese produced from *Bacillus licheniformis* was cloned into the expression host *Escherichia coli* M15 [pREP4] and subsequently immobilized. The immobilized rhodanese was then employed to detoxify cyanide-contaminated water.

Materials and Methods

Collection of industrial wastes

As industrial wastes, two soil and two effluent samples were collected from different areas in the Iron and Steel Factory in Helwan, Cairo, Egypt, and five soil samples were collected from the industrial area in 6th of October City, Cairo, Egypt. All samples were labeled, and collected in autoclaved containers and polyethylene bags.

Isolation of the predominant bacteria from industrial wastes

Soil suspensions were prepared immediately following the collection of industrial waste samples. Serial dilution was applied to dilute the amount ofbacterial cell to be plated. Serial dilutions up to 10^{-6} and 10^{-7} were carried out. Using the spread plate and pour plate methods (Sanders, 2012), 100 µl of suspension from tubes with 10^{-6} and 10^{-7} dilutions were inoculated individually onto Petri plates containing Luria-Bertani and Tryptic Soy agar media. In molecular microbiology, LB medium is frequently employed to culture microbial strains in the lab. This medium included enzymatic or chemical digests of plant, yeast or animal tissues, and it was rich in organic chemicals that several microorganisms can use to develop (Egli, 2009). Tryptic Soy Agar (TSA) is a generally known medium for culturing different microorganisms (Drelich *et al*., 2011). Inoculated plates were incubated for 48 h at 37°C in an inverted position. All samples were performed at least in duplicate. After incubation, the developed microbial colonies were picked, purified, and subcultured individually on slants of the same medium for further studies.

Screening of cyanide-degrading microorganisms

This step was performed to select microbial isolates that could degrade cyanide. Using the streak plate method, each microbial isolate was sub-cultured on a composed basal medium (as a selective medium), containing agar (1.5%), yeast extract (0.5%), peptone (1%), sodium chloride (0.5%), and potassium cyanide (0.3%) as an inducer (Oluwatosin *et al*., 2017), and incubated at 37 °C for 72 h. All samples were prepared in triplicate. After incubation, the plates were examined, the isolates that could grow in the presence of potassium cyanide were selected, and preserved on slants of LB medium at 4°C for identification.

Rhodanese activity assay

The rhodanese enzyme activity assay was conducted quantitatively to select the rhodanese-producing bacteria. RhdA (rhodanese) activity was determined calorimetrically according to the method reported by Sorbo (1953) with slight modifications, using sodium thiosulfate as a sulfur donor and cyanide as a sulfur acceptor substrate. In a test tube, a mixture consisting of 0.5 ml of 50 mM borate buffer at pH 9.4, 0.2 ml of 0.25 M KCN, 0.2 ml of 0.25 M $Na_2S_2O_3$ and 1 ml of an enzyme extracted from the different selected isolates was prepared. Then, the mixture was incubated for 1 h at 37°C, and enzyme reaction terminated by adding 0.5 ml of 15% formaldehyde, followed by the immediate addition of 1.5 ml of sorbo reagent (10 g ferric nitrate + 20 ml nitric acid + 80 ml distilled water). For the control, the enzyme activity was inhibited immediately by adding 15% formaldehyde (0.5 ml) and sorbo reagent (1.5 ml). The absorbance was read at 460 nm (Sorbo, 1953) using a visible spectrophotometer (JENWAY, Model 6300, UK).

Identification of the most promising rhodanese-producing bacterium

The bacterial isolate that had the highest rhodanese enzyme activity was selected for additional investigations. The promising isolate was identified based on morphological, cultural, biochemical, and molecular characterizations. At first, the bacterial isolate was stained with Gram stain to identify its morphological type according to Bartholomew and Mittwer (1952).

MALDI-TOF MS identification

The MALDI-TOF MS identification method was employed to identify the type species of the promising isolate. The bacterial sample was prepared for MALDI-TOF-MS. A small visible amount of bacterial cells was transferred to the sample spot of stainless steel MALDI-TOF MS target. Then, the cells were spread on the whole area of the spot to form thin film. 1 µl of

70% formic acid was applied and left to dry at room temperature. This was followed by overlaying with 1 µl of the HCCA matrix solution, placed inside a fume hood cabinet to dry, and the target was inserted into the the VITEK® MS (bioMérieux Inc., France) (Schumann and Maier, 2014).

Scanning electron microscope (SEM) examination

Scanning electron microscope examination (SEM) was used to examine the morphological characteristics of the selected bacterial isolate. A drop of bacterial suspension was placed on a glass slide and left to air dry. The cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.2) for 30 min at room temperature, and rinsed with sodium phosphate buffer (pH 7.2). Dehydration was performed using a graded acetone series (30% to 100%) with 5 min incubation at each step (Khan *et al*., 2016). For enhanced imaging contrast, the sample was covered with a gold film using a sputter coater (Emitech K550X, England, UK), and set on aluminum stubs. Further analysis was carried out using a scanning electron microscope model Quanta 250 FEG microscope equipped with an EDX unit at the Egyptian Mineral Resources Authority, Giza, Egypt.

Molecular identification via sequencing of 16S rRNA

The genomic DNA was extracted from the bacterial sample following the recommended manufacturer's instructions (GeneDireX® Genomic DNA Isolation Reagent Kit (Blood/Cultured Cell/Tissue) Cat. No. NA022-0100) with a final elution volume of 100 µl. The extracted DNA was stored at -20˚C until performing polymerase chain reaction (PCR) analysis. Two universal 16S rRNA bacterial primers; 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R(5´-TACGGYTACCTTGTTACGACTT-3´) were used to amplify the 16S rRNA gene using 2 µl of genomic DNA isolated from the bacterial isolate. The PCR reaction begun with an initial denaturation step at 95˚C for 5 min. This was followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing step occurred at 59˚C for 30 sec, and extension was carried out at 72˚C for 90 sec. After the cycling process, a final extension was performed at 72˚C for 10 min to ensure complete elongation of all amplified products. Subsequently, PCR products were visualized using a 1% agarose gel stained with Xpert Green DNA Stain (grisp, Ref: GSO1.0001). Then, the PCR product was subjected to nucleotide sanger sequencing (Gomes and Korf, 2018) in both directions.

Rhodanese gene isolation

In order to clone the *rhdA* gene of promising isolate into pJET1.2/blunt cloning vector, which was a blunt-ended and linearized cloning vector capable of accepting inserts (Thermo Scientific, Cat. Nos. K1231, Lithuania), *rhdA* was isolated and amplified by PCR with two designed primers, mainly *rhdA* forward 5′- GAGCTCATGACGATTTCGGGATTTATCTTTG-3′ and *rhdA* reverse 5′-CTGCAGTCAAATATCGTCCTCGGCTCCA-3′, using an isolated *B. licheniformis* encoded B47 genomic DNA as a template. The primers were designed using DNAStar Lasergene Software, based on nucleotide sequence of the rhodanese gene described by Krawczyk *et al*. (2016). Primers were designed to introduce *Sac*I and *Pst*I restriction sites (underlined) at the 5′-primer ends, respectively. PCR program started with an initial denaturation step at 95˚C for 3 min. This was followed by 30 cycles of amplification, each consisting of three key steps: denaturation at 95˚C for 1 minute, annealing of primers at 62˚C for 1 minute, and extension at 72˚C for 90 sec. A final extension step at 72˚C for 5 min with an incubation phase at 4˚C was carried out, allowing preservation of the amplified DNA products. The amplified *rhdA* gene was purified using Gene JET Gel Extraction Kit (Thermo Scientific, Cat. #K0691), and then cloned into pJET1.2/blunt Cloning Vector (Thermo Scientific, cat. Nos. K1231). Next, the resulting plasmid pJET*rhdA* was propagated into *Escherichia coli* DH10β (Thermo Fisher Scientific, Cat. No. 18297010) as previously reported by Sambrook *et al*. (1989). The plasmid construct pJET*rhdA* was isolated by using Plasmid miniPREP Kit (Simply™, Inc., Cat. No. SN005-0100), and sequenced in both directions using the designed *rhdA*-specific primers. The digestion reaction was performed in 20 µl containing 3 µl of pJET*rhdA* recombinant plasmid DNA, 1 µl of *Sac*I, 1µl of *Pst*I, 2 µl of 10X Fast Digest Buffer, and 13 µl of deionized distilled water. The reaction was incubated at 37˚C for 45 min. The mixture was examined *via* performing electrophoresis on a 1% agarose gel in 1x TAE buffer.

Bacterial expression of rhodanese protein

The *Sac*I and *Pst*I *rhdA* gene fragments were cloned into the expression vector pQE-30. The ligation reaction was performed in a total volume of 20 l containing 13 µl of purified digested *rhdA* (14 ng/µl), 1.5 µl of Purified digested PQE-30 vector (50 ng/µl), 2 µl of T4 DNA Ligase (Thermo Scientific, REF- K1422), 2 µl of 5X rapid ligation buffer, and 1.5 µl of deionized

distilled water. The reaction was incubated at 22˚C for 1 h. Individual clones were examined by colony PCR. For protein expression, the isolated recombinant plasmid designated as pQE*rhdA* contained the *rhdA* full-length gene in frame with a 6xHis tag. It was subsequently transformed into the expression host *E. coli* M15 [pREP] cells. Then, the cells were grown in LB medium plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated at 37˚C as reported by Sambrook *et al*. (1989). Individual clones containing the recombinant pQE*rhdA* were examined by colony PCR using pQE-30 Forward Sequencing Primer (5′-CCCGAAAAGTGCCACCTG-3′) and pQE-30 Reverse Sequencing primer (5′-GTTCTGAGGTCATTACTGG-3′). The PCR reaction begun with an initial denaturation at 95˚C for 5 min, followed by 30 cycles of denaturation (95˚C, 30 sec), annealing (50˚C, 30 sec), and extension (72˚C, 1.5 min). An extension at 72˚C for 5 min, and finally, the reaction was preserved at 4˚C.

Time course of rhodanese protein expression

An individual colony of *E. coli* M15 [pREP] containing pQE*rhdA* was used to inoculate 5 ml of LB broth supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin, and grown overnight at 37˚Cin a shaking incubator at 150 rpm. 1 ml of an overnight culture of the selected potent isolate was used to inoculate 10 ml LB broth containing 100 µg/ml ampicillin and 25µg/ml kanamycin. The culture was incubated in a shaker at 37˚C and 200 rpm, until it reached the mid-log growth phase OD_{600}~ 0.6, at that point isopropyl thio-*β*-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. At various time intervals (0, 2, 3, 4, 5, 6, and 7 h), 1 ml was taken from each sample, centrifuged for 7 min at 8000 rpm, and the resulting cell pellets were rinsed with deionized distilled water, treated with 2X sample loading buffer, boiled for 10 min at 95˚C. and immediately chilled on ice for sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Youssef and Al-Omair, 2008).

Analysis of protein expression

The protein profile was determined using 12% SDS-PAGE stained with Coomassie blue as previously described by Laemmli (1970). For western blot analysis, the protein bands were transferred onto a polyvinylidene fluoride (PVDF) transfer membrane (Thermo Scientific, Cat. No. 88518, USA) using a trans-blot apparatus (Thermo Fisher Scientific, Power Blotter Station, Model PB0010, China). Afterwards, the membrane was blocked in Tris buffer saline (TBS) containing 5% bovine serum albumin (BSA) and 0.05% Tween-20 overnight at 4˚C with gentle shaking. The membrane was rinsed twice with TBST buffer (Tris buffer saline (TBS) + 0.05% Tween-20), and then 3 times with TBS, 5 min for each. The membrane was incubated in anti-His mouse monoclonal antibody (1:5000) diluted in 1% BSA of TBS buffer for 2 h at room temperature. Then, the membrane was washed as mentioned previously, followed by incubation in anti-mouse IgG alkaline phosphatase conjugate (1:10000) diluted in 1% BSA of TBS buffer for 2 h at room temperature, and then washed as previously stated. The membrane was treated with Nitro Blue Tetrazolium Chloride (NET)/ 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) in alkaline phosphatase solution and left in the darkness until purple bands appeared, indicating the positions of target proteins (Towbin *et al*., 1979).

Preparation of cleared expressed protein from E. coli M15 (pREP4) (pQErhdA) lysates

The recombinant rhodanese was extracted from the expression host in the cleared lysate. Briefly, 1 L LB broth containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) was inoculated with 10 ml of an overnight-grown bacterial culture, followed by induction with isopropyl thio-*β*-D-galactopyranoside (IPTG) (0.5 mM) for 5 h. The cells were harvested by centrifugation (4,200 × g for 30 min), and the pellet was placed on ice for 15 min. Afterward, the pellet was resuspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and phenyl methyl sulfonyl fluoride (PMSF) was added at a final concentration of 1 mM as a protease inhibitor. Then, lysozyme (1 mg/ml) was added and incubated on ice for 30 min, sonicated on ice using six 10-second bursts with a 10-second cooling period between each burst, and centrifuged at 10000 x g for 30 min at 4˚C. The resulting supernatant containing soluble proteins was withdrawn (Qiaexpressionist, 2002). For further purification of the lysate, it was dialyzed overnight against several changes of a buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) at 4˚C. The cleared lysate was stored at –80 ˚C after the addition of 10% glycerol. The protein profile of the cleared lysate was determined using 12% SDS-PAGE.

Estimation of protein concentration and specific activity of recombinant rhodanese

Total soluble protein concentrations of the recombinant rhodanese and *B. licheniformis* encoded B47 rhodanese were determined by a colorimetric assay using BSA as a standard, where the protein absorbance was extrapolated from a standard curve (Bradford, 1976). The enzyme activity of recombinant rhodanese was estimated as previously described.

Immobilization of recombinant rhodanese

Recombinant rhodanese was immobilized by using a mixture of sodium alginate and gelatin according to a reported method by Youssef and Al-Omair (2008), with slight modifications. Briefly, by mixing gelatin (3%) with sodium alginate (5%) in water, a gelatin-alginate combination was created. Typically, 0.5 g sodium alginate and 0.3 g gelatin were dissolved in 8 ml distilled water, subsequently sterilized, and then cooled. 2 ml of recombinant rhodanese (the protein concentration of extracted recombinant rhodanese was 2.87 mg\ml) were added to the mixture and stirred for 15 min. After being withdrawn by a syringe, the slurry was dropped into a cold 0.5 M $CaCl_2$ solution (4°C) dropwise. The resulting beads were left in $CaCl_2$ solution for 2 h at 4°C to harden. The beads were washed with sterilized distilled water and stored at 4 °C.

Biodegradation of cyanide using immobilized recombinant rhodanese

Briefly, 0.1 g of immobilized recombinant rhodanese beads was added to 1 L of water sample containing cyanide with approximate concentration of 0.141 mg/L. The reaction mixture was incubated for 4 h at 37 °C. Aliquots were withdrawn every 60 min from the reaction mixture. The immobilized recombinant rhodanese beads were separated from the aliquots. The beads were collected and washed with sterilized distilled water and stored at 4 °C. Cyanide concentration in the different aliquots was measured in the Central Laboratory for Environmental Quality Monitoring, National Water Research Center, EL-Kanater, Egypt. The percentage (%) of removed cyanide was measured according to the following equation reported by Ademakinwa *et al.* (2021):

$$\%\,Cyanide\ removal = 100 - \frac{(CN)_t}{(CN)_0}\ \%$$

Where $(CN)_0$ refers to initial cyanide concentration before adding immobilized recombinant rhodanese beads, $(CN)_t$ refers to remaining cyanide contcentration at a defined time after adding beads of immobilized recombinant rhodanese.

Statistical analysis

The results were inserted, validated, and analyzed statistically using one way analysis of variance (one-way ANOVA) test on IBM SPSS Statistics (version 27.0.1) software, followed by Tukey test for means comparisons, with confidence level of 95.0% (p = 0.05).

Results

Screening of cyanide-degrading bacteria and rhodanese-producing bacteria

Fifty bacterial strains were isolated from different industrial wastes. Subsequently, they were subcultured on KCN-containing medium. Only 17 bacterial isolates grew in presence of cyanide, out of which a single isolate was selected for further study, which recorded maximum rhodanese activity and encoded as B47 based on its appreciable rhodanese enzyme production, as shown in Figure 1. The enzyme activity results were analyzed statistically and B47 isolate was the most promising bacterium producing rhodanese (0.25967 µ mol thiocyanate/ml).

Identification of the bacterial isolates

The bacterial isolate designated B47 was subjected to morphological and biochemical characterization. The B47 isolate was shown to be a Gram-positive bacterium as a result of the Gram-staining test. As shown in Figure 2, the morphology of the bacterial cell was investigated using a SEM. The bacterial cells appeared as bacilli (rod-shaped) with endospores and were arranged individually or as pairs. MALDI-TOF profiles identified the selected strain as *Bacillus licheniformis*.

For molecular characterization, the amplicon of the 16S rRNA gene of B47 was sequenced. The sequence result was analyzed by the nucleotide BLAST of the National Center for Biotechnology Information (NCBI) database for identification based on sequence similarity. The NCBI BLAST result indicated a 98.88% similarity between isolate B47 and the previously reported strain *Bacillus licheniformis* (Accession number KC895912).

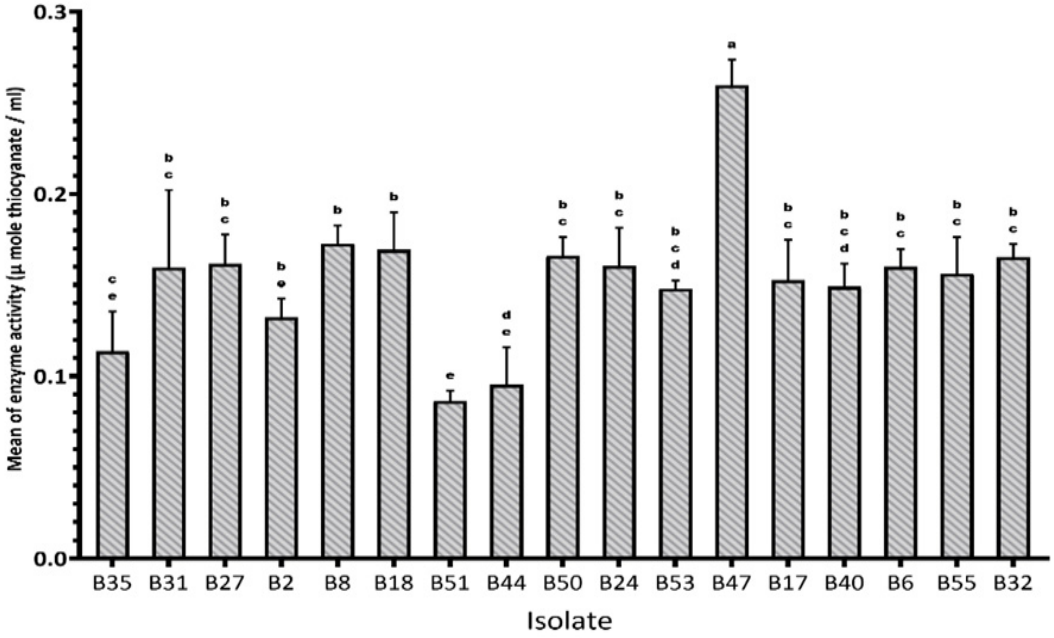

Figure 1: *Determination of rhodanese enzyme activity. The bars represent the mean value of rhodanese activities ± standard deviation (±SD) recorded from different 17 isolates. Isolate B47 recorded the maximum rhodanese activity. The data were analyzed by ANOVA (F = 12.750), p value < 0.05 (p < 0.05 was considered statistically significant (95% confidence interval)), followed by the Tukey test for means comparison. A significant difference among tested strains is indicated by the existence of different letters on the bars, while the presence of a common letter shows that there is no significant difference between the strains (p < 0.05).*

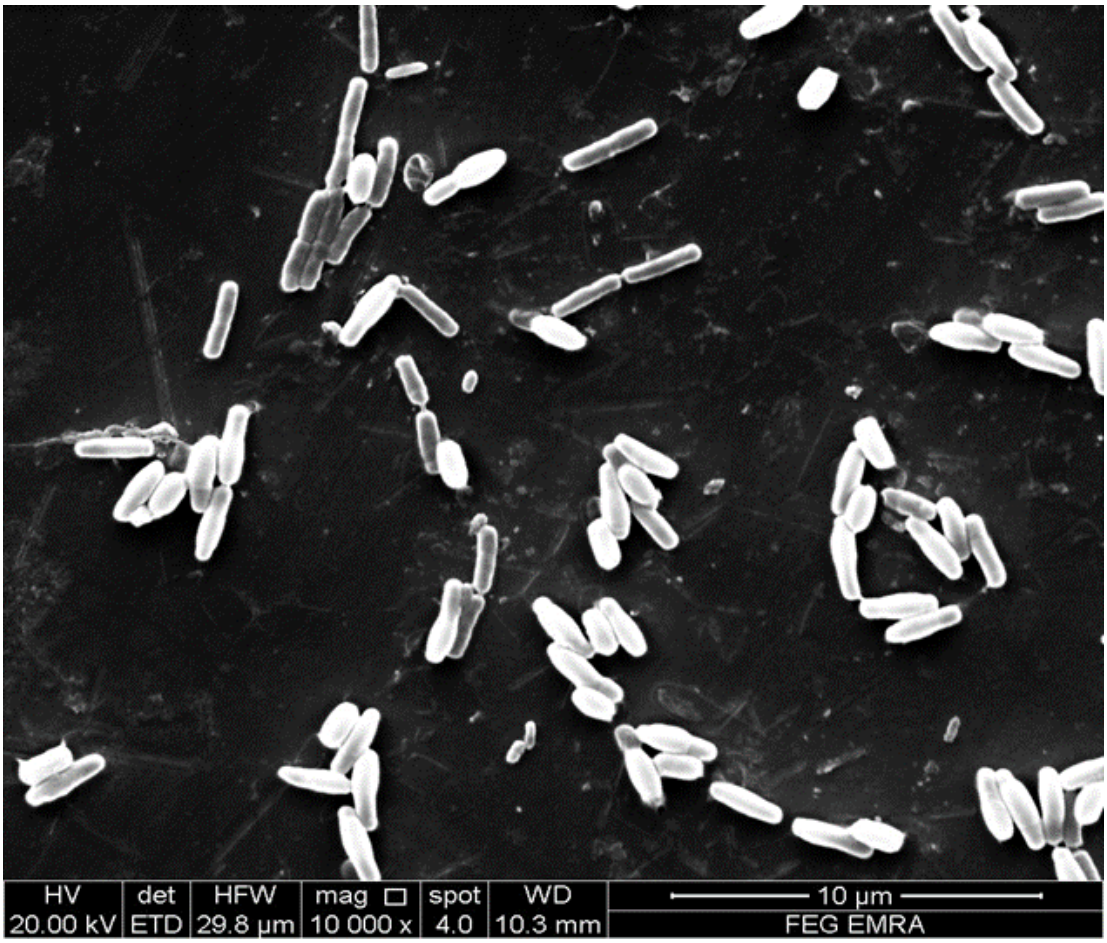

Figure 2: *Examination of the most promising isolate B47 with the highest rhodanese activity under the Scanning Electron Microscope (SEM) at magnification x10000.*

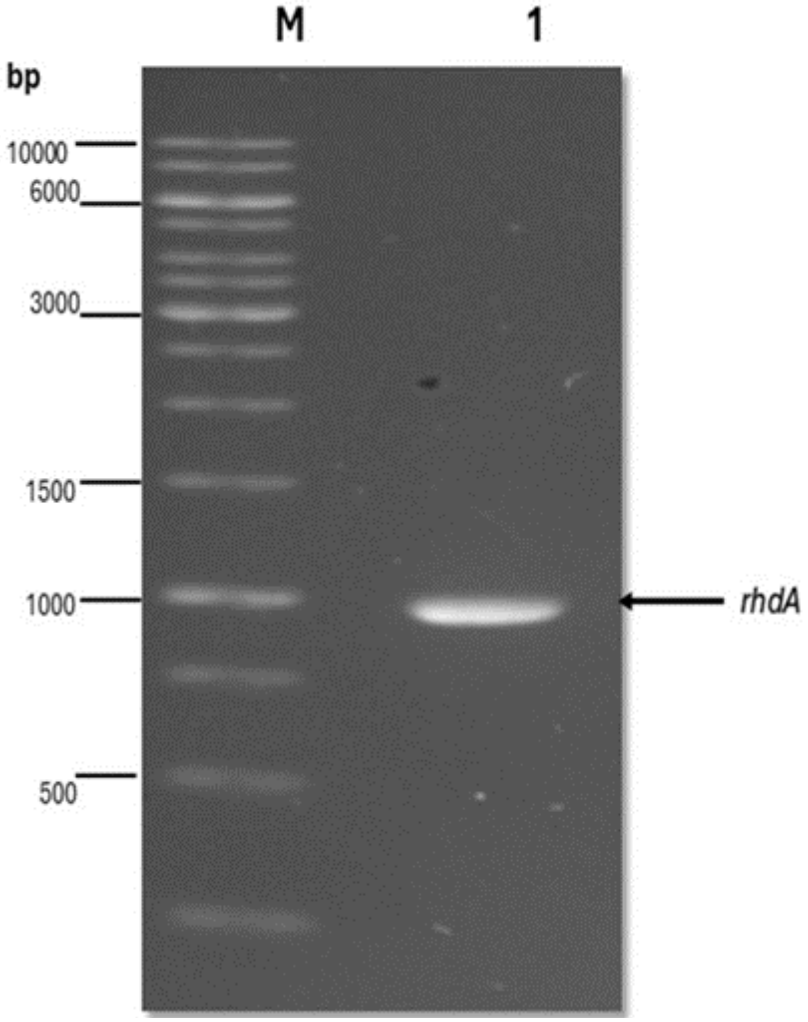

Figure 3: *PCR amplification of rhdA gene full-length using B. licheniformis encoded B47 DNA template. M: 1 Kb DNA ladder (Thermo Scientific™ GeneRuler, REF. SM0313, Lithuania), and Lane 1: the rhdA amplicon at the expected size of ~954 bp.*

Rhodanese gene isolation and cloning

Rhodanese gene fragment of 954 bp corresponding to the full *rhdA* coding sequence from *B. licheniformis* encoded B47 was successfully amplified by PCR utilizing specific primers (Figure 3), and was cloned into pJET1.2/blunt cloning vector. The sequencing results indicated that the cloned sequence began with an ATG start codon and terminated with a TAG stop codon. BLASTn analysis of the nucleotide sequence revealed sequence similarity to a set of sequences. It revealed 99% identity with the rhodanese gene of *B. licheniformis* strain PB3 with an accession number CP025226. The full *rhdA* coding sequence encoded a protein consisting of 318 amino acids with a molecular weight of 35.98 kDa. Furthermore, nucleotide sequence of recombinant *B. licheniformis* rhodanese was deposited in the GenBank database under an accession number PQ421362.

The different rhodanese protein sequences were collected from the GenBank database, focusing on bacterial rhodanese. The rhodanese protein sequences of different bacterial genera were aligned in MEGA11 employing MUSCLE, and a phylogenetic tree was constructed based on amino acid sequence alignments in MEGA11 using a Maximum Likelihood method; selecting WAG model, and estimated gamma distribution parameter.

Phylogenetic analysis of rhodanese sequences (Figure 4) revealed their evolutionary and functional diversity across bacterial species, and confirmed the existence of some conserved areas among rhodaneses from different bacterial species, irrespective of their relatedness. The rhodanese sequence of interest, PQ421362, obtained from *B. licheniformis* clusters closely related with other *Bacillus* species, supported by high bootstrap percentages (72–99%), indicating evolutionary conservation and functional similarities among rhodanese proteins within the genus *Bacillus*.

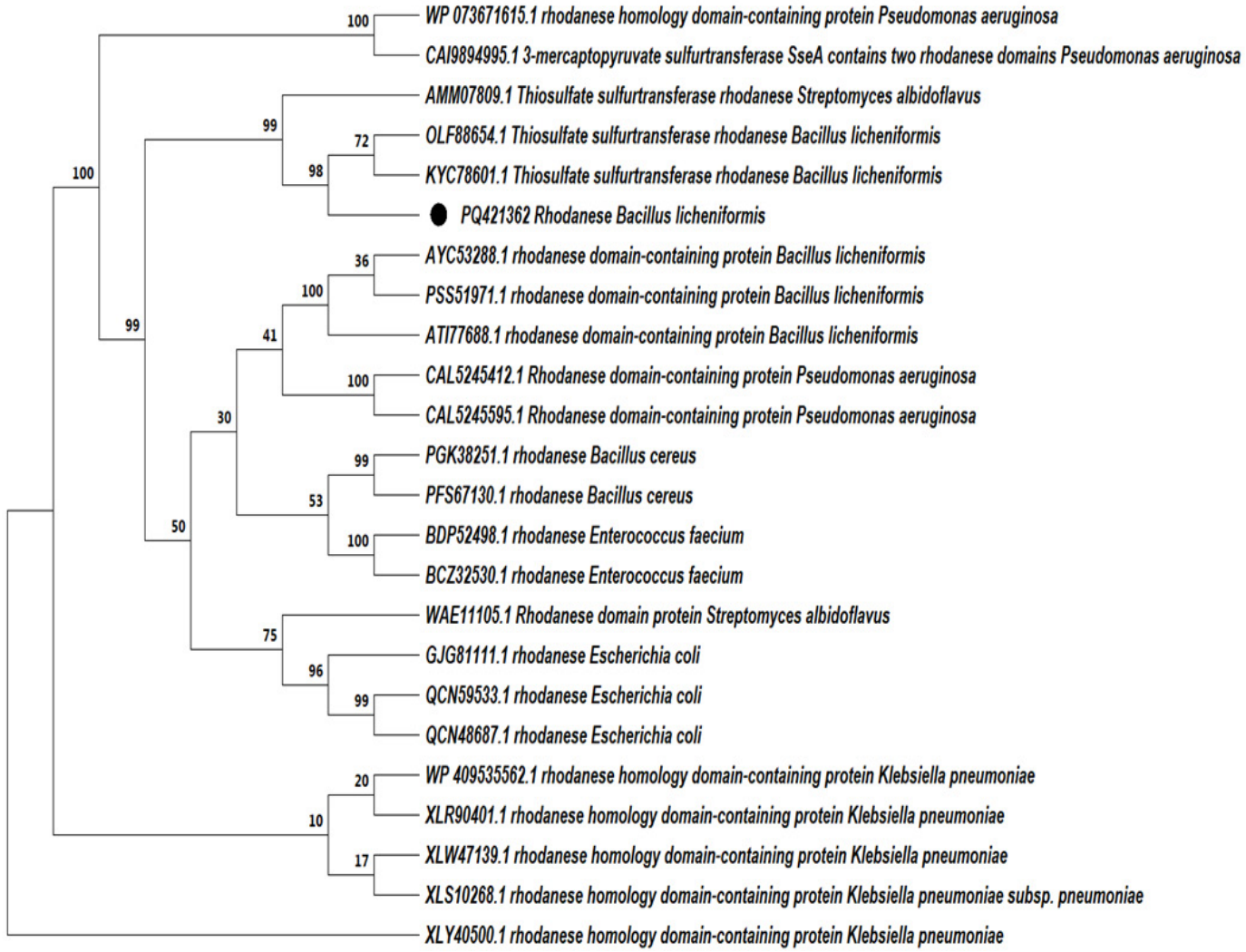

Figure 4: *A phylogenetic tree showing rhodanese similarity among different bacterial species. A maximum likelihood analysis was performed with 1000 bootstrap replicates; with bootstrap values indicated above the branches. The isolated B. licheniformis rhodanese sequence (GenBank accession number PQ421362) is marked with a black circle.*

Overexpression of the recombinant rhodanese gene

Expression of the *rhdA* gene in *E. coli* M15 (pREP4) (pQE*rhdA*) resulted in high level of protein production with molecular weight of 35 KDa, which was consistent with the predicted molecular mass of *B. licheniformis* RhdA obtained after the addition of 0.5 mM IPTG-induction at 37°C, and maximal expression was reached after 7 h post induction (Figure 5a). Western blotting analysis with anti-His antibody against r-RhdA recognized a 35 kDa band (Figure 5b); thereby, confirming the identity of r-RhdA, while the presence of a distinct purple band at approximately 36.65 kDa confirmed the successful expression of the target protein.

Assessment of specific activity of recombinant rhodanese

The expressed recombinant rhodanese in form of cleared lysate was generated and analyzed using 12% SDS-PAGE gel, as illustrated in Figure 6. The total protein and specific activity of recombinant rhodanese were measured. The specific activity of recombinant rhodanese (0.155 RU/mg) was 4.08-fold more than that of *B. licheniformis* rhodanese (0.038 RU/mg).

Biodegradation of cyanide using immobilized recombinant rhodanese

In order to evaluate the role of recombinant rhodanese (r-RhdA) in detoxification of cyanide, the enzyme was immobilized within calcium alginate-gelatin mixture. The immobilized r-RhdA beads were incubated with wastewater containing approximately 0.141 mg/L of cyanide at 37 °C. After incubation. the remaining amount of cyanide in water sample was 0.04 mg\L, indicationg the achievement of a biodegradation efficiency of 71.6%. These results suggested that the immobilization technique is well-suited for enhancing the functionality of recombinant rhodanese in cyanide detoxification.

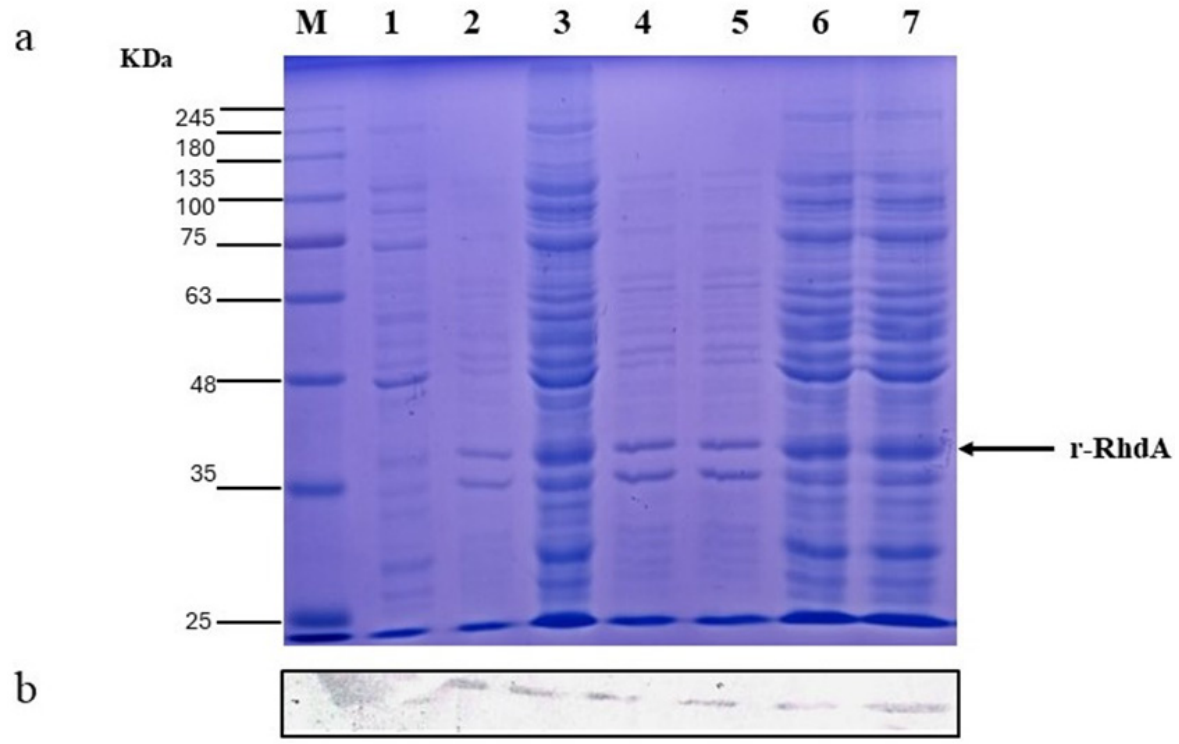

Figure 5: *Expression of recombinant rhodanese in E. coli M15[pREP4]. (a) 12% SDS-PAGE, and (b) Western blot analysis. Lane M: pre- stained protein ladder (Gang Nam-STAIN™, Cat. No. 24052, iNtRON Biotechnology, Inc., Korea), Lane 1: recombinant plasmid at zero time of induction, Lane 2: recombinant plasmid after 2 h of induction, Lane 3: recombinant plasmid after 3 h, Lane 4: recombinant plasmid after 4 h, Lane 5: recombinant plasmid after 5 h, Lane 6: recombinant plasmid after 6 h, and Lane 7: recombinant plasmid after 7 h.*

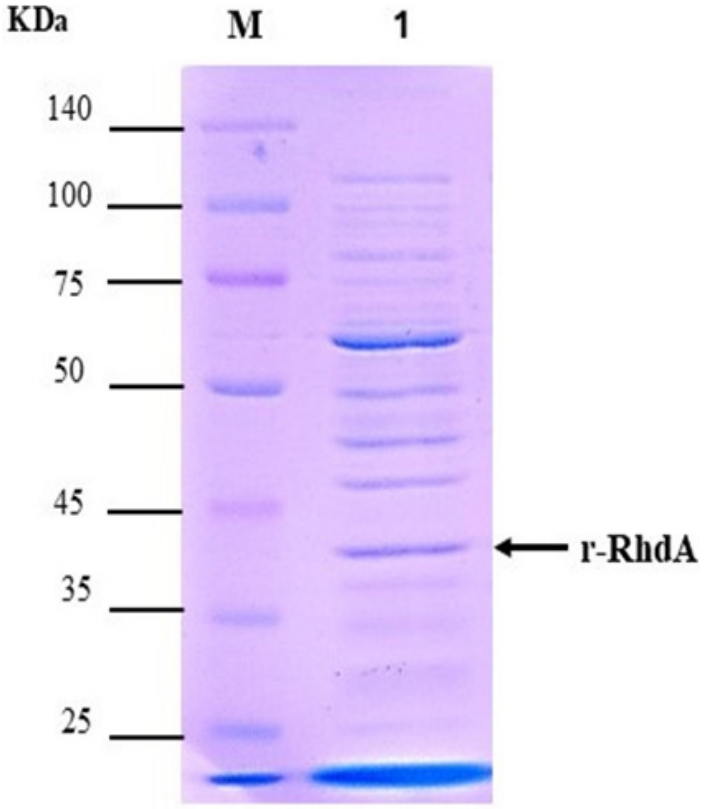

Figure 6: *A 12% SDS-PAGE of the cleared lysate of expressed rhdA protein in E. coli M15[pREP4] under native conditions. Lane M: Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, Cat. No. 26634, Lithuania), Lane1: cleared lysate of expressed r-RhdA at ~36.65 KDa.*

Discussion

In this study, the bacteria were successfully isolated from industrial wastes to investigate their potential for producing rhodanese, an enzyme with critical bioremediation applications used in detoxifying of cyanide; a highly toxic environmental contaminant. Meanwhile, we reported the successful cloning, expression, and characterization of the expressed rhodanese enzyme in *E. coli* M15, along with its subsequent immobilization.

Cyanide exerts its toxic effects by binding to iron in blood, blocking oxygen transport, and causing asphyxiation (Thabet *et al.*, 2023). This toxicity is primarily associated with the halt of aerobic cellular metabolism (Schaffer *et al.*, 2025). Rhodanese activity has been discovered in several bacterial species, such *as Enterococcus faecium* and *E. gallinarum* (Khota *et al.*, 2023), and *Klebsiella oxytoca* (Itakorode *et al.*, 2024b). Rhodanese has a critical role in cyanide detoxification. It catalyzes the transfer of sulfur to cyanide, forming thiocyanate that is substantially a less toxic compound.

In the present study, fifty bacterial isolates that were obtained from industrial wastes were sub-cultured on a medium supplemented with potassium cyanide (KCN) as a selective agent to isolate the cyanide-degrading bacteria. Cyanide-degrading bacteria are capable of utilizing cyanide as a nitrogen source (Luque-Almagro *et al.*, 2005). Out of the fifty isolates, only 17 isolates demonstrated growth on KCN-containing medium within 72 h. The extended lag phase observed in their growth was attributed to the cyanide concentration in the medium, indicating that they required a short time for adaptation (Kandasamy *et al.*, 2015). Cyanide-degrading bacteria employ four primary ways for biodegradation of cyanide; mainly hydrolytic, reductive, oxidative, and substitution/transfer (Ebbs, 2004). In turn, an enzyme assay was performed on these 17 isolates to select the most promising rhodanese-producing isolate. As a result, a single B47-encoded bacterial isolate exhibited the highest rhodanese activity. The selected isolate was Gram-positive, spore-forming, and rod-shaped, and its cells were arranged individually and in pairs. The results of comparative phenotypic and genotypic analyses identified the B47-encoded isolate as *B. licheniformis*. The use of two techniques, MALDI-TOF MS and 16S rRNA gene sequencing together improved the microbiological identification accuracy by harnessing the strengths of both technologies (Meng *et al.*, 2025). The sequencing result of the isolated gene was compared to other available sequences of the rhodanese gene in the GenBank database using the BLASTn tool, revealing a 99 % identity with the rhodanese gene of *B. licheniformis* strain PB3, with an accession number of CP025226. This fragment encoded a protein consisting of 318 amino acids with a molecular weight of 35.98 kDa.

Phylogenetic analysis of rhodanese indicated that the sequence of interest, PQ421362, from *B. licheniformis* clusters was closely related with other *Bacillus* species, evidenced by high bootstrap values. This strong clustering indicated evolutionary conservation and functional similarities among rhodanese proteins within the genus *Bacillus*. This strong conservation highlights the critical roles of rhodanese in survival and environmental adaptation of *Bacillus* species, particularly in industrial waste environments. Similarly to a prior study showed that, across species, a phylogenetic analysis indicates that rhodanese's primary structure is highly conserved. There are 85 differences but 70% amino acid similarity between bovine and chicken rhodanese (Miller *et al.*, 1991). The native molecular weight of rhodanese of *Klebsiella oxytoca* JCM 1665 wild type was 35.1 KDa (Itakorode *et al.*, 2024a), and the size of purified rhodanese protein from *Azotobacter vinelandii* was 30 KDa (Colnaghi *et al.*, 1996).

In this study, SDS-PAGE analysis displayed a band corresponding to the expected size of r-RhdA. Additionally, western blotting using commercial monoclonal antibodies against the His_6 epitope identified a 35.98 kDa band, thereby confirming the identity of *B. licheniformis* RhdA. As reported in a pervious study, these variations in molecular weights among bacterial rhodanese enzymes demonstrate the presence of conserved region within the gene responsible for rhodanese synthesis (Itakorode *et al.*, 2024a), these variations in molecular weights among bacterial rhodanese enzymes demonstrated the presence of a conserved region within the gene responsible for rhodanese synthesis. Moreover, as per the phylogenetic tree, there was a notable variation in amino acid sequence and a conserved region among the bacterial species, to keep the major function and catalytic activity of rhodanese. In this respect, the recorded specific activity of crude rhodanese from *B. licheniformis* encoded B47 was higher than that

produced by *K. edwardsii* (Oluwatosin *et al.*, 2017).

The specific activity of recombinant rhodanese in the lysates of *E. coli* M15 expression host cells was higher than that of the crude extract from *B. licheniformis* encoded B47. Specifically, the rhodanese activity was 4.08-fold higher in lysates of IPTG-induced *E. coli* M15 (pREP4) (pQE*rhdA*) cells. This increased activity may result from the use of robust, inducible promoters in the *E. coli* expression system, leading to higher enzyme production compared to native wild-type bacteria. Another possible reason could be a conformational difference between recombinant rhodanese produced in *E. coli* and native rhodanese from *B. licheniformis*. A similar observation was reported by Miller *et al.* (1992) that examined IPTG-induced expression of rhodanese in *E. coli* BL21 and found that the recombinant enzyme had 12% greater specific activity than bovine rhodanese. The authors explained that the reason for this difference was unclear and could be attributed to conformational differences. Similarly, Li *et al.* (2008) reported that the recombinant cellulase activity peaked at 4 h after induction, reaching a level nearly three times higher than that of the wild-type strain.

The recombinant rhodanese was immobilized to enhance its efficiency for cyanide removal from water samples using a distinct approach involving calcium alginate-gelatin composites. As reported by Mogharabi *et al.* (2012), gelatin was incorporated into the alginate matrix during immobilization to tighten the large pore sizes of the alginate beads, thereby minimizing enzyme leakage. Actually, this strategy enabled the immobilized recombinant rhodanese beads to achieve a cyanide biodegradation efficiency of 71.6%.

A previous study showed that the highest cyanide removal efficiency from wastewater using mixed bacterial culture was 41.12% (Saravanan *et al.*, 2019). Comparative analyses indicated that the use of immobilized enzymes, as opposed to microorganisms, provides a more effective approach for cyanide biodegradation. Several studies have demonstrated that free microbial cells are highly susceptible to external environmental stressors, whereas immobilized enzymes exhibit increased resistance to such conditions and can be readily removed upon completion of the reaction (Nadaroglu and Sonmez, 2016; Li *et al.*, 2019).

In another study, an immobilized consortium of *Trichoderma saturnisporum* and *T. citrinoviride* recorded the highest cyanide degradation of 99.9% within 48 h (Rishi *et al.*, 2023). In comparison, the currently used method combined two fungal strains, resulting in high cyanide biodegradation, but it took a very long time to reach maximum degradation. We suggest that using an immobilized enzyme may achieve faster cyanide degradation. To examine if an immobilized r-RhdA offers this advantage, we propose conducting experiments that run beyond 4 h, such as up to 24 h to fully test this possibility. In this respect, the efficiency of cyanide biodegradation using an immobilized rhodanese in a calcium alginate-gelatin mixture was higher, compared to that achieved with immobilized rhodanese in calcium alginate only, as reported by Ademakinwa *et al.* (2021). These obtained results suggests that incorporating gelatin into calcium alginate during immobilization offers a more effective approach to cyanide bioremediation, compard to using calcium alginate alone, as it reduces enzyme leakage and enhances biodegradation efficiency.

Conclusions and Recommendations

In the current study, a *rhdA* gene was successfully isolated from *B. licheniformis* and cloned into the pQE-30 vector under the T5 promoter. The r-RhdA was expressed with a 4.08-fold increase in specific activity compared to the crude extract, which showed activity of 0.155 RU/mg and was subsequently immobilized into calcium alginate gelatin composites, achieving a cyanide biodegradation efficiency of 71.6%. These findings highlight the potential of immobilized r-RhdA as a sustainable bioremediation tool for cyanide-contaminated fluids. Furthermore, the construction of *E. coli* M15 (pREP4) (pQE*rhdA*) for r-RhdA production demonstrated the feasibility of microbial rhodanese for cyanide detoxification under laboratory conditions and its possible application in natural environments. This approach is an ecofriendly alternative to traditional ways of treating cyanide with chemicals and physical methods. Despite these promising findings, several limitations must be acknowledged. Firstly, the complete cyanide removal was not achieved. Secondly, we didn't use a commercial enzyme as a control for cyanide breakdown. Thirdly, the repeated usage of immobilized recombinant rhodanese was not studied. To fully establish the applicability of recombinant rhodanese, we recommend future investigations

focusing on achieving complete removal of cyanide. We plan to study the effect of various factors on cynide detoxification using microbial rhodanese enzyme, including different cyanide concentrations, different cyanide derivatives, combination of cyanide with other pollutants, and different immobilization techniques. Additionally, in the future, we recommend the use of omics technologies to develop and gain more knowledge about cyanide biodegradation.

Novelty statement

The novelty of this study lies in using *E. coli* M15 (pREP4) as an expression host to achieve high level of recombinant rhodanese production. This recombinant rhodanese was subsequently immobilized for potential application in the bioremediation of cyanide-polluted water.

Author's Contribution

MB: Methodology, Analysis, and Writing.
WE: Data analysis, Interpretation, and Reviewing.
MSM: Conceptualization, Interpretation, and Reviewing.
MSK: Conceptualization, Supervison, and Reviewing.
All authors contributed to the final manuscript and discussed the outcomes.

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Ethical approval
Not applicable.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI was used for data collection, analysis, interpretation, or manuscript writing. All content was created by the authors.

Conflict of interests
The authors have declared no conflicts of interest.

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