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Short Communication

The Optimization of Sulphide Oxidizing Bacteria (SOB) for Oil Corrosivity Reduction at Indramayu Coast, The Northern Coastal Area of West Java, Indonesia

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Abstract

Crude oil production triggers the formation of hydrogen sulphide, also known as souring, which is extremely toxic and corrosive to the environment. It additionally give an adverse consequence to aquatic, terrestrial, and human existence. Studies of hydrogen sulphide reduction in sediments polluted by crude oil have been carried out recently to investigate the capability of indigenous Nitrate-Reducing Sulphide Oxidising Bacteria, hereinafter referred to as NR-SOB, as bioremediation agents. The experiments utilised hydrogen sulphide with 200 µM concentration combined with NO_3 with different concentrations of 100 µM, 200 µM, and 300 µM. Measurements of the hydrogen sulfide concentrations were observed up to 48 hours within the experimental period. The SOB used in this study were taken from Balongan Bay at Indramayu coast using Nansen bottle to carry out water sample. The sulphide-oxidising ability of SOB was then evaluated at room temperature in control environment. Methylene blue method was applied to monitor the sulphide concentration. The results showed a complete removal of hydrogen sulphide concentrations in 48 hours accompanied with gradual drops of nitrate in all experiment series. Sulphide oxidation rate was detected to appear between 6.8 and 10.2 fmol/cell/hour. Measurements of cell abundance after 48 hours showed 6.2 x 105, 7.5 X 105, and 8.2 X 105 cell/ml from Experiments I, II, and III respectively. Using MSS selective medium, the bacteria were identified as *Thiobacillus denitrificans* and *Arcobacter* sp. Overall, the isolated NR-SOB from the coast of Balongan Bay, Indramayu proves to be a promising candidate for sulphide controls and mitigation

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1. Introduction

Sulphide generation, resulting from both naturally and anthropogenic activities, has been long regarded as a major problem to its environment. It is prone to occurring not only in engineering facilities such as sewer, oil reservoirs, aquaculture, wastewater treatment plants, but also in natural environment like sediments, volcanic and hydrothermal vents. Waste water contaminated by sulphide not only negatively impact the aquatic and terrestrial life, but also affects human life (Zaib *et al*., 2022). This type of sulphide formation is attributed to the group of bacteria called Sulphate Reducing Bacteria (SRB), whose jobs is to reduce the release of sulphate in anaerobic environment by using sulphate as an electron acceptor to produce sulphide and free form of hydrogen sulphide (H_2S) (Liu *et al*., 2018). The release of free hydrogen sulphide (H_2S) from sediment into water column is viewed as the extreme consequence of anaerobic water column reactions and it occurs after the oxidative capacity of bottom waters is exhausted. Hydrogen Sulphide (H_2S) is certainly undesirable due to its high toxicity, unpleasant odor, and severe corrosiveness (Austigard *et al*., 2018). Exposure of hydrogen sulphide (H_2S) was reported to decrease the survival rate of benthic communities by 30% due to tissue hypoxia, disturbance in aerobic metabolism, and the exposure is also able to cause pulmonary oedema and sudden unconsciousness (Austigard *et al*., 2018). Some elevated hydrogen sulphide (H_2S) concentrations have been recorded in other areas such as in the Namibian shelf waters which correlates with high macrofaunal mortality rate and in China exposure of sulphide is related to immunology and stress response of the ark shell (Currie *et al*., 2018; Wang *et al*., 2019). Moreover, the presence of hydrogen sulphide in sewers and oil field facilities have been damaging the existential structures in the environment by initiating corrosion, and billions of financial lost have been reported.

Several efforts to address this issue have been performed for years, yet they were all still in the phase of exploratory research. The most common method is to add biocides to suppress the growth of SRB (Vaithiyanathan *et al*., 2018). Generally, biocides used to control SRB includes glutaraldehyde, tetrakis (hydroxymethyl) phosphonium sulphate (THPS), benzalkonium chloride, formaldehyde, and sodium hypochlorite (Basafa and Hawboldt, 2019). However, this approach could potentially lead to generation of biocide-resistant strain of bacteria that would create another challenging problem (Basafa and Hawboldt, 2019). Another strategy to tackle the issue is by nutrient addition, particularly nitrate, where it promotes the growth of another group of bacteria called Nitrate-Reducing Sulphide-Oxidizing Bacteria (NR-SOB) that will metabolically convert sulphide to relatively less toxic sulphate (Basafa and Hawboldt, 2019; Dolfing and Hubert, 2017). Sulphide oxidation done by chemolithoautotrophic-denitrifying bacteria can lead to a formation of sulphur or sulphate, depending on the physiological conditions (Cui *et al*., 2019; Jørgensen *et al*., 2019; Kiragosyan *et al*., 2019). When nitrate is injected to the well or water column of compartment that is rich in sulphide, NR-SOB will start reducing nitrate and change it into nitrite, which later acts not only as hydrogen sulphide scavengers, but also an effective inhibition agent for SRB (Watsuntorn *et al*., 2017). Thus, the final products are sulphate and nitrogen gas.

NR-SOB typically can be found as indigenous bacteria in anaerobic environment. Several studies reported of the prevalence of isolation of NR-SOB in oil fields, sediment of intensive aquaculture, and hot springs (Kumar *et al*., 2018; Watsuntorn *et al*., 2017). However, in open ocean waters, little is known since episodic appearances of sulphuric plumes have only been reported recently. The coast in the northern part of West Java, Balongan Bay Indramayu, is home to Indonesia's biggest oil refinery, where risks of contamination occur frequently. Yet, no studies on the potential isolation of NR-SOB from this contaminated sediment have been reported. This research focuses on batch cultures taken from the bay coast area to identify nitrate reducing sulphide oxidizers (i.e., several bacteria groups will be used for this research) and to study sulphur and nitrogen products with various nitrate concentrations.

2. Materials and Methods

2.1 Materials

Laboratory experiments were used as the method to conduct this research. The inoculum was isolated from contaminated sediments in Balongan bay, Indramayu. Artificial seawater and specific modified version of Coleville Synthetic Brine (CSB), called MSS (Medium Screening Sulphide) for facilitating exclusive growth of NR-SOB, were used according to the research that had been published previously (Callbeck *et al*., 2019). Our MSS contained 2g of KH_2PO_4, 2g of KNO_3, 1g of NH_4Cl, 0,8g of $MgSO_4.7H_2O$, 2ml of mineral solution with pH 6, 5g of $Na_2S_2O_3.5H_2O$, 1g $NaHCO_3$ and an addition of 15g solid medium culture. Throughout the whole experiments, nitrogen gas was also used to prevent aerobic condition. Uv-Vis Spectrophotometer and Gas Chromatography Mass Spectroscopy (GC-MS)

were used for our parameter measurement.

2.2 Method

2.2.1 Incubation

The treatment in this research was carried out by adding 200 µM of H_2S concentrations of sea water (testing medium) with various nitrate concentrations of 100 µM (Experiment I), 200 µM (Experiment II), and 300 µM (Experiment III) by 10% v/v bacterial inoculum derived from the previously grown liquid culture stock. The experiments were done in an-aerobic condition by adding N_2 gas to omit oxygen (Figure 1). As for the measurements of gasses, an adequate amount of sample was then inserted to vial bottles based on the treatment time of each observation (0, 3, 6, 12, 24, and 48 hours within the experiment period) and $HgCl_2$ was added in each time step accordingly.

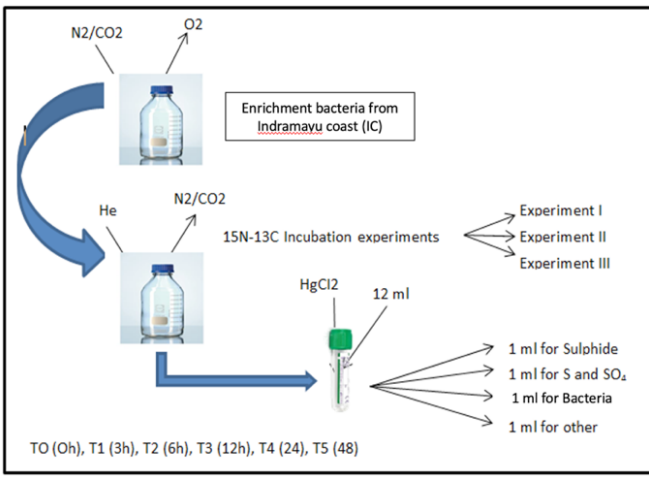

Figure 1. Incubation Process

2.2.2 Identification and parameter measurement

Bacteria identification was performed through two phases, namely morphology test by gram staining, and biochemical test using Kit API 20A of 20 tests. Fluorescent In Situ Hybridization was also performed to further see the bacterial characteristics (Hu and Wu, 2021). Parameters that are measured in this research are H_2S, NO_3, and the bacteria abundance in each treatment. Hydrogen Sulphide concentration was measured colorimetrically using methylene blue method (Wang *et al.*, 2021). Colorimetric determination was also conducted to measure NO_3 and bacterial abundance using UV-Vis Spectrophotometer at 540 nm and 600 nm, respectively (Studt *et al.*, 2020). Gas chromatography spectroscopy was used to understand the pattern of nitrate reduction products such as N_2O and N_2 gasses.

3. Results and Discussion

3.1 Bacterial Growth

Controls of hydrogen sulphide especially in oil reservoirs, have been performed by manipulating the environment, such as having an addition of nitrate, in order for its microbiological population structures to shift from the SRB dominated environment to NR-SOB homogenised one. Typically, an addition of nitrate will promote the growth of NR-SOB and consequently suppress SRB. Isolation procedure was employed to exclusively isolate NR-SOB from the contaminated sediment using a modified version of Coleville Synthetic Brine (CSB), a commonly used medium for NR-SOB isolation, called MSS (Medium Screening Sulphide) for NR-SOB. The medium was incorporated with a redox indicator for a colour change as the NR-SOB actively grew (Callbeck *et al.*, 2019). The isolated inoculum of NR-SOB would then be transferred to a liquid culture medium for the incubation experiment shortly thereafter.

The growth of NR-SOB in batch experiments with the addition of nitrate revealed a clear pattern of gradual shift in the population density from the lowest nitrate concentration (100 µM) to the highest one (300 µM) (Figure 2). Its peak growth took place at 48-h (T_5) with a measured value of 8.2 x 10^5 cell/ml, whereas its initial concentration at 0-h (T_0) was only measured at 2.1 x 10^5 cell/ml. Therefore, the bacterial growth in Experiment III is 1.27 x 10^4 cell/ml. The lowest bacterial growth occurred in Experiment I with the concentrations of H_2S and NO_3 measuring at 200 µM and 100 µM respectively. Bacteria abundance on experiment I after 48-h (T_5) is 6.2 x 10^5 cell/ml. Meanwhile the bacteria abundance on experiment I is 8.2 x 10^3 cell/ml/h. The values matched the previously published research by Nemati *et al.* (2001) when the ratio of SRB and NR-SOB appeared more distinct as the nitrate addition triggered an increase up to 20 mM. It is important to note that NR-SOB will metabolically oxidize sulphide to sulphate or elemental sulphur by utilizing nitrate as their sole electron acceptor in anaerobic condition (Lahme *et al.*, 2019; Veshareh *et al.*, 2021). Thus, growth in population was also expected. It is also reported that, after NR-SOB cultures with SRB and sulphide were added to the batch medium (Kamarisima *et al.*, 2018), instant depletion of nitrate occurred, accompanied with the discontinuation of sulphide production. This reaction was associated with the rapid growth of NR-SOB in the presence of nitrate.

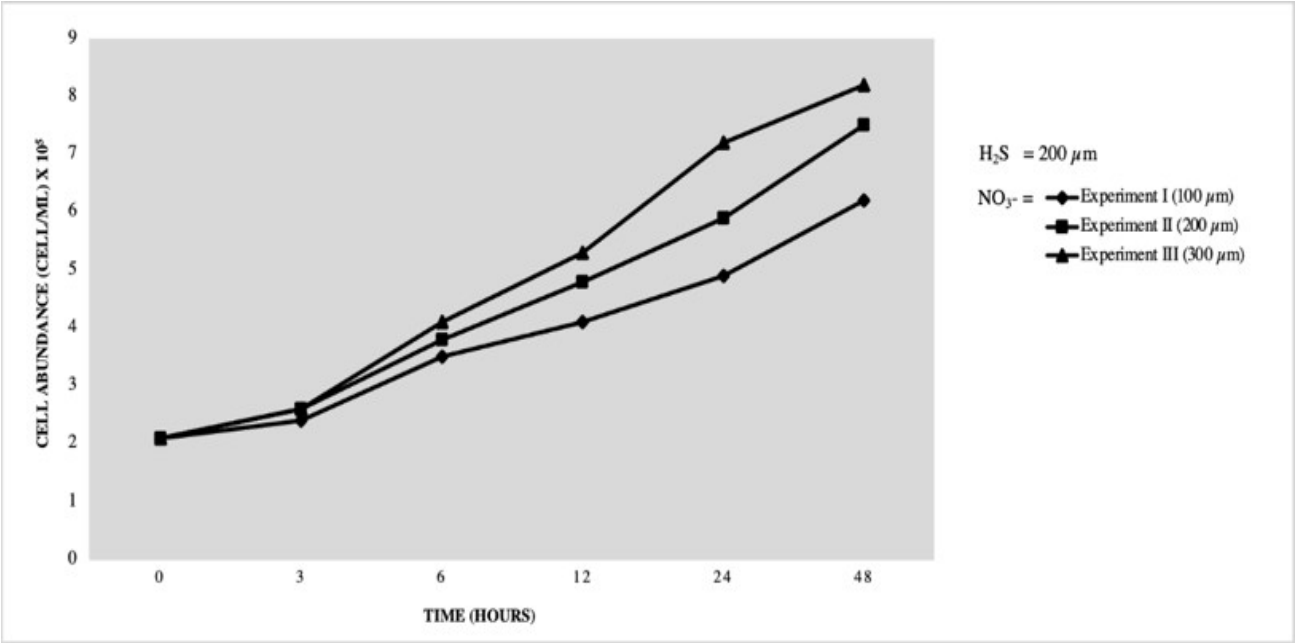

Figure 2. Bacterial growth in artificial sea water

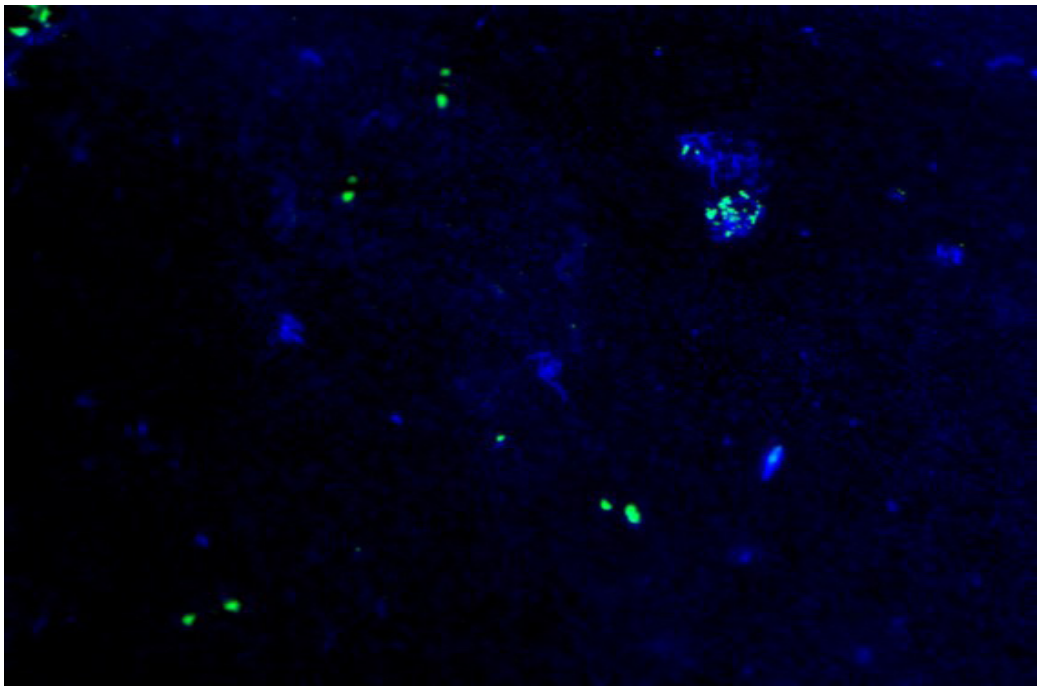

Figure 3. In situ hybridization

Bacterial growth is determined by the availability of nutrients such as C, N, and P, and while their presence is considered limited factors in determining the bacteria activities, the presence of those compounds in the sea water medium is known to be very scarce. Therefore, the bacteria in sea water medium with 200 µM H_2S can only be used along with 100 µM, 200 µM, and 300 µM of NO_3. Another fact to consider during the calculation was that the 300 µM NO_3 from the bacterial growth experienced a lag phase between 0 and 3-h, and another lag phase at the 48th hour, followed by a stationary phase and declination thereafter. Though in general substrate supports bacteria growth, these bacteria were unable to grow due to the accumulation of toxic metabolism byproducts.

3.2 Bacterial Characterisation

The isolates were characterized both morphologically and biochemically to give a glimpse of species compositions of the NR-SOB consortia. It became

apparent that the bacteria consisted of a large number of gram-negative bacteria. Our biochemical 20A API Test revealed that the isolates shared almost the same characteristics with *Arcobacter* sp. and *Thiobacillus denitrificans*. The functional gene sequence analysis and fluorescence in situ hybridization indicated that the detoxification, performed with chemolithotrophic oxidation of sulphide and nitrate, was mainly catalysed by two discrete populations of gammaproteobacteria, the blue one, and epsilonproteobacteria, the green one (Figure 3).

Both gamma and epsilon proteobacteria are well-known for their ability in oxidizing sulphuric compounds (Patwardhan *et al*., 2018). They were identified in deep sea hydrothermal vent environment as predominant primary producers both in symbiotic and free-living system with inorganic sulphur as their source of energy (Yamamoto and Takai, 2011). Gammaproteobacteria are known to live in microaerobic environment, where its optimum metabolism occurred in the co-existence of reduced sulphur and oxygen (Guerrero-Cruz *et al*., 2021; Gupta *et al*., 2022).

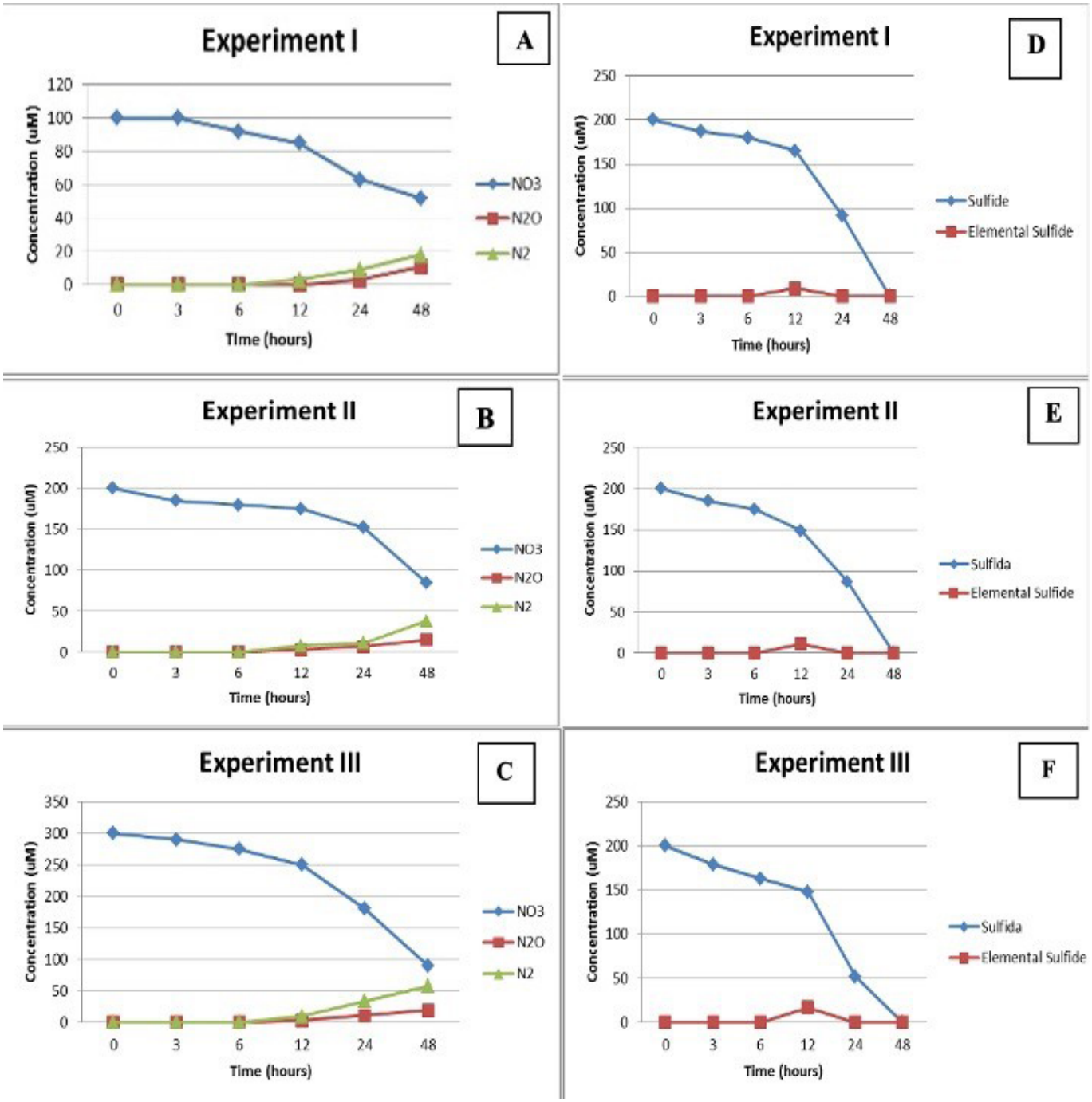

Figure 4. Nitrogen removal from sulphidic batch culture in various concentrations (A) 100 µM (B) 200 µM, and (C) 300 µM, along with the sulphide oxidation rates in (D) 100 µM, (E) 200 µM, and (F) 300 µM during the initial nitrate concentrations.

Meanwhile, the epsilonproteobacteria were also found to be able to utilize sulphur both for the electron donor and acceptor in two kinds of metabolic pathway, i.e. using hydrogenase and polysulphide reductase enzymes to perform hydrogen oxidizing sulphur respiration, and using Sox Multienzyme Complex System (Yamamoto and Takai, 2011). Sox pathway will utilize different types of proteins, e.g. SoxYZ, SoxXA, SoxB, and SoxCD, that are essential to conduct a complete oxidation from sulphide to sulphate (Wu *et al*., 2021). Similar to this, the gammaproteobacteria group has also utilized the Sox Multienzyme Complex System, but in the absence of SoxCD protein that operated as sulphur dehydrogenase (Yamamoto and Takai, 2011).

Several other species of gammaproteobacteria and epsilonproteobacteria were also identified as a symbiont of higher organisms in sediments, and deep sea. Epsilonproteobacteria *Sulfvorafum* sp. and *Nitratriptor* sp. for example were isolated from the sediments of mid-Okinawa (Mino and Nakagawa, 2018). *Thiomicrospira crunogena* from the vents of Galapagos Rift, *Endoriftia persephone* from Riftia symbiont, *Ruthia magnifica* from Calyptogena symbiont also reported as gammaproteobacteria are present in sulphide rich environments (Mangiapia *et al*., 2017). Nevertheless, both groups have a large impact on the sulphur cycle and productivities in deep sea ecosystems and anaerobic conditions.

3.3 NR-SOB Activities

In anaerobic conditions, where oxygen is not present, microorganisms utilize other terminal electron acceptor such as nitrate, sulphate or iron (Rubio-Rincón *et al*., 2017). However, in natural system, its occurrence is limited by various physicochemical factors, thus reverting all the processes. An addition of terminal electron acceptor will then promote its metabolic activity and growth as it was indicated in this research as well as in other studies similar to ours (Cai *et al*., 2021; Fan *et al*., 2020). The physicochemical factors such as temperature and pH will influence the metabolic activity of NR-SOB as they govern the rate of the reaction and the speciation of sulphur and nitrate species in the water (Watsuntorn *et al*., 2017).

Nitrate was found to experience a slight decrease in the first few hours followed by a noticeable depletion afterwards reaching a total of 70% reduction at 48h observation (Figure 4A-4C). When nitrate was injected to the culture, it readily acted as electron acceptor for the NR-SOB to oxidize H_2S (Lahme *et al*., 2019), it can be depicted by alignment decrement of both nitrate and H_2S in the observed experiments, accompanied by generation of nitrate reduction final product which is N_2 gas via nitrite intermediate. This was also observed in Zhang *et al*. (2020) where a sudden plunge of nitrate concentration occurred as soon as NR-SOB was added to the medium. The same pattern was also detected during the practical application of nitrate injection to an oil field reservoir where progressive reduction occurred as it was being utilized by the NR-SOB (Kamarisima *et al*., 2018). This nitrate reduction was accompanied by a steady generation of nitrate final products, e.g., N_2O and N_2, indicating that a denitrifying mechanism occurred after its reduction (Figure 4A-4C).

Hydrogen sulphide present in the water acted as electron donor for NR-SOB, where then the oxidation adjusts the transformation to a final product, either sulphate or elemental sulphur via Sox pathway (Grabarczyk and Berks, 2017). This electron from the sulphide oxidation would then be utilized to reduce nitrate to N_2O and N_2 with the help of nitrite that acted as intermediate species (Greene *et al*., 2003). N_2 and N_2O productions started to occur after 12h as they were also confirmed by GC-MS, N_2 productions were measured higher than N_2O's in all experiments, indicating that denitrification to N_2 gasses was dominating in the process (Figure 4A-4C).

The decline of H_2S concentrations that occurred in all experiments (Figure 3). Generally, all experiments showed the same trends of decreasing sulphide. Initial screening at 0 to 6 h showed low oxidation ability. Subsequently, the oxidation was measured higher after 6h. The illustrations show where sulphide was removed approximately 54% to 74% in the first 24hours, progressing to a total of 100% removal in 48 hours thereafter. The sulphide oxidation rate was found ranging between 6.8 and 10.2 fmol/cell/hour. This total removal of all sulphide in all batch experiments also explained the unfinished nitrate reduction where approximately 30% of nitrate remained in the medium. Since the hydrogen sulphide was all used up, there were no electron donors left for nitrate reduction, hence leaving the nitrate unreduced.

After 12h, elemental sulphur was detected at a very low concentration of approximately 9 to 17 µM (Figure 4D-4F). It indicated that sulphate was the main product of sulphide oxidation. Sulphide oxidation by chemolithoautotrophic denitrifying bacteria can lead to the formation of either sulphur or sulphate depending on its physiological condition. Facultatively, chemolithoautotrophic sulphide-oxidizing bacteria are widespread in marine environments (Cúcio *et al*., 2018). They do

not only mediate the oxidative part of sulphur cycle, but also the reductive part of nitrogen cycle as it is also in conformity with the stoichiometry equation (De Anda *et al*., 2018).

$$5H_2S + 8KNO_3 \text{ --> } 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O$$

The results above are in concurrent with Findlay *et al*. (2020) research where sulphate is a dominant final product from sulphide oxidation. In a natural experiment where coexistence of SRB and NR-SOB occurred, the relationship between two groups is largely governed by the chemical equilibrium of sulphate, sulphide, nitrate, and nitrite. An addition of NR-SOB to the system followed by even a relatively small amount of nitrate was able to terminate SRB activities from reducing sulphate. This was postulated by the generation of nitrate intermediate product, nitrite, that acted as the natural biocides for SRB by inhibiting their sulphite reductase (Fida *et al*., 2021). While SRB stopped reducing sulphate, NR-SOB started to oxidize sulphide, in the presence of nitrate, in order to produce sulphate. A sufficient amount of nitrate should be present in order to have a complete oxidation of sulphide (Sun *et al*., 2019). Eventually, as the oxidation continued to occur, nitrate concentration dropped until there was no more suitable amount left. Then again, SRB found its way to start reducing the produced sulphate.

4. Conclusion

In conclusion, the NR-SOB isolate from Balongan Bay, Indramayu proves to be an excellent candidate for the sulphide removal with denitrification as the main process. The sulphide oxidizers used in batch culture are *Thiobacillus denitrificans*, and *Arcobacter* sp. The experiments performed in control environment with various nitrate concentration additions successfully oxidized sulphide up to 100% within 48 hours with a considerable rate ranging between 6.8 to 10.2 fmol/cell/hour. This study provide an assistance for the government of Indonesia or the relevant authorities including industries in resolving the problem of contamination in aquatic and coastal environment.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow: Y.N.I and T.K.P.; Conceptualization, Y.N.I. and R.P.; methodology, Y.N.I. and J.M.; validation, Y.N.I. and T.K.P.; formal analysis, K.F.; investigation, R.P.; resources, Y.N.I and R.P.; writing-original draft preparation, Y.N.I. and K.F.; writing – review and editing, J.M.; supervision, K.F.; project administration, Y.N.I.; funding acquisition.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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