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Eco-friendly Industrial wastewater treatment: Potential of mesophilic bacterium, *Pseudomonas putida* (ATCC 49128) for hydrogen sulfide oxidation

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ABSTRACT

The effectiveness and environmental friendliness of biological sulfide oxidation have endeared it to many researchers, mainly for its potential to offer the best alternative for the evacuation of various forms of sulfide. The present study was conducted to assess the potential of gammaproteobacteria, *Pseudomonas putida* (ATCC 49128), to biodegrade sulfide significantly in a suspended medium of batch reactor type. Sulfide oxidation efficiency was measured spectrophotometrically under defined operational conditions of temperature, agitation, aeration, and influent sulfide concentrations. Sulfide reduction rates were observed at three disproportionate sulfide concentrations of 100 ppm S^{2-} L^{-1} d^{-1}, 300 ppm S^{2-} L^{-1} d^{-1}, and 500 ppm S^{2-} L^{-1} d^{-1}. The simple statistical analysis was employed in the interpretation of the data and presented graphically. The results indicated that it was possible to realize sulfide removal efficiency of 45–70% within the first 6 h of start-up and 96–100% in 24 h period. On the other hand, the corresponding exponential cell growth recorded was 3.91, 3.80, and 3.61 in 100 ppm, 300 ppm, and 500 ppm, respectively. This also translates to cell biomass synthesis (cell dry weight) of 0.61 g/L, 0.58 g/L, and 0.50 g/L in 100, 500, and 300 ppm, respectively. In conclusion, it can be deduced that this inoculum can utilize different sulfide concentration for its growth and biosynthesis and thus can be employed to treat sulfide contaminated wastewater in a suspended growth form.

1. INTRODUCTION

Hydrogen sulfide (H_2S), or popularly sulfide, is a notable environmental pollutant considerably produced from numerous domestic and industrial wastewater sources. A toxicity effect of H_2S is not restricted to human and environment alone but extended to microbial community capable of degrading it. It is highly toxic compounds that can be formed in an aqueous system which contains both organic matter and sulfate. According to Enning and Garrelfs [1], sulfide buildup in industrial systems may cause several side effects such as corrosion of concrete sewer pipes (mainly, due to microbiologically-induced corrosion by sulfate-reducing bacteria), releasing pungent malodors, toxicity due to sulfide gas, and negative effect on subsequent receiving waters, hence necessitating its mitigation from the surrounding [2].

The impacts caused by these industrial pollutants and growing concern for environmental issues have led to the search for new methods of treatment and development of new approaches that are able to reduce sulfide to a permissible discharge level. The classical approach to sulfide removal recorded some tremendous successes, albeit was associated with some set of drawbacks, such as high energy requirements, huge capital investment for handling and maintenance as well as production of secondary pollution [3-6].

However, biological sulfide oxidation (BSO) has the potential to give a perfect different option for the evacuation of low- and high-level sulfide from both fluid and gas streams, alongside the recuperation of sulfur [7,8], which is of greater economic value. Besides, BSO is the cost-effective, efficient, flexible, and more importantly, eco-friendly technology used to treat sulfide-laden wastewater. There are many different approaches to BSO based on immobilized and suspended batch reactor types (BRT).

The present study was therefore set to ascertain the potential of this mesophilic bacterium to BSO, which until now was rarely documented, although some significant discoveries were made related to growth optimization conditioned in different substrates medium. In addition to this, some findings were available in the use of this

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mesophilic bacterium in the treatment of wastewaters laden with phenol, petrochemical effluent, and some heavy metals. Pilot study related to sulfide reduction using synthetic water with exogenous carbon and nutrient sources under defined operational parameters of BRT by *Pseudomonas putida* against different simulated sulfide concentrations has not been assessed previously. However, simple as it may appear, it is believed that this could serve as an indicator to achieve BSO in a cheap, simple, and eco-friendly approach. Therefore, the finding from this work could be utilized to further sulfide oxidation research studies using this pure culture.

2. METHODOLOGY

2.1. Test Organism and Cultivation

A pure culture wild-type strain of *P. putida* (ATCC 49128) was used for this study. The nutrient broth was purchased from Merck (Darmstadt, Germany) and prepared according to the manufacturer's instruction. Thereafter, it was sterilized in an autoclave (H+P Varioklav Steam Sterilizer ESCO, Japan) at 121°C for 15 min, cooled in a water bath to 47°C, and later dispensed in bottles. The stock culture was maintained throughout the experimental process using a periodic subculture at least fortnightly on nutrient agar (NA) and stored at 4°C in a refrigerator until use [9,10]. To prepare the pre-culture, 1–3 loopful [11] of cells from a 24 h actively growing culture on a NA plate was dispensed in bottles containing sterile nutrient broth (10% w/v) and incubated at 37°C (Mummert-Germany/BE 600) for 24 h. Afterward, the inoculum was transferred into a 500 ml Erlenmeyer flask containing 150 ml of 30% w/v nutrient broth. The inoculation was aseptically performed in a laminar flow cabinet to avoid contamination, and the flask was sterilized by passing it over a Bunsen flame before and after inoculation. To ensure proper bacterial growth, the inoculation was carried out in triplicates.

2.2. Media and Synthetic Wastewater

The media and additional composite exogenous carbon and nutrients sourced formulation were in accordance with the methods described by Fajardo *et al.* [12], with little modifications. Concisely, the synthetic wastewater contained all the essential constituents for bacterial growth was used. The solution containing in DI water is as follows: 7.5 g sucrose, 7.5 g NaS.7H_2O, 3.5 g $NaHCO_3$, 3.6 g KH_2PO_4, 5.5 g $NaNO_3$, 5.46 g KNO_3, and 0.08 g $MgSO_4$. The solutions were thoroughly mixed, and top up with tap water to balance other microelements required, and the pH was adjusted and maintained at 8.5 using standard buffer solution. In addition, the selected pH range was found to be an equidistance point where the most reactive sulfide ions existed, hence ensure high oxygen affinity, and thus facilitate for an effective oxidation. Sodium sulfide at a concentration range of 200, 300, and 500 ppm was added at a specific time interval of 1 h after startup to enable that the culture acclimatized to the new environment.

2.3. Analysis Methods

For sample analysis, 2.5 ml aliquots were withdrawn periodically at fixed intervals throughout the 8 h run. Analysis of the samples for the quantification of sulfide depletion was as described elsewhere [13]. The methyl blue method in Hach (2800DR) spectrophotometer was used to analyze sulfide [14].

2.4. Experimental Setup

The experiment was conducted in a 2 L laboratory-scale BRT BIOTRON (LiFlus GX, Intran, Korea). Before startup, the fermenter was stocked with media (with the exception of Na2S. 9H_2O) and sterilized with the buffer solution and other accessories at 121°C for 15 min. After cooling, the calibrated reactor was inoculated with *P. putida* isolate (150 ml) 10% v/v. The operation was carried out batch-wisely. The temperature of the medium was maintained at required range using a thermostat water jacket from water bath [15]. Complete homogeneity was maintained inside the reactor with double Rushton mechanical turbine with one foam breaker operated at an agitation of 150 rpm. Aeration was achieved using an air compressor (HIBLOW HP-80, Japan) from the reactor base at 50 vvm. Furthermore, dissolved oxygen was maintained within the range of 20–5 mg/l of the least value to the end of the experimental cycle. Thermostat control was used to maintain the temperature of the reactor medium at the optimum of 36°C throughout the experimental processes. Likewise, the BRT was operated at a retention time of 24 h and each experimental cycle with a four periodic sampling for analysis. The oxidation rate of the system was estimated using the Eq. (1).

$$RE(\%) = \frac{\Delta S}{S_0} * 100 \quad (1)$$

Where RE is the removal efficiency, ΔS is the difference in sulfide concentration gradient between the influent and the sulfide concentration at time *t*, and S_o is the initial sulfide concentration.

3. RESULTS AND DISCUSSION

Results from Figures 1-4 and Tables 1-3 indicated the relative impact of different concentrations of hydrogen sulfide on the growth of *P. putida* (ATCC 49128) measured as exponential cell growth and cell biomass increase (cell dry weight [CDW]). This also corresponds to appreciable sulfide reduction rate and utilization over 24 h experimentation. The mechanism of electron transport systems involved in BSO is utilized as a source of energy generation and biosynthesis. It was reported that the rate of substrates conversion directly corresponds to increase in cell density [16] which is affected by the sulfide loading rates, oxygen availability, and product types [17], although the kinetic of BSO rate was reported to be a little more independent to oxygen concentration [18]. Microbial cell growth and biomass synthesis through sulfide utilization were shown to be favored more when the equilibrium shifted toward sulfur production, which usually occurs at low oxygen-deficient medium and higher substrates concentration.

An appreciable sulfide utilization indicated by its oxidation was recorded within the first 6 h of inoculation, with 130 ppm/h (65%), 220 ppm/h (73.33 %), and 390 ppm/h (78%) in 200 ppm, 300 ppm, and 500 ppm, respectively. This was also compensated by an exponential cell biomass growth range of 2.62–3.91 and cell biomass increase measured by CDW of 0.02–0.59 cell g/l [Tables 1-3].The process of biological hydrogen sulfide oxidation to either sulfate or elemental sulfur through dissociation of H_2S to HS^- or S^{2-} has been indicated to cause a rise in pH of the medium, eventually reversing the presumed inhibitory effects and hence resumption of cell growth and sulfide oxidation drastically. Hydrogen sulfide serves as an electron donor during the oxidation process, as well as a substrate to the biodegrading consortium in addition to its role as the primary nutrient source. Depending on the concentration level, sulfide tends to be inhibitory to the isolates growth, especially at high level, although this could be taken care of through oxygen dosing rate.

Furthermore, BSO and cell biomass increase was consistently significant and sustained through the 24 h period in all the three different concentrations. Consequently, sulfide was significantly reduced by 190 ppm/h (95%), 285 ppm/h (95%), and 495 ppm/h (99%),

Table 1: Growth and sulfide oxidation of *P. putida* (ATCC 49128) in 200 ppm

Time	OD (600 nm)	CDW	Exponential cell growth	H_2S red. rate	H_2S removal	ppm H_2S/g cell
0	0.03	0.00	0.00	200	0.00	0.00
1	0.06	0.02	0.69	115	42.5	5,700
6	0.81	0.32	3.3	70	65	220
12	0.8	0.31	3.28	110	45	350
18	1.5	0.59	3.91	50	75	80
24	1.5	0.59	3.91	10	95	17

P. putida: Pseudomonas putida, CDW: Cell dry weight

Table 2: Growth and sulfide oxidation of *P. putida* (ATCC 49128) in 300 ppm

Time	OD (600 nm)	CDW	Exponential cell growth	H_2S red rate	H_2S removal	H_2S/g cell
0	0.03	0.01	0.00	300	0.00	0
1	0.02	0.01	0.01	155	48.33	15,50
6	0.73	0.28	3.19	80	73.33	280
12	0.67	0.26	3.1	140	53.33	531
18	1.02	0.4	3.52	60	80	150
24	1.34	0.5	3.8	15	95	30

P. putida: Pseudomonas putida, CDW: Cell dry weight

Table 3: Growth and sulfide oxidation of *P. putida* (ATCC 49128) in 500 ppm

Time	OD (600 nm)	CDW	Exponential cell growth	H_2S red. rate	H2S removal	H_2S/g cell
0	0.04	0	0.00	500	0	0
1	0.03	0	0.01	325	35	32,500
6	0.75	0.3	2.93	110	78	380
12	0.55	0.2	2.62	130	74	620
18	1.05	0.4	3.27	30	94	73
24	1.48	0.6	3.61	5.0	99	8

P. putida: Pseudomonas putida, CDW: Cell dry weight

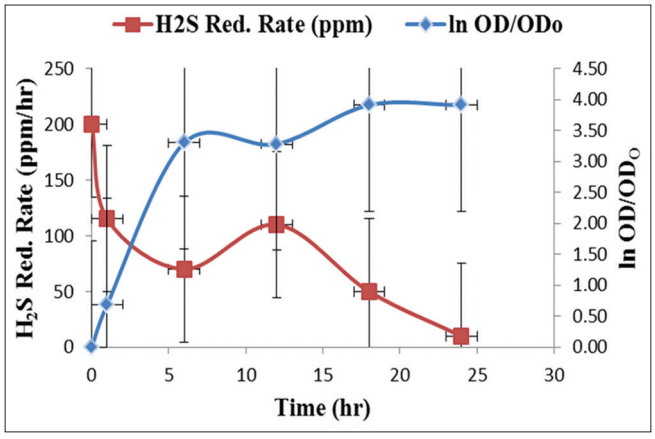

Figure 1: *Pseudomonas putida* (ATCC 49128) growth and removal in 200 ppm sulfide concentration.

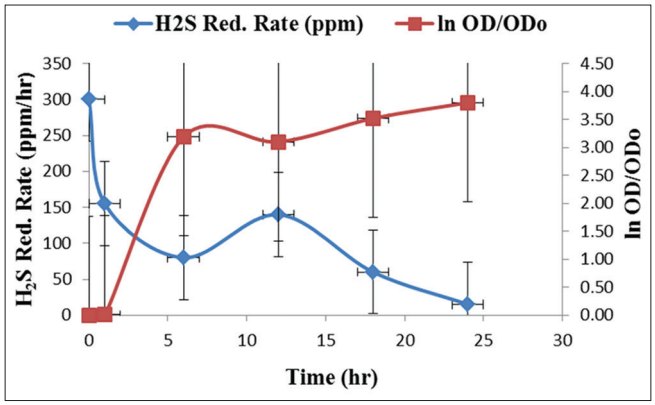

Figure 2: *Pseudomonas putida* (ATCC 49128) growth and removal in 300 ppm sulfide concentration.

in 200 ppm, 300 ppm, and 500 ppm, respectively [Figure 4]. This sulfide oxidation also corresponds to highest rates of growth observed [Figures 1-3 and Tables 1-3]. It was reported that microaerobic nature (low oxygen dosing level) coupled with high sulfide concentration facilitate sulfur formation [19,20], and this was further affirmed in the finding by [21], who suggested that high substrate concentration and low oxygen level favored sulfur selectivity. Overall, BSO was consistent in all the three different concentrations, although with some few variations probably due to the impact of the substance on metabolic activities of the isolate. This assertion could be deduced from the sulfide reduction rate in relation to cell biomass accumulation (CDW), which is given as the ratio of concentration sulfide to cell biomass (ppm H_2S/g cell). This inverse relationship indicated an increase in CDW as sulfide concentration is further depleted, probably signaling sulfide uptake, and assimilation by the growing and dividing

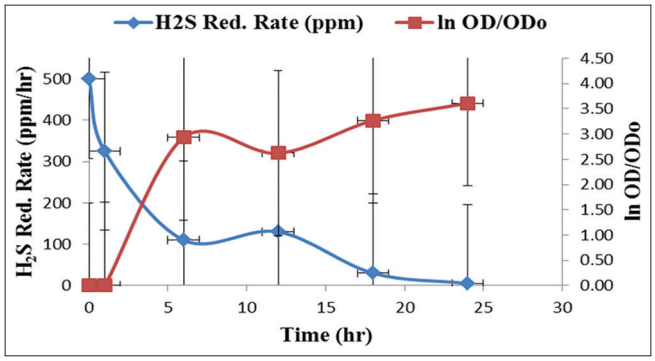

Figure 3: *Pseudomonas putida* (ATCC 49128) growth and removal in 500 ppm sulfide concentration.

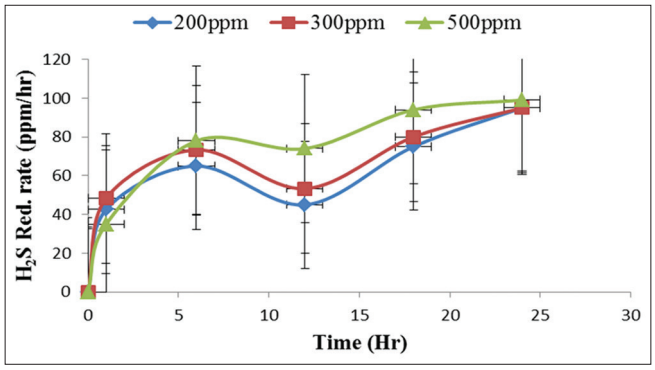

Figure 4: Sulfide removal rate at different concentration over 24 h period.

cells [Tables 1-3]. This finding is supported by the studies of Bin Mohd Azoddein *et al*. and Mosquera *et al*. [22,23].

It was reported that BSO used to be a self-spontaneous process which used to be as fast as other biological reactions. However, the rate of BSO is dependent on pH, the concentration of the reactants (H_2S/SO_4^{2-}), as well as the presence of catalyzing heavy metals [23]. The pH mainly determines the product type, elemental sulfur, thiosulfate, or sulfite as well as sulfide within the range >7 or less. Biological oxidation of sulfide is an energy-yielding mechanism, where more energy is generated in sulfide oxidation to sulfur than oxidation of elemental sulfur to sulfate. The rates of sulfide reduction seem to proceed faster at lower concentration [12] compared to higher concentration. However, higher sulfide concentration can become inhibitory [24], at a certain stage as well as lead to large residual metabolites. However, on exhaustion of sulfide, chemolithotrophic bacteria (BSO) use sulfur as an alternative source of energy with the expression of sulfur-oxidizing ability (sox) gene cluster system [25]. These are a collection of genes that allow the isolate to utilize sulfur *in vitro* in the absence of sulfide. Furthermore, sulfide biological oxidation and cell biomass synthesis increase consistently through the 24 hour period in all the three different concentrations. Furthermore, a synergistic comparative cell growth and sulfide oxidation (i.e., reduction in sulfide concentration) clearly demonstrated utilization of sulfide for biomass synthesis [Figure 1-3]. Therefore the relationship between sulfide utilization and growth agrees with Monod growth model for microbial cell and related literature [26,27].

4. CONCLUSIONS

P. putida (ATCC 49128), has been known for its biodegradability potential of both domestic and refractory industrial wastewater. But its application directly to sulfide oxidation was not so much popular. Despite the hypoxic nature of the experimented medium, the suitability of this strain to sulfide bioremediation process was undisputable, manifested by high rate of sulfide depletion of 99% in 500 ppm sulfide, 95% in 300 ppm and 200 ppm, within 24 hour period respectively. Furthermore, this strain displayed its reliability in reducing sulfide level within the first six hours by 78% in 500 ppm, 73% in 300 ppm and 65% in 200 ppm, respectively. On the other hand, overall cell exponential growth was contrastingly higher in 200 ppm with 3.91, followed by 3.80 300 ppm and 3.61 in 500 ppm. While bacterial cell dry weight followed the same pattern with 0.61 g/L, 0.58 g/L and 0.50 g/L in 200 ppm, 500 ppm, and 300 ppm, respectively. But the reason behind this abnormal trend was not quite understood. Although it was probably due to the limited inhibitory effect of sulfide, which allowed for faster growth in 200 ppm, over the other ranges, since it is the only varying limiting factor

5. ACKNOWLEDGMENTS

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