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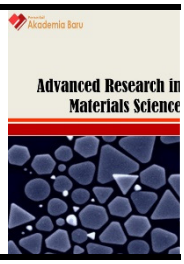

Assessment of sulfide concentration effects in a micro-oxygenated condition on the growth and removal by pseudomonas putida (ATCC 49128)

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Mani Malam Ahmad [1,*], Abd.Aziz Mohd Azoddein [1], Mior Ahmad Khusairi bin Mohd Zahari [1], Mazrul Nizam bin Abu Seman [1], Mohammad Saedi Jami [2], Faten Ahada Binti Mohd Azli [1]

[1] Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang (UMP), Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia
[2] Faculty of Engineering, Department of Biotechnology Engineering, International Islamic University, Malaysia (IIUM), Gombak, 50728, Kuala Lumpur, Malaysia

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ABSTRACT

Micro-oxygenation has recently being gaining popularity as a successful biological sulfide oxidation technique from inflicted wastewater. In this study the application of orbital shaker as an alternative digester for sulfide bioxidation was experimented using Pseudomonas putida (ATCC 49128), under an oxygen tension or micro-oxygenated condition. Growth and sulfide reduction efficiency was measured spectrophotometrically under optimum physical conditions of pH, temperature, acclimatization time and agitation. Sulfide reduction was overwhelmingly recorded at three different sulfide loading rates of 200mM S^{2-} L^{-1} d^{-1}, 300mM S^{2-} L^{-1} d^{-1} and 500mM S^{2-} L^{-1} d^{-1} with corresponding appreciable cell growth measured at OD600nm. The obtained results indicated that it was possible to realized sulfide removal efficiency of 96% to 100% within 24hrs, as well 45% to 70% within the first 6hrs of inoculation, with an overwhelming removal of 100% after 18hrs in 200mM and 300mM. On the other hand, the corresponding growth was 3.00 OD600nm, 2.824 OD600nm and 2.456 OD600nm in 500pmm, 200ppm and 300ppm concentration respectively. Based on this finding, it was clear that this inoculum can be employed to treat sulfide contaminated wastewater even at higher range under micro-oxygenated environmental condition.

1. Introduction

Bioremediation includes stimulating the native microbial populations or introducing microorganisms from external sources that have been known to degrade a particular contaminant, or have been engineered to do so. The environment necessary for the growth of these

* *Corresponding author.*
E-mail address: mmahmadu@gamil.com (Mani Malam Ahmad)

microorganisms must be created [9]. These techniques have more advantages than the chemical and physical methods, including treatment cost. Bioremediation strategies involved the use of microbes to transform the harmful pollutants into harmless products. Bacteria and fungi are often used in the biochemical decomposition of wastewaters to stable end products. More microorganisms, or sludge are formed and the portion of the waste is converted to carbon dioxide, water and other end products. This is achieved by enhancing the conditions (pH, nutrients and aeration) of the indigenous microbes to carry out the bioremediation process [23-10]. Hydrogen sulfide either in liquid or gaseous forms, is an odorous substance with rotten egg smell produce naturally by reduction of either sulfate or sulfur containing inorganic material by a wide range of both aerobic and anaerobic microbes [31].

Hydrogen sulfide generation by anaerobic microorganisms in sewer systems is generally associated with biogenic corrosion of concrete and release of odours to the urban atmosphere [6-39]. Depending on the exposed level, sulfide can cause several negative health effects such as coma, irritated eyes, and respiratory system irritation, impairment of the human physique, neural system and major organs like the liver and the kidney and even death. In addition, high levels of gaseous forms of sulfide are particularly dangerous in the presence of particulate matter, because it slowly adsorbs onto fine atmospheric particles and easily transported very deep into the lungs, where it remains for a long time [24-38]. Wastewater containing sulfur compounds presents a serious discharge problem due to their poor biodegradability, high toxicity and ecological aspects [2]. The impacts caused by these industrial pollutants and growing concern for environmental issues [25] have led to the search for new methods of treatment, and development of new materials that are able to reduce these environmental problems to a permissible level [21].

Biological sulfide oxidation (BSO) has the potential to give a perfect different option for the evacuation of low level hydrogen sulfide (H_2S) from both fluid [16] and gas streams, alongside the recuperation of sulfur [18]. The products of sulfide oxidation, other than sulfur, such as sulfate and thiosulfate are highly water soluble and difficult to separate. Therefore, it was argued that most of the sulfide removal studies focused mainly on the partial oxidation of sulfide to sulfur that could be efficiently separated from the waste stream. The biological removal of hydrogen sulfide (H_2S) is based on the biochemical oxidation of sulfide to elemental sulfur (S^0) or/and sulfate (SO_4^{2-}) [14], as well as thiosulfate ($S_2O_3^{2-}$) [6].

Under oxygen limiting (microaerobic) conditions, at oxygen concentrations below 0.1 mg L^{-1}, where sulfur was reported to be the major realized product of the sulfide oxidation (Equ. 1), with a partial oxidation to thiosulfate [11-33-30], with maximum elemental sulfur producing around 85% at 0.5mol O_2/S^{2-} ratio. While, at this point the rate was expanded by two fold, sulfide was totally oxidized to sulfate (Equ.2). Formation of elemental sulfur, sulfate or thiosulfate, depend mainly on oxygen concentration, sulfide concentration and inoculum size [14-16]. Indeed, from the practical perspective, sulfur formation is favoured, since it can conceivably be recouped. Plus, the lower measure of oxygen required for the oxidation to sulfur contrasted with sulfate infers lower vitality utilization [14]. It was also indicated that, depending on the substrate and operational conditions (mainly oxygen content available), microorganisms responsible for the H_2S oxidation belong to very large and different genera and [4-5-27-38]. Although, research findings showed the importance of utilizing a micro-oxygenation to anaerobic process; however, it was faulted regarding a possible process failure due to the damage oxygen could cause to strict anaerobes, due to some bacterial strains, though with few contrary views [14-21].

$$2H_2S + 2O_2 \longrightarrow 2S^0 + 4OH^- \quad (1)$$

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$$4H_2S + 8O_2 \longrightarrow 4SO_4^{2-} + 8H^+ \quad (2)$$

Microaerobic [14-15] or Micro-oxygenation [27]; are some of the few terms used to describe the dosing or introduction of limited supply of air/oxygen into a fermenter. Hydrogen sulfide was found to be successfully oxidized up to 94% -98% from anaerobic digesters in a biogas form [6-27]. Indeed, this approach saves a cost by providing an alternative to additional units of biodigesters to an earlier BOS at the headspace to further oxidized the sulfate produced by merely introducing not more than 6% of air to both fed-batch and continuous reactor [6]. In addition, this process was reported in a parked-bed reactor, but with potential negative effects of partial oxidation of the soluble substrate as well as clogging of pipes and walls of the reactors with oxidized products such as sulfur [14].

There were so many works done on the effects of dissolved oxygen as well as micro-oxygenation on the bioxidation of sulfide to elemental sulfur or sulfate in different types of reactors. Example of such including, open and closed batch type [26], UASB reactor [15], AFBR [34], Biofilm airlift suspension reactor [19] and Microaerobic desulfurization unit [27] among other ones. But literature related to BOS in an oxygen tension environment of orbital shaker has been very scarce or even not accessible before the present research work. Despite the limited aeration as well as dissolved oxygen tensed medium, a chemolithoheterotrophic mesophilic bacteria *Pseudomonas putida* (ATCC 49128) was able to grow and reduce sulfide level in a different stock of sulfide solution by an overwhelming percentage of between 75% to 100% level. It was also, indicated that these organisms were responsible for reducing oxidized nitrite and nitrate, as well as simultaneously sulfide oxidation under minimum nutrient and aeration rate [23]. As well the efficiency of the BOS more or less depend on the varying concentration of oxygen in the medium and other stress phenomenon, such as nutrient, temperature, pH, contaminant concentration and composition among others [6].

This study is aimed to experiment the influence of limited air supply to BOS in a liquid medium using a limited nutrient concentration under a defined operational parameters of a shake flask. The result of this finding was quite interesting as not many literatures exist related to micro-oxygenation in a liquid medium as well the use of this approach in an orbital shake flask.

2. Materials and methods

2.1. Media and bacterial inoculum

The bacterial isolate used in this study was obtained from stock culture bank at the faculty of chemical engineering, University Malaysia Pahang. Nutrient broth made up of peptone (5%) and meat extract (3%) was the media used for the initial growth and biodegradation experiment. And entire chemical and nutrient used in this research were of analytical grade BD 234000, Merck Malaysia Sdn. Bhd, hence need no further preparation.

2.2. Biodegradation studies

A stock solution of 10000 *m*M to be utilize was prepared be dissolving 7.5g (w/v) of Sodium sulfide (Na2S.9H2O), in 1000ml. From the stock solution, a standard working solution of 200*m*M, 300*m*M and 500*m*M were made through appropriate serial dilution [7]. To each of the three out of four Erlenmeyer flasks containing 150ml NB and 20ml inoculum; a different concentration of sulfide in the range of 200*m*M, 300*m*M and 500*m*M were added, the last flask was left without adding any sulfide, and an additional fifth flask contained only nutrient broth serving as a blank solution or control. The pH of each of the three inoculated flasks was adjusted and maintained to near neutral using 0.5M HCL and 1M NaOH. The entire four flasks with exception of the blank solution were placed in an

orbital shaker and adjusted to 180rpm agitation, temperature 36^0C for one day (24hours) and a small opening was made to each flask, so as to allow for microaeration. While the last flask containing the blank solution was placed in a refrigerator and stored at -4^0C to avoid any contamination. This experiment was repeated twice, to ensure the near accuracy of the observed results. The experimental set up in the shaker was as follow:
SAMPLE A: NB 180 ml (control)
SAMPLE B: NB 180ml + 20ml NB (*Pseudomonas putida*)
SAMPLE C: NB 180ml + 20ml NB (*Pseudomonas putida*) + 200 *m*M
SAMPLE D: NB 180ml + 20ml NB (*Pseudomonas putida*) + 300 *m*M
SAMPLE E: NB 180ml + 20ml NB (*Pseudomonas putida*) + 500 *m*M

2.3. Analytical procedure

For growth and sulfide reduction analysis, Aliquots were withdrawn at 0hr, 1hr, 6hr, 12hr, 18hr and 24hr respectively. 2.5ml of each sample including the blank were drawn and measure growth using U-VIS Spectrophotometer (Hatachi, U-1800), at OD600nm wavelength. While sample drawn for sulfide was analyzed Spectrophotometry using methyl blue method in Hach (2400DR) Spectrophotometer.

3. Results and discussion

Results indicated the relative effects of different concentration of hydrogen sulfide on the growth of *p. putida* (ATCC 49128), over the period of 24 hours experimentation. During the first six hours of inoculation, an overwhelming growth (lag phase and early exponential growth) was recorded. This is attributed response of the isolate to the new environmental conditions which were similar to the recently acclimatized one, as well the present uptake of substrate at an early stage during which sulfide was used as electron donor in biosynthesis for growth and maintenance, after which the growth decreased due to sulfide accumulation. Overall growth within this six-hour period ranges between an averages OD600nm of 0.158 to 1. 789. The decreased in cell growth was due to inhibitory effects of free soluble form and undissociated H2S which permeate cell membranes and form cross-links between polypeptide chains, thus altering cell proteins as well as coenzyme activities [21-35] and sulfide assimilation. In addition, H2S react with metals and precipitated as metal sulfides which inhibit bacteria from the trace metals essential for activation of their enzymes [1-3-28-30-35]. Previous studies indicated growth during the first 4-6 hours of inoculation, which decreases thereby mainly due to depletion of nutrients or accumulation of toxic metabolites in addition the earlier started reasons. The process of biological hydrogen sulfide oxidation to either sulfate or elemental sulfur through dissociation of H2S to HS^- or S^{2-}, S^0 or $SO4^{2-}$ cause a rise in pH of the medium, eventually reversing the inhibitory effects, hence resumption of cell growth drastically. As indicated from the result, that growth inhibition was found to be higher in sample containing 500*m*M sulfide concentration, while the least in 200*m*M. This finding agrees well with other reported results regarding increase in inhibitory effects with increase in sulfide concentration [8-12], (Fig. 1, 2, 3, 4; Table 1, 2, 3). Overall exponential/log phase growth of 2.5OD and above was achieved during 18hrs of inoculation, with 500mM indicated a steady smooth curve. This growth pattern could be due to the effects of sulfide concentration under the low air/oxygen dosing which favoured sulfate formation to elemental sulfur, thus presenting available medium for biosynthesis.

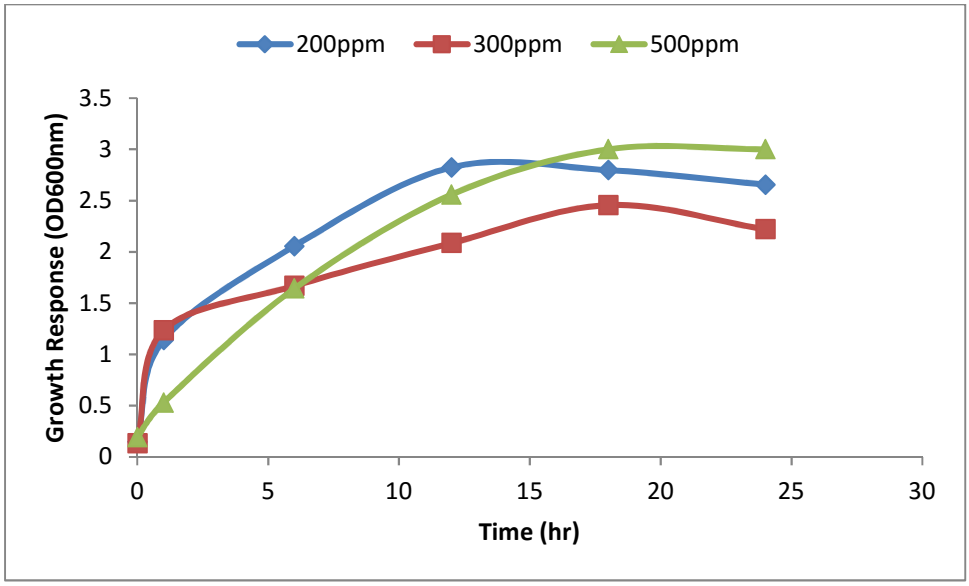

Fig. 1. Comparative Growth Rate (Response) of *B. cereus* ATCC 14579 at Different Sulfide Concentration

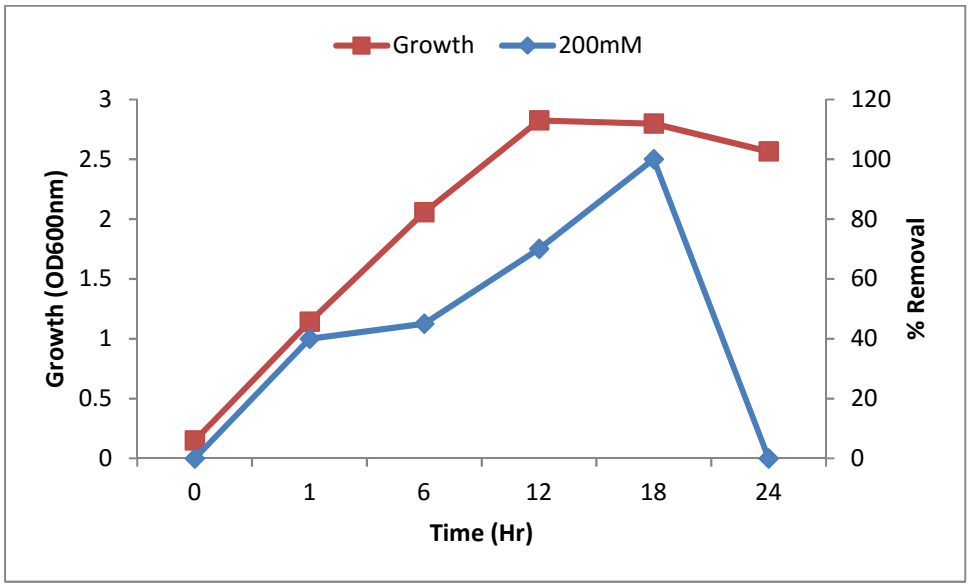

Fig. 2. P. putida (ATCC 49128) Growth & Removal in 200mM Sulfide Concentration

Table 1
Growth & Removal of Pseudomonas putida (ATCC 49128) at 200ppm

S/N	TIME (hr)	INITIAL SULFIDE CONCENTRATION (*m*M)	SULFIDE REMOVAL (%)	GROWTH (OD600nm)
1	0	200	-	0.149
2	1	120	40	1.140
3	6	110	45	2.056
4	12	60	70	2.824
5	18	00	100	2.797
6	24	-	-	2.565

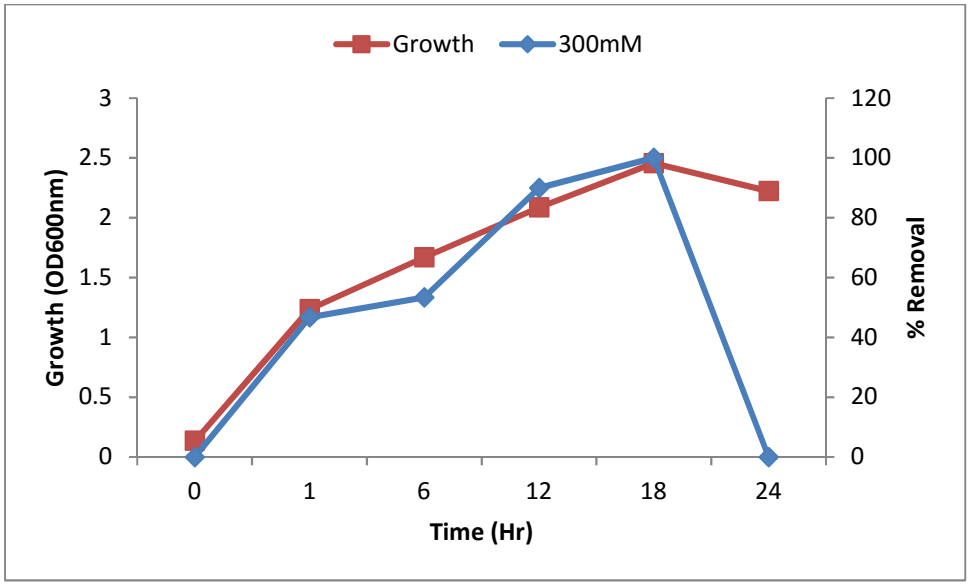

Fig. 3. P. putida (ATCC 49128) Growth & Removal in 300mM Sulfide Concentration

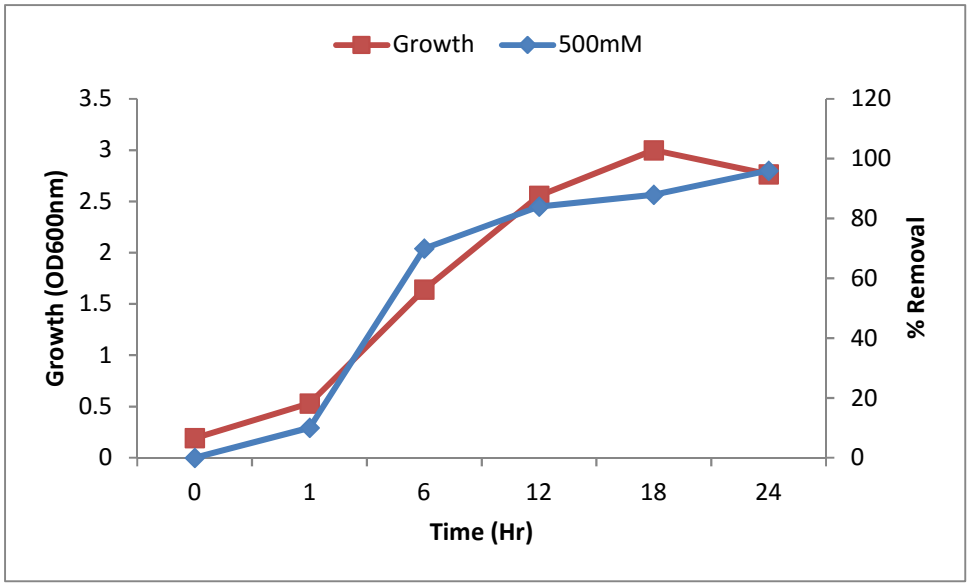

Fig. 4. P. putida (ATCC 49128) Growth & Removal in 500mM Sulfide Concentration

Table 2
Growth of & Removal Pseudomonas putida (ATCC 49128) at 300ppm

S/N	TIME (hr)	INITIAL SULFIDE CONCENTRATION (*m*M)	SULFIDE REMOVAL (%)	GROWTH (OD600nm)
1	0	300	-	0.134
2	1	160	46.67	1.234
3	6	140	53.33	1.668
4	12	30	90	2.086
5	18	00	100	2.456
6	24	-	-	2.223

Table 3
Growth & Removal of *Pseudomonas putida* (ATCC 49128) at 500ppm

S/N	TIME (hr)	INITIAL SULFIDE CONCENTRATION (*m*M)	SULFIDE REMOVAL (%)	GROWTH (OD600nm)
1	0	500	-	0.192
2	1	450	10	0.530
3	6	150	70	1.642
4	12	80	84	2.557
5	18	60	88	3.000
6	24	20	96	2.765

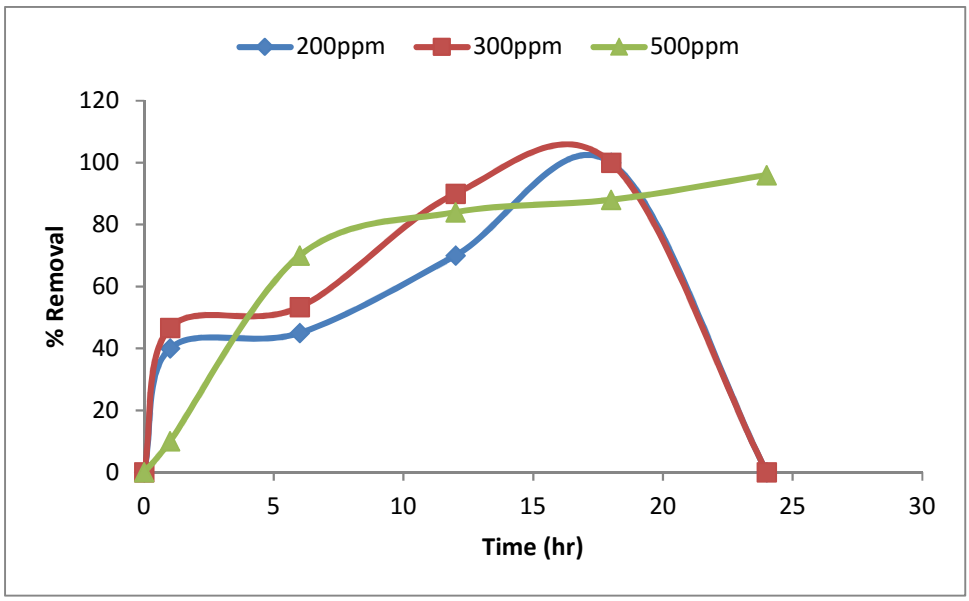

Fig. 5. Sulfide Removal Rate at Different Concentration over 24 hour Period

It was also reported in some text that higher sulfide concentrations led to a larger formation of intermediary sulfur compounds. According to [41], hydrogen sulfide is an acid gas dissolving in alkaline solution much more than in acid medium, making pH in alkaline medium more favourable for sulfide reduction by SOB. While at lower pH range the SOB activity is greatly affected due to the presence of unionized sulfide (Fig. 5). In addition, it can be seen that there was a general removal rate pattern for the three different concentrations at almost the same time period of initial 6hrs, with 200mM and 500mM reflecting a typical Monod growth curve, though it was more pronounced in the curve of 500ppm, from where a paused or steady removal was observed. Such patterns were previously attributed to either exhaustion of some growth limiting factors or accumulation of metabolic residue/ inhibition effect of sulfide. However, it was observed that sulfide conversion resumed afterward, hence the decline could be due to the accumulation of metabolites which subsequently serve as secondary source of nutrient [20-37]. Furthermore, this scenario could be explained by diauxic growth pattern which is the case in this study where the abnormal patterns could be due to the exhaustion of one constituents of growth mixture and the inhibitory effects of the other factor on the enzymatic activities relating to the other substrate [13]. In this regard H_2S, could have an inhibitory effect on the metabolic enzyme acting the media (nutrient broth), which cause its accumulation, thus unable to biosynthesize by the inoculum [17-20]. In addition, the accumulation or reduced removal rate in this case could possibly be associated to the exhaustion of limited oxygen supplied to the medium, which normally affect sulfide oxidation rate [14]. Sulfide oxidation, seem to proceed under micro-oxygenated oxidation, but with formation of elemental

sulfur mainly or sulfate through intermediary thiosulfate which later converted to sulfate. Therefore, removal rate is minimally affected by oxygen dosing, but growth rate used to be impaired apparently due to this change in products formation, that are catabolized differently thus biosynthesis occurs at different rate. Overall, the finding in this work is overwhelmingly similar to some of the previous findings, specifically by [6], who recorded 98.8% to 99.8%, with a longer period than the current study.

4. Conclusion

Based on the reports of numerous literatures, regarding the potential or otherwise wider acceptability of microaerobic approach to sulfide reduction; the present work has further proved the reliability of low oxygen/air dosing in BOS using orbital shaker by *P. putida* (ATCC 49128). Furthermore, orbital shaker has also been identified as an alternative fermenting medium for sulfide oxidation, especially low level contaminated wastewaters. This strain was able to reduce sulfide level in the solution by at least 40% in the first one hour of start up to 0% sulfide level after 18hours in 200mM and 300mM, with almost only 10% sulfide level remained in 500mM after 24hours.

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