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BIOLOGICAL SULFIDE OXIDATION AND ITS IMPACT ON CELL BIOMASS SYNTHESIS BY MESOPHILIC BACTERIUM *BACILLUS CERUES* (ATCC 14579)

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ABSTRACT

Inhibitory effects of hydrogen sulfide could either be due to toxicity to living tissue or precipitation of sulfide salts which inhibit its utilization for cell growth. Growth, is undoubtedly affected by certain conditions, among which are nutrient types and availability, as well as the operational parameters of the fermenting medium. In this study, *Bacillus cereus (*ATCC 14579) was tested for potential sulfide biodegradation in a suspended growth medium of orbital shake flasks using a single milieu composition under defined operational parameters. Growth and sulfide oxidation efficiency were 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 200 ppm S^{2-} L^{-1} d^{-1}, 300 ppm S^{2-} L^{-1} d^{-1} and 500 ppm S^{2-} L^{-1} d^{-1} with corresponding appreciable cell growth measured at $OD_{600\ nm}$. Results indicated that it was possible to realise sulfide removal efficiency of 95% to 99% using this strain in an orbital shake flask within 24 hrs, as well as 65% to 78% within the first 6 hrs of inoculation. Overall, sulfide was reduced by 95% in 200 ppm and 300 ppm, while 99% in 500 ppm, respectively. While, the corresponding exponential cell growth recorded was 3.91, 3.80 and 3.61 in 200 ppm, 300 ppm and 500 ppm, respectively. This also translate to cell biomass synthesis (cell dry weight) of 0.61 g/L, 0.58 g/L and 0.50 g/L in 200 ppm, 500 ppm and 300 ppm. Based on this finding, it was clear that this inoculum can utilize different sulfide concentration for growth and biosynthesis; thus can be employed to treat sulfide contaminated wastewater in a suspended growth form under simple nutrient composition and operational conditions.

Keywords: Inhibition, Sulfide, Oxidation, Growth, Removal, *Bacillus cereus*

1.0 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 any aqueous system which contains both organic matter and sulfate.

Enning & Garrelfs, (2014) reported that sulfide build up in industrial systems may cause several side effects like corrosion of concrete sewer pipes (mainly, due to microbiologically-induced corrosion by sulfate reducing bacteria), releasing unpleasant malodors, toxicity due to sulfide gas, and negative effect to subsequent wastewater. It is argued that wastewater containing sulfur compounds poses a challenging problem due to their recalcitrant nature of poor treatability, high toxicity and ecological aspects. Conventional physicochemical approach to sulfide removal need huge capital investment for handling and maintenance (Chung, *et al.,*, 1996; Kim, *et al.,* 2008; Nur Hafizah *et al.,*, 2016; Tang, *et al.,*, 2009; Zytoon, *et al.*, 2014).

Biological sulfide oxidation (BSO) on the contrary 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 (Liang, et al., 2015; Mathew, 2014). The predominance of either elemental sulfur or sulphate as the final product of the oxidation is mainly oxygen dependent mechanism; thus, in oxygen deficient conditions (micro-oxygenation), elemental sulfur is the main product (Diaz, *et al.,*, 2011; Wang, *et al.,*, 2016). Formation of elemental sulfur, sulfate or thiosulfate, depend mainly on oxygen concentration (Eq. 1,2,3,4), sulfide concentration and inoculum size (Krayzelova *et al.*, 2015; Krayzelova, *et al.,*, 2014; Liang *et al.*, 2015). Suspended form of BSO using chemolithotrophs in an aqueous medium is a popular option over the other BSO techniques which require exogenous carbon source and attachment surfaces (Zytoon, *et al.*, 2014). In the initial phase of BSO, sulfite is produced through electron transport framework exchange from sulfide to the cell and subsequently to the terminal electron acceptor. In the most far reaching mechanism, *sulfite oxidase* exchanges electrons from sulfite specifically to cytochrome c with subsequent generation of an energy molecule, *Adenosine triphosphate* (ATP). Thereafter, sulfite oxidation is facilitated through an inversion action of an enzyme *adenosine phosphosulfate reductase*. This response gives rise to a strong phosphate bond which leads *adenosine monophosphate* (AMP) changed to *adenosine diphosphate* (ADP). At the point when thiosulfate is utilized as electron donor, it dissociated into sulfur and sulfite, both of which are then oxidized to sulfate (Tang, *et al.,*, 2009).

$$2HS^- + O_2 \longrightarrow S^0 + 2OH^- \tag{1}$$

$$2HS^- + 2O_2 \longrightarrow S_2O_3^{2-} + H_2O \tag{2}$$

$$2HS^- + 3O_2 \longrightarrow 2SO_3^{2-} + 2H^+ \tag{3}$$

$$2HS^- + 4O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \tag{4}$$

Sulfide oxidation rate is estimated based on the difference between the initial concentration S_o and a concentration S_t at a given time t divided by S_o, while the percentage removal is determined by multiplying the outcome by 100 and is given as:

$$RE(\%) = \left(\frac{S_o - S_t}{S_o} \right) x100 \tag{5}$$

Bacillus cereus (ATCC 14579) β-hemolytic, spore-forming and facultative anaerobes, naturally found in soil and vegetation, growing under mesophilic temperature range of 20 -45 ^{o}C. Being a heterotroph, it possesses the ability to utilize nitrate as electron acceptor through autotrophic denitrification process (Borah & Yadav, 2014; Bujang *et al.*, 2013; Vinothini, *et al.*, 2015). It was reported that some of these bacterial strains have been identified to anaerobically oxidize inorganic sulfur compounds such as sulfide, sulfur, thiosulfate and sulfite by using nitrate and nitrite as electron acceptor that is finally reduced to nitrogen gas (Fajardo *et al.*, 2014; Li,*et al.*, 2009; Moon, *et al.*, 2004; Zhou *et al.*, 2011). However, its application to BSO is rare or even unavailable. Growth pattern of this isolate is reported to be slowed with prolonged lag phase upon exposure to limiting factors popularly known as oxidative stress or stress phenomenon (Hornstra, *et al.*, 2006; Mols & Abee, 2011; Schagger, *et al.*, 2004). The oxygen deficient nature of shake flasks medium of near anoxic was quite tolerated by this isolate that enable it to oxidize sulfide to appropriate form depending on the prevailing physicochemical conditions.

The present study was set to ascertain the novel experimentation of this new mesophilic bacterium to biological sulfide oxidation, 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 on the use of this mesophilic bacterium in treatment of wastewaters laden with phenol, petrochemical effluent and some heavy metals. Pilot study related to sulfide oxidation under nutrient constrains and other operational physical parameters of orbital shake flasks for *B. cereus* growth and biosynthesis against different simulated sulfide concentrations is not reported elsewhere. However, simple as it may appear, it is believed this could serve as an indicator to achieving 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.

2.0 MATERIALS AND METHOD
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 chemicals and nutrients used in this research were of analytical grade (grade (BD 234000, Merck (Malaysia) Sdn. Bhd)), hence need no further preparation.

Microbiological preparation of the inoculum
Enriched culture media was prepared in accordance with the manufacturer's guidelines. Typically, 8 g of nutrient broth was dissolved in 1000 ml of deionized water in Schott bottles and shaken vigorously until it dissolved. The solution was then heated on a hot plate and sterilized in an autoclave at 121 ^{o}C for 15 minutes; the sterilized media was then placed in a water bath to cool the media to 47 ^{o}C before pouring into various 20 ml sampling bottles. Inoculation of bacterial strain was done by suspending 1-3 loops from the stock culture (Shea *et al.* 2013) into a 20 ml freshly prepared nutrient broth 10% (wv^{1-}). The seeded culture was incubated at 37 ^{o}C for 24

hours at a vigorous shaking of 180 rpm. After 24 hours, the inoculum was transferred into four 500 ml Erlenmeyer flask containing 150 ml nutrient broth 30% (vv^{1-}) of the original volume of the shake flask (Standbury, *et al.* 1984). The samples were then placed inside a shaker and calibrated for agitation and temperature accordingly for each run. The experiments were run under the selected different ranges of nutrient concentration and residence/acclimatization time. All the processes were aseptically done inside a biological close cabinet to avoid contamination.

Biodegradation studies

A stock solution of 10000 ppm to be utilized was prepared by dissolving 7.5 g (wv^{-1}) of Sodium sulfide (Na$_2$S.9H$_2$O), in 1000 ml. From the stock solution, standard working simulated solutions of 200, 300 and 500 ppm were made through appropriate serial dilution (Emami, *et al.* 2004). To each of the three out of four Erlenmeyer flasks containing 150 ml NB and 20 ml inoculum in a different concentration of sulfide in the range of 200, 300 and 500 ppm was added. The last flask was left without adding any sulfide which served a control and an additional fifth flask contained only nutrient broth was serving as a blank solution. A 0.5 M buffer was used to maintain the medium pH at 8.5, which is within the reported tolerable limit of this isolate as well to minimize the risk of H$_2$S gas release to the surrounding using. The entire four flasks with the exception of the blank solution were placed in an orbital shaker and adjusted to 180 rpm agitation, 36 ^{o}C for a day, while the last flask containing the blank solution was placed in a refrigerator and stored at -4 ^{o}C 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 follows:

SAMPLE A: NB 180 ml (**blank**)
SAMPLE B: NB 180 ml + 20 ml NB (*Bacillus cereus*)- **control**
SAMPLE C: NB 180 ml + 20 ml NB (*Bacillus cereus*) + 200 ppm
SAMPLE D: NB 180 ml + 20 ml NB (*Bacillus cereus*) + 300 ppm
SAMPLE E: NB 180 ml + 20 ml NB (*Bacillus cereus*) + 500 ppm

Analytical procedure

For growth and sulfide reduction analysis, 2.5 ml aliquots were withdrawn at 0 hr (initial), 1 hr, 6 hr, 12 hr, 18 hr and 24 hr. Growth was measured using UV-VIS Spectrophotometer (Hatachi, U-1800), at wavelength measurement of 600 nm. This range is usually selected when a huge growth is expected so as to remain within the linear part of the relation between cell number and optical density without any need for a dilution to get a reliable value. Optical density is an indirect method for measuring bacterial growth which is based on the mechanism of light passing through a suspended medium. This concept is based on the fact that, as the cells grow the suspension become more turbid, hence the less percentage of light transmitted. However, UV-VIS spectrophotometer is working based on absorbance of light by the suspended medium, which is directly proportional to the increase in cell number and inversely proportional to percentage light transmission (%T). Bacterial biomass or cell dried weight was determined by withdrawing 2 ml sample from exponentially growing cells simultaneously with sample for OD analysis. Sample was centrifuged at 12,000 rpm for 15-20 minutes (Bratbak & Dundas, 1984). Supernatant was discarded and solid washed in a saline solution and centrifuged again then oven dried at 100 ^{o}C–110 ^{o}C for 1hr. The mass was measured and drying repeated until a stable

weight was obtained, while methyl blue method in Hach (2400DR). Spectrophotometer was used to analyse sulfide (Kleinjan et al., 2005; Wang, *et al.,* 2016).

3.0 RESULTS AND DISCUSSION
**Effect of Sulfide Concentration level on Bacterial Growth and Removal**

Results from Figures 1- 3 and Table 1-3 indicated the relative impact of different concentrations of hydrogen sulfide on the growth of *B. cereus* (ATCC 14579) measured as exponential cell growth and cell biomass increase. This also corresponds to appreciable sulfide reduction rate and utilization over a period of 24 hours 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 correspond to increase in cell density (Mora et al., 2014) which is affected by the sulfide loading rates, oxygen availability and product types (Alcántara *et al*., 2004), although the kinetic of BSO rate was reported to be a little more independent to oxygen concentration (Nielsen, *et al.,* 2006). Microbial cell growth and biomass synthesis via sulfide utilization was shown to be favored more when the equilibrium shifted toward sulfur production, which usually occurs at low oxygen deficient medium and higher substrates concentration. During the first six hours of inoculation, an overwhelming growth (lag phase and early exponential growth) was recorded. This is attributed to response of the isolate to the new environmental conditions which were similar to the recently acclimatized one, as well present uptake of sulfide at an early stage during which it was used as electron donor to kick start exogenous carbon source utilization for biomass synthesis. However a slight decrease in growth was observed which could be attributed to accumulation of toxic waste due to aggressive metabolism at this early growth phase. This phase is characterized by synthesis of carbon source utilization genes(Schultz & Kishony, 2013), but not ribosomal and amino acid biosynthesis genes which are responsible for cellular division and other late physiological mechanisms.

An appreciable sulfide utilization indicated by its oxidation was recorded within the first six hours of inoculation, with 130 ppm/hr (65%), 220 ppm/hr (73.33) and 390 ppm/hr (78%) in 200 ppm, 300 ppm and 500 ppm, respectively. This was also compensated by an exponential cell biomass growth range of 2.62 to 3.91 and cell biomass increase measured by cell dry weight of 0.02 cell g/L to 0.59 cell g/L (Table 1-3).. Bacterial cell growth within the initial 6-8 hours of inoculation was shown to be the fastest phase due to efficient exchange of electrons between donors and acceptors as well as available nutrient source for rapid cell division and absence of accumulated metabolites residue that used to slowed cell growth (Azoddein, et al., 2016; Azoddein, *et al.,* 2015). However, this sudden growth was followed by a decrease in cell growth probably due to inhibitory effects of free soluble form and undissociated H_2S which permeate cell membranes and form cross-links between polypeptide chains, thus altering cell proteins, coenzyme activities (Lorna Guerreroa et al., 2015). This activity is suspected to not only affecting cell growth but sulfide utilization, which may also cause a decreased in reduction rate and assimilation. Previous studies indicated growth during the first 4-6 hours of inoculation, which decreases mainly due to depletion of nutrients or accumulation of toxic metabolites in addition to the earlier stated reasons. 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 inhibitory effects, hence resumption of cell growth and sulfide oxidation drastically. As indicated from the result, that growth inhibition was found to be higher in sample containing 500 ppm sulfide concentration, while the least in 200 ppm (Fig. 4). This finding agrees well with other reported results regarding increase in inhibitory effects with increase in sulfide concentration (Greben, *et al.*, 2005; Kousi, *et al.*, 2015).

Furthermore, sulfide biological oxidation and cell biomass increase was consistently significant and sustained through the 24 hour period in all the three different concentrations. This may probably be due to sulfide being a complimentary nutrient source in addition to poorly rich medium consisting of only nutrient broth. Consequently, sulfide was significantly reduced by 190 ppm/hr (95%), 285 ppm/hr (95%) and 495 ppm/hr (99%), in 200 ppm, 300 ppm and 500 ppm, respectively. This sulfide oxidation also corresponds to highest rates of growth observed (Figures1-3andTable1-3). It has been proven that microaerobic nature (low oxygen dosing level) coupled with high sulfide concentration facilitate sulfur formation (Janssen, *et al.,* 1999; Tang, *et al.,* 2009), thus high oxidation rate as in the case of 500 ppm, although, this was further affirmed in the finding by Van den *et al.,* (1993) where they suggested high substrate concentration and low oxygen level favoring the sulfur formation. Overall, BSO was consistent in all the three different concentrations, although with some few variations which are believed to be due to the impact of the substance to 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 ration of concentration sulfide to cell biomass (ppm H_2S/g cell). This inverse relationship indicated an increase in cell dry weight as sulfide concentration is further depleted, probably signaling sulfide uptake and assimilation by the growing and dividing cells (Table 1-3). This finding is supported by the (Abd.Aziz *et al*, 2016; Mosquera, *et al.,* 2014).
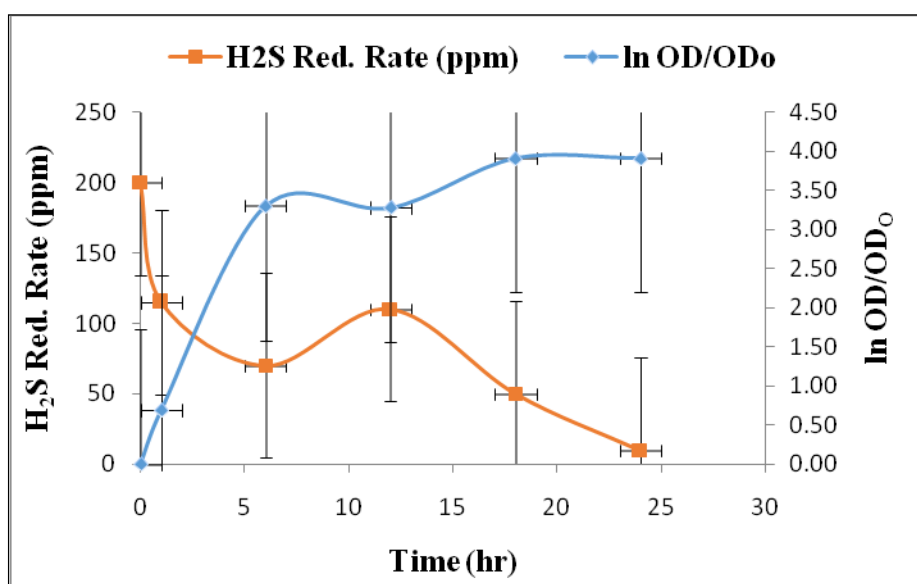
Figure 1: *B. cereus* (ATCC 14579) Growth & Removal in 200 ppm sulfide concentration

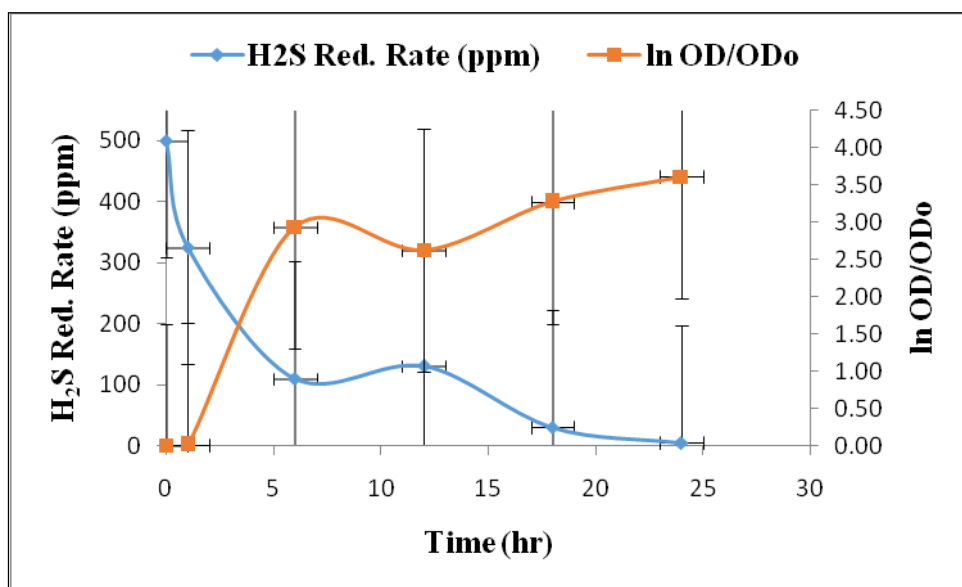**Table 1:** Growth & sulfide oxidation of *Bacillus cereus* (ATCC 14579) at 200 ppm

| Time	OD (600 nm)	Cell dry wt (g/L)	Expon. Cell growth (lnOD/OD$_o$)	H$_2$S Red. Rate (ppm/hr)	H$_2$S Removal (%)	ppm H$_2$S/g cell
0	0.03	0.01	0.00	200	0.00	0.00
1	0.06	0.02	0.69	115	42.5	5,700
6	0.81	0.32	3.30	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	0.61	3.91	10	95	16
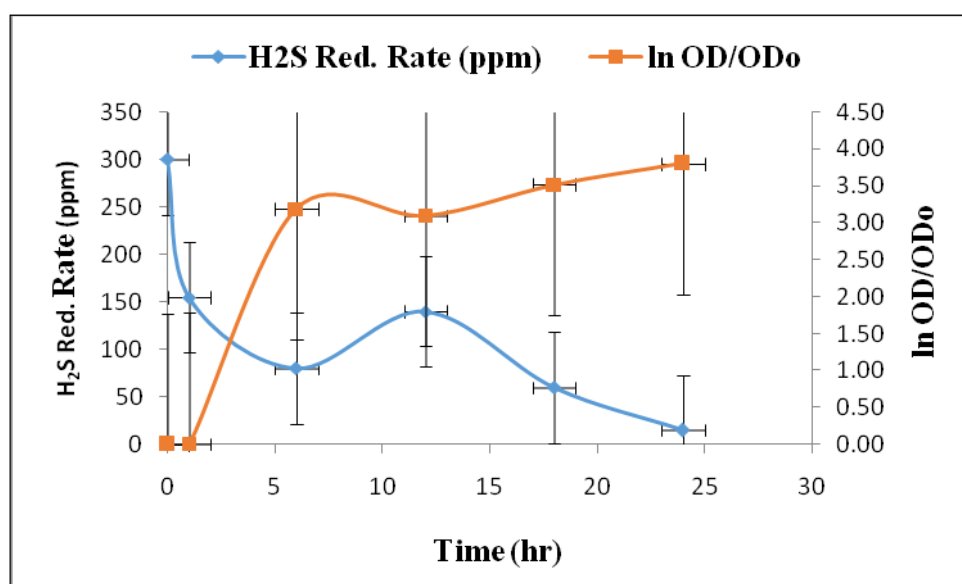
Figure 2: *B. cereus* (ATCC 14579) Growth & Removal in 300 ppm sulfide concentration

Table 2: Growth & ulfide oxidation of *Bacillus cereus* (ATCC 14579) at 300 ppm

Time	OD (600 nm)	Cell dry wt (g/L)	Expon. Cell growth (lnOD/ODo)	H$_2$S Red. Rate (ppm/hr)	H$_2$S removal (%)	ppm H$_2$S/g cell
0	0.03	0.01	0.00	300	0.00	0.00
1	0.02	0.01	0.01	155	48.33	15,500
6	0.73	0.28	3.19	80	73.33	280
12	0.67	0.26	3.10	140	53.33	531
18	1.02	0.40	3.52	60	80	150
24	1.34	0.50	3.80	15	95	30

Figure 3: *B. cereus* (ATCC 14579) Growth & Removal in 500 ppm Sulfide Concentration

Table 3: Growth & Sulfide oxidation of *Bacillus cereus* (ATCC 14579) at 500 ppm

Time	OD (600 nm)	Cell dry wt (g/L)	Expon. Cell growth (lnOD/ODo)	H_2S Red. Rate (ppm/hr)	H_2S removal (%)	ppm H_2S/g cell
0	0.04	0.01	0.00	500	0.00	0.00
1	0.03	0.01	0.01	325	35	32,500
6	0.75	0.29	2.93	110	78	380
12	0.55	0.21	2.62	130	74	620
18	1.05	0.41	3.27	30	94	73
24	1.48	0.58	3.61	5.0	99	8.0
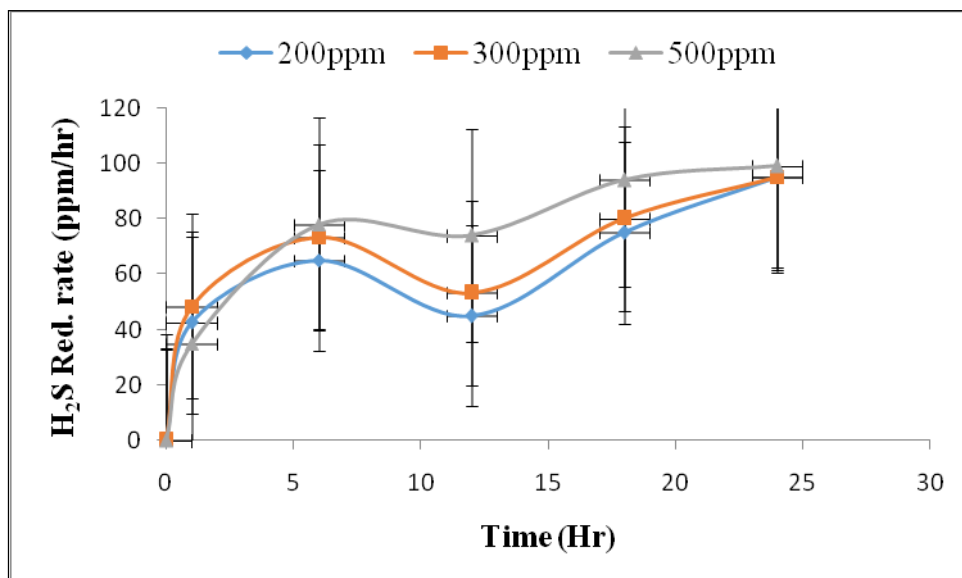
Figure 4: Sulfide Removal Rate at Different Concentration over 24 hour Period

Effect of sulfide bioxidation on bacterial cell biomass synthesis (cell dry weight)
Figures 5-7, showed the finding of *B. cereus* (ATCC 14579) cell dry weight (CDW) in g/L, which is ideally supposed to be 0.39 the value of OD 600 nm. The trend in bacterial cell biomass synthesis is better explain by a calibration curve which is indicated by plotting OD against the CDW. The linear pattern of the plots from this finding fitted well to the supposedly relationship between amounts of light absorbance measured at wavelength (600 nm) of optical density and the increase in cell biomass over time also measured as cell dry weight. Based on the scatter plots; the regression expressed by correlation coefficient (R-squared) value of 0.9998, 0.9987 and 0.9999 were estimated for cell biomass increase at 200 ppm, 300 ppm and 500 ppm, respectively. This result is further strengthened by equation line of best fit, from which the average percentage of OD value that corresponds to maximum CDW (*y*) could be established. In all the results; cell dry weight was observed to be directly proportional to optical density value. This finding was upheld by Azoddein, *et al.* (2015). It can be concluded that, all the model equations fitted well, indicating a significant positive correlation between cell biomass with optical density in an ascending order of 0. 61 g/L, 0.58 g/L and 0.5 g/L in 200 ppm, 500 ppm and 300 ppm, respectively.
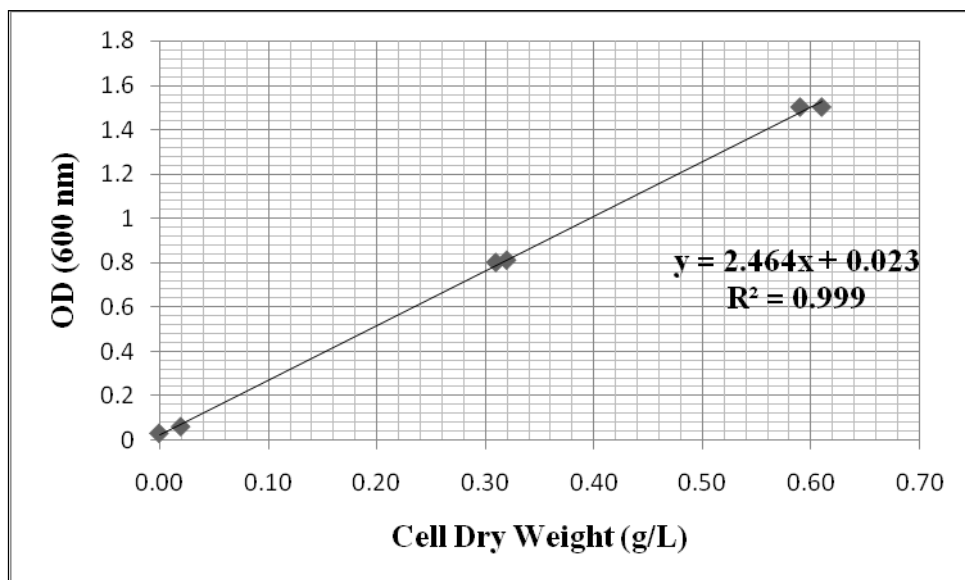
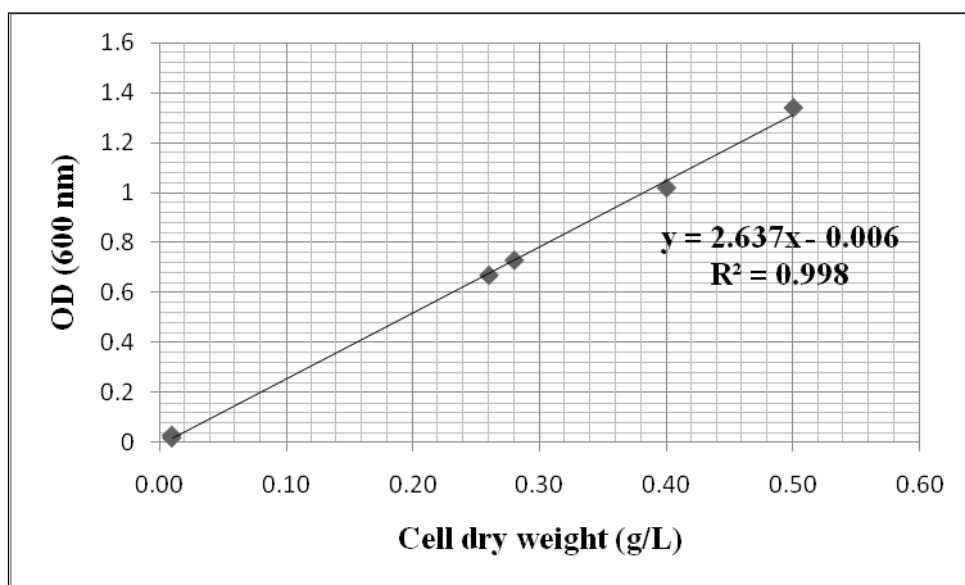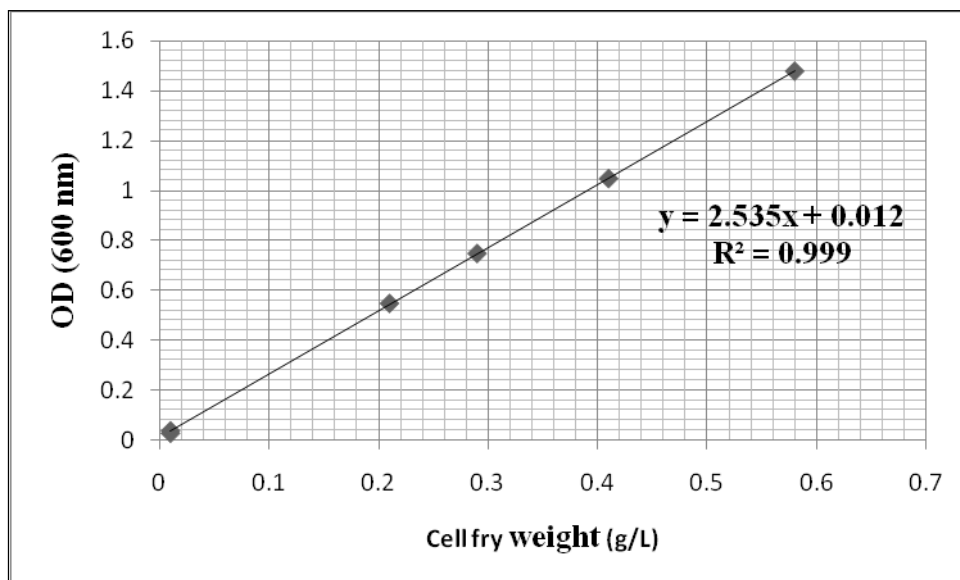**Figure 5:** Bacterial cell dry weight curve in 200 ppm sulfide concentration

Figure 6: Bacterial cell dry weight curve in 300 ppm sulfide concentration

Figure 7: Bacterial cell dry weight curve in 500 ppm sulfide concentration

4.0 CONCLUSIONS

Bacillus cereus (ATCC 14579*)*, 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. The findings from this research have further confirmed the suitability of this strain in bioremediation process. Although, the condition under which this experiment was carried out was with limited oxygen availability, yet this inoculum was able to utilize and reduce sulfide at three different loading rates by almost 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 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.

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