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Microbial interactions in response to sulfide effect on mesophilic bacterial mixed culture (BMC) growth

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ABSTRACT

The inhibitory effect of hydrogen sulfide has been indicated to activate a sequence of response mechanism through microbial mutual interactions. Bacterial mixed-culture (BMC) consortium has been found to reduce the complexity of oxidative stress through mutual interactions of helper bacteria thereby facilitating the growth of the disadvantaged inoculum. The potential sulfide bioxidation of strain one (S1) and strain two (S2) mixed-culture bacterial consortiums were tested at different sulfide level within a 24-hour period. The present study was therefore able to decipher the potential of BMC to oxidation of a fragmented sulfide concentration in an oxygen deficient condition of an incubator shake flasks using a single nutrient composition under defined operational parameters of pH, temperature, acclimatization time and agitation. Results indicated an overwhelming oxidation of varying sulfide concentration which is an energy source for biomass synthesis. This correspond to an average cell biomass increase at optical density greater than one after 6 hrs of seeding, which later almost doubled at 24 hrs period. Furthermore, BMC degraded on average, 97.50%, 96.67% and 93% of the different concentrations of sulfide in 24 hrs of inoculation, respectively. Sulfide oxidation and uptake were in ascending order 200 ppm S^{2-} L^{1-} d^{1-} > 300 ppm S^{2-} L^{1-} d^{1-} > 500, ppm S^{2-} L^{1-} d^{1-}. Overall, the result signifies the synergistic complementary effects of helper bacteria in the consortium which lessen the oxidative stress, thus ensuring the growth and removal in both isolates. The mixed-culture consortium, S1 and S2, showcased an expeditious growth and sulfide degradation in chemically deficient medium and operational parameters of shake flasks, demonstrating that the consortium would be applicable to handle wastewater laden with H_2S..

Keywords:
Microbial interactions, Inhibition, Sulfide, Bacteria Mixed-Culture, Bioxidation

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1. Introduction

Hydrogen sulfide (H_2S), or popularly sulfide, is a notable environmental pollutant considerably produced from numerous domestic and industrial wastewater sources. The maximum human threshold permissible exposure limit (PEL) to H_2S is 10 ppm for 7-8 h periods according to OSHA [5], and its excess must be removed for safety reasons. Depending on the exposed level, sulfide can cause several negative health effects such as coma, irritated eyes, respiratory system irritation, impairment of the human physique, neural system as well as major organs like liver and kidney and at certain level death. Its accumulation in industrial set up may result in several damage and loss of efficiency to the systems such as corrosion of concrete sewer pipes (mainly, due to microbiologically-induced/influenced corrosion by sulfate reducing bacteria), releasing unpleasant malodors, toxicity due to sulfide gas, and negative effect to subsequent wastewater [11, 15]. The wastewater containing sulfur compounds poses a challenging problem due to their recalcitrant nature of poor treatability, high toxicity and ecological aspects. 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 can reduce sulfide to a permissible discharge level. Physical and chemical approaches to sulfide treatment in both domestic and industrial sources of biogas and aqueous forms have been in use, such as absorption, ozone/chemical oxidation, and incineration, precipitation and electrochemical [2, 9]. Although these processes recorded some tremendous successes, these conventional techniques have some drawbacks, such as high energy requirements, treatment and high disposal costs and production of secondary pollutions [26, 42, 19, 6, 33]. The shortcoming is mainly due to irreversibility of the process mechanisms which necessitated the use of large catalyzing agents and energy sources to facilitate the reactions. However, this is quite contrary to biological approach (bioremediation), which employs the use of reversible enzymatic catalyzing processes.

Biological sulfide oxidation (BSO) using sulfide oxidizing bacteria, has the potential to give a perfect different option for the removal of both low and high level hydrogen sulfide from both fluid and gas streams, with greater potential alongside the recovery of sulfur as an economical approach [24, 29]. The initial phase in 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). Thereafter thiosulfate served as electron donor, which lead to its dissociation into sulfur and sulfite, both of which are then oxidized to sulfate [38, 43]. In addition, one of the pre-requisite for successful conversion of sulfide to elemental sulfur over sulfate in particular; is the potential of BMC to survive limited nutrient and oxygen concentration as well as ease the ease of sulfur recovery from the microcosm [36]. Due to difficulty in recuperation, sulfide removal studies focus mainly on the partial oxidation of sulfide to sulfur that could be efficiently separated from the waste stream depending on the aeration rate and sulfide concentration level [20, 8]. Sulfide oxidation from biogas is being identified with issues such as oversaturation of waste stream with sulfide [21], as well as low form of elemental sulfur production. Contrastingly, BSO from aqueous medium is a consistent approach with gradual removal rate that lessen toxicity effects of oversaturation coupled with high recuperation to elemental sulfur. Furthermore, most of the recent BOS involve an integrated simultaneous process

of denitrifying sulfide removal (DSR) using Autotrophic-Heterotrophic denitrification-nitrification [4, 12, 23].

Application of bacterial mixed-culture (BMC) was reported to be more effective than a single pure culture, especially in biodegradation of highly recalcitrant wastewaters, despite being influenced as well by certain growth limiting factors [32, 45, 49], biotic factors [3] and aggression of indigenous microbes [16, 32, 35]. Sulfide bioxidation using BMC either to elemental sulfur or sulfate was indicated to rely heavily on the initial sulfide loading rate and oxygen dosing rate as well [25]. It was argued that, the efficacy of BMC in terms of growth and removal is mainly due to the complementary impact of helper bacteria. Under this condition, the supporting isolate utilizes a substance believed to be inhibitory to the other partner (A), thus lowering the oxidative stress, allowing the disadvantaged isolate (B) to grow, on its own part, (B) now produce essential amino acid which is required by an auxotrophic isolate (C) which in return could synthesis growth factors needed by (A) [14]. Indeed, the co-facilitate uptake of essential growth substrates and other factors enhance the tolerance of the isolates to stress phenomenon for better growth of all the isolates (Fig. 1). Mixed-culture microbial consortium has successfully been utilized in a simple and integrated approach to various types of wastewaters treatment. The use of *Pseudomonas sp.* and *Bacillus strains* were reported in a handful of literatures [7, 45]. However, the use of specifically *P. putida* (ATCC 49128)/S1 and *B. cereus* (ATCC 14579)/S2 as mixed-culture in sulfide oxidation are not reported elsewhere. In addition, not many citations were at sight for the selected BMC utilization in sulfide bioxidation especially in aqueous medium of orbital shake flasks.

The present study was set to ascertain the novel experimentation of this new consortium mesophilic bacterial mixed culture to biological sulfide oxidation, which was rarely documented, although some literatures were available on their application to some bioremediation aspect [45]. Pilot study related to sulfide oxidation under nutrient constrains and other operational physical parameters of orbital shake flasks for S1 and S2 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 mixed culture consortium.

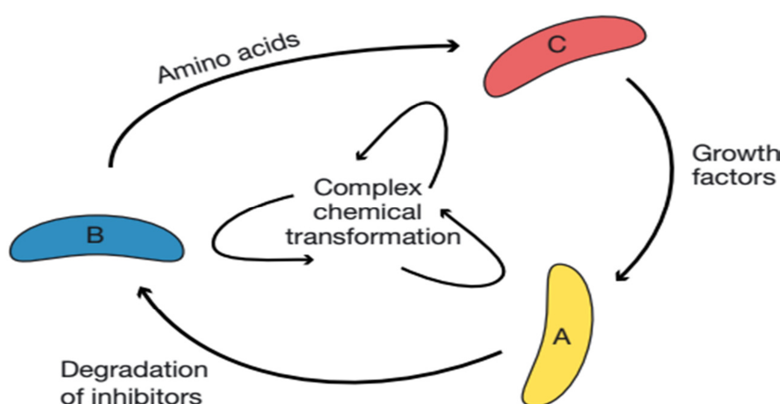

Fig. 1. Microbial interactions between different microorganisms (A, B, and C) in a mixed culture [14]

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2. Materials and methods

2.1. Inoculum and growth media

A BMC; S1 and S2 and the ingredients growth/nutrient broth (5% of peptone meat and 3 % of extract meat) used in this study were obtained from Merck (Malaysia) Sdn. Bhd as local agent dealing with the bacteria, sourced from Microbiologic, 217 Osseo Ave. North, St. Cloud, USA. Enriched culture media was prepared in accordance to the manufacturer's guidelines. Typically, 8 g of nutrient broth was dissolved in 1000 ml of deionized water (DI) in Schott bottles and shaken vigorously until it dissolved. The solution was heated on a hot plate and sterilized in an autoclave at 121 °C for 15 minutes; the sterilized media was then placed in a water bath to cool the media to 47 °C before pouring into various 20 ml sampling bottles.

2.2. Equipment

Equipment used for this research studies were auto-clave H+P Varioklav Steam Sterilizer ESCO, Shaker (B. Braun, German model), microbiological incubator (Mermmert-Germany/BE 600), UV-Visible Spectrophotometer (U-1800, Hitachi), pH Meter (Mettler Toledo) and analytical Balance (Mettler Toledo).

2.3. Seeding of the inoculums into prepared media

Inoculation of bacterial strain was done by suspending 1-3 loops [38] from the stock culture into a 20 ml freshly prepared nutrient broth (NB)10% ($w\ v^{-1}$). The seeded culture was incubated at 37 °C for 24 hours at a vigorous shaking of 180 rpm. After 24 hours, the inoculum was transferred into a 500 ml Erlenmeyer flask containing 150 ml of nutrient broth with 30% (vv^{-1}) of the original volume of the shake flask [39]. The inoculation process was aseptically performed inside a laminar flow to avoid any contamination; as well the flask was passed over a Bunsen burner flame before seeding and after. This inoculation was done three times each to ensure proper bacterial growth [1].

2.4. Biodegradation studies

A stock solution of 10000 ppm hydrogen sulfide to be utilized was prepared by dissolving 7.5 g (w/v) of sodium sulfide ($Na_2S.9H_2O$), in 1000 ml. From the stock solution, standard simulated working solutions of 200 ppm, 300 ppm and 500 ppm were made through appropriate serial dilution [10]. To each of the three out of four 500 ml 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 NB served 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_2S gas release to the surrounding using. The entire four flasks except for 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 followed:
SAMPLE A: NB 180 ml (blank)
SAMPLE B: NB 180 ml + 20 ml NB (S1 + S2)- Control

SAMPLE C: NB 180 ml + 20 ml NB (S1 + S2) + 200 ppm
SAMPLE D: NB 180 ml + 20 ml NB (S1 + S2) + 300 ppm
SAMPLE E: NB 180 ml + 20 ml NB (S1 + S2) + 500 ppm

2.5. Analytical procedure

Analysis for the samples in this experiment were as described elsewhere according to standard [46]. 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 and slit distance of 2 cm. 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 (Galushko, 2015). 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). Sulfide component on the other hand was analysed spectrophotometrically using methyl blue method in Hach (2400DR).

3. Results and discussion

Hydrogen sulfide serves as an electron donor during bioxidation 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. The experiments were started up without adding the sulfide solution until after attaining optical density level of 0.2 which correspond to one hour, the inoculum acclimatization to its new environment. Results from Fig. 2, Table 1, indicated the relative effects of different concentration of hydrogen sulfide on the growth of S1 and S2 mixed-culture consortium, over the period of 24 hours. During the first six hours of inoculation, an overwhelming growth (early exponential growth) was recorded, more vividly in 200 ppm and the control sample. 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 [37], but not ribosomal and amino acid biosynthesis genes which are responsible for cellular division and other late physiological mechanisms. Overall growth within this six hour period ranges between an averages OD of 0.195 to 0.997 (Fig. 2, Table 1). An appreciable sulfide utilization indicated by its oxidation was recorded within the first six hours of inoculation, with 40%, 60% and 68% in 200 ppm, 300 ppm and 500 ppm, respectively (Fig. 3). This was also compensated by an exponential cell biomass growth range of 0.82 to 1.33 (Table 1, Fig. 2). The decline in cell growth was probably due to many factors which include exhaustion of substrates, accumulation of toxic metabolites. In addition to inhibitory effects of free soluble form and undissociated H_2S which permeates cell membranes and form cross-links between polypeptide chains, which altered cell proteins as well as coenzyme activities, [26, 48] and sulfide assimilation. Previous studies indicated that the growth during the first 4-6 hours of inoculation was mainly due to depletion of nutrients or

accumulation of toxic metabolites. An OD value >1 was observed for all the set up at around 12 hrs after start up, thereby maintaining a steady growth of 1.680, 1.699, 1.726 and 1.706 in 200 ppm, 300 ppm, 500 ppm and the control experiment, respectively (Table 1). This steady growth was mainly due to the fact that the experiment was conducted under low aeration rate which maintained the reactions at a slow phase with partial oxidation to elemental sulfur and thiosulfate as the main products, with rare sulfate production. It can be seen that the inhibitory effect in this study was minimal; due to the complementary co-metabolic impact of mixed-culture consortium which exerts different rate of biochemical activities to the contaminant, as well minimizes production of toxic wastes compared to single culture [30]. A similar biodegradation potential of mixed-culture over a single culture was reported in many literatures [32, 17, 22, 41]. Sulfide Removal rate of up to 99% and above was also reported in some texts [36].

Table 1
Growth rate under three different sulfide concentrations

TIME (hr)	**GROWTH AT DIFFERENT SULFIDE CONCENTRATION (OD600 nm)**			
	200 ppm	300 ppm	500 ppm	Control
0	0.133	0.207	0.246	0.192
1	0.178	0.315	0.419	0.420
6	1.335	0.824	0.831	1.559
12	1.134	1.140	1.122	1.156
18	1.414	1.368	1.358	1.428
24	1.680	1.699	1.726	1.706

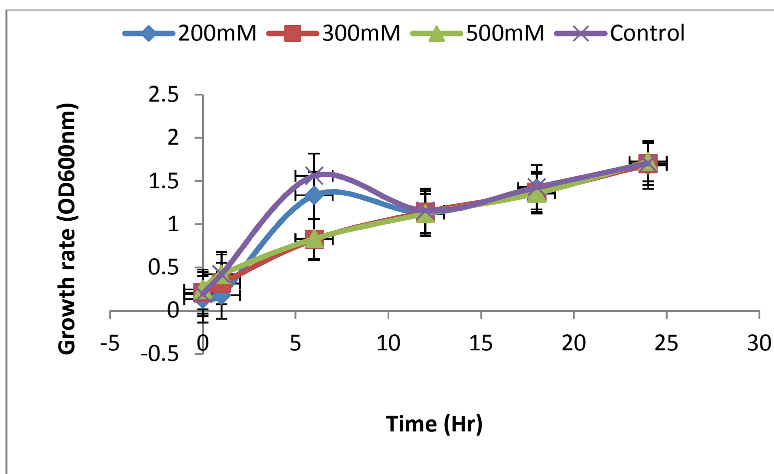

Fig. 2. Growth rate under three different sulfide concentrations

Table 2
Percentage Sulfide removal in three different concentrations

TIME (hr)	**% SULFIDE REMOVAL RATE UNDER DIFFERENT CONCENTRATION**		
	200 ppm	300 ppm	500 ppm
0	100	100	100
1	35.00	36.67	56.00
6	40.00	60.00	68.00
12	65.00	66.67	76.00
18	93.00	91.67	84.00
24	97.50	96.67	93.00

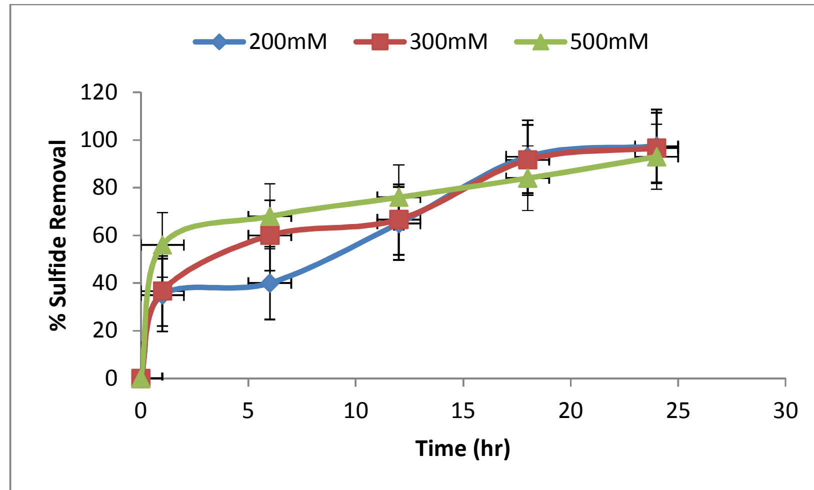

Fig. 3. Percentage Sulfide removal from three different concentrations

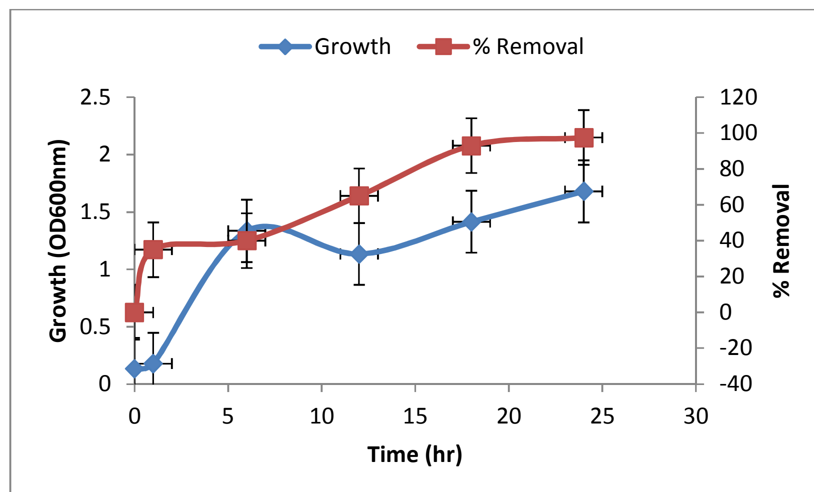

Fig. 4. Mixed-culture Growth & Removal in 200 ppm Sulfide Concentration

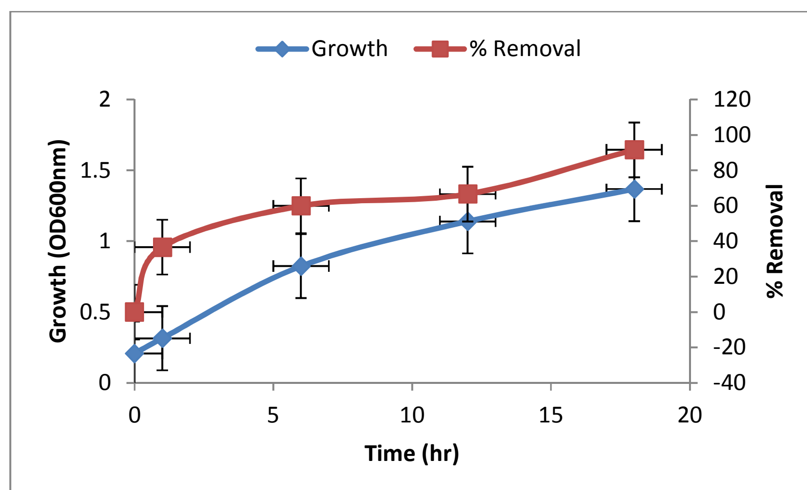

Fig. 5. Mixed-culture Growth & Removal in 300 ppm Sulfide Concentration

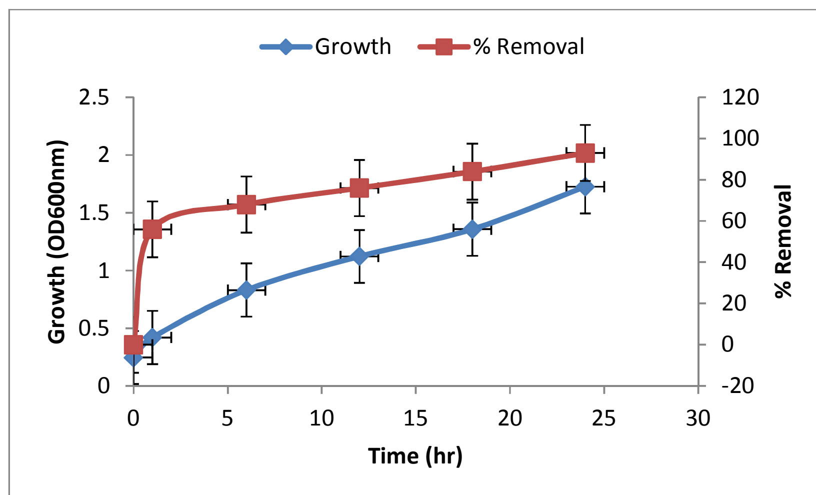

Fig. 6. Mixed- culture Growth & Removal in 500 ppm Sulfide Concentration

Biological sulfide oxidation is a self-spontaneous process which used to be as fast as other biological reactions. However, the rate of biological sulfide oxidation is dependent upon pH, concentration of the reactants (H_2S/SO_4^{2-}), as well as presence of catalysing heavy metals [27]. The pH mainly determines the product type, elemental sulfur, thiosulfate or sulfide as well as bisulfide or sulfide within the range >7 or less. Biological oxidation of sulfide is 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 in lower concentration [12] compared to higher concentration. Higher concentration of sulfide can become inhibitory [43] at certain stage as well as lead to large residual metabolites. However, upon 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* [13]. These are collection of genes that allow these isolates to utilize sulfur in-vitro in the absence of sulfide. Furthermore, sulfide biological oxidation and cell biomass was increase consistently 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 continuously utilized as indicated with an average oxidation level of 97.50%, 96.67% and 93% in 200 ppm, 300 ppm and 500 ppm, respectively (Table 2, Fig. 3). This sulfide oxidation also corresponds to highest rates of growth observed. However, these figures were more consistent than findings from similar work with a single pure culture of *Pseudomonas putida* (ATCC 49128) [28]. This consistency has affirmed the supposed impact of microbial interactions effects of helper bacteria to sulfide oxidation and growth. It has been proven that microaerobic nature (low oxygen dosing level) coupled with high sulfide concentration facilitate sulfur formation [18, 42], thus high oxidation rate as in the case of 500 ppm, although [44], reported contrary opinion where they suggested high substrate concentration and low oxygen level favouring the sulfur formation. Furthermore, a synergistic comparative cell growth and sulfide oxidation (i.e. reduction in sulfide concentration) clearly demonstrated utilization of sulfide for biomass synthesis (Fig. 3-6). Therefore, the inverse relationship between sulfide utilization and growth agrees with Monod growth model for microbial cell and related literatures [31, 34].

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4. Conclusion

Based on the findings from this study, it was obvious that BMC has proven to be a good candidate for bioxidation of sulfide and other bioremediation application. Growth and sulfide oxidation was consistently achieved without a noticeable pause in growth and reduction, which may probably cause by toxicity of metabolites and other oxidative stresses. Therefore, BMC could be recommended when a consistent sulfide treatment is required in place of drastic high level reduction that may in the long term be halt by stress phenomenon such as inhibition effects, toxic metabolic residues among others.

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