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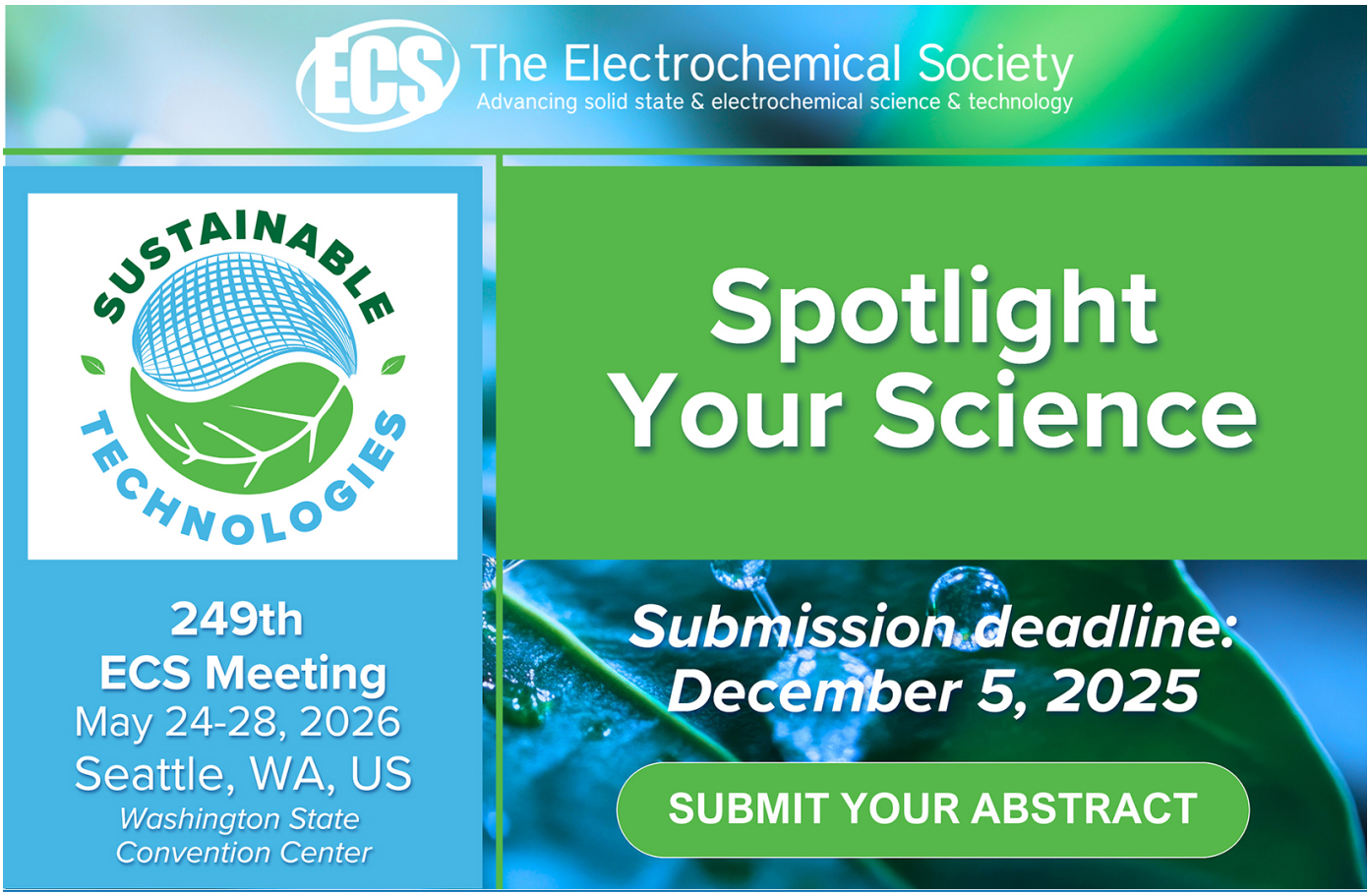

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Microbiological Removal of Hydrogen Sulphide from Natural Rubber Latex Processing Wastewater by *Acidithiobacillus thiooxidans* strain UniMAP-AIN01

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Abstract. *Acidithiobacillus thiooxidans* is an acidophilic chemoautotrophic bacterium which capable to convert the toxic hydrogen sulphide in wastewater into non-toxic compounds. The *Acidithiobacillus thiooxidans* strain UniMAP-AIN01 was previously isolated from a local natural rubber latex processing mill. In this study, the *A. thiooxidans* strain UniMAP-AIN01 was subjected to its growth performance evaluation in the wastewater of natural rubber latex. The sulphur content in the wastewater was utilized as energy source and support their growth. It was discovered that the *A. thiooxidans* strain UniMAP-AIN01 has long doubling time of 2 days in thiosulphate medium, while log phase lasted until 6th day of incubation. Regression analysis of the growth kinetic was established with the aid of POLYMATH software. The precision value obtained as follows; linear regression, R^2 of 0.9811, adjusted linear regression, Adj R^2 of 0.9764, root mean square deviation, RMSD of 0.0015, and variance of 1.992×10^{-5} indicate the data is highly correlated and error is insignificant. Assessment on hydrogen sulphide removal efficiency using locally isolated *A. thiooxidans* strain UniMAP-AIN01 revealed 90% of hydrogen sulphide removal was achieved within a week.

1. Introduction

High and continuous demand for natural rubber is the key reason why rubber latex is acknowledged as one of the worlds' vital commodities. Malaysia was once the top rubber producer and exporter in the world which led to the establishment of many rubber processing facilities. Typically, natural rubber latex is collected from the *Hevea brasiliensis* tree and transported to these processing facilities before turning it into usable products. In the processing facility, the raw rubber latex undergoes a series of refining steps that use a voluminous amount of water and chemicals while it parallelly generates an enormous amount of wastewater. Particularly, the use of sulphuric acid for coagulating the rubber latex results in the generation of a high level of sulphate in the wastewater of rubber processing facilities. High level of sulphate containing effluent could pose threats to natural sulphur cycle if it is released to

nearby stream without appropriate treatment. Currently, the most common wastewater treatment method in rubber processing facilities is anaerobic treatment as it is cheap and highly efficient. However, this process results in hydrogen sulphide formation as the naturally occurring sulphur-reducing bacteria reduces sulphate into sulphides and elemental sulphur. This gas could be removed by a process called chlorination by adding chlorines to remove sulphide. Nevertheless, the use of a large amount of chlorine for H_2S removal adversely affects the environment. Besides that, another issue to be noted in the chlorination method is some of the hydrogen sulphides will only be partially oxidized. This comes with the risk of reverse conversion back into hydrogen sulphide by the sulphur-reducing bacteria.

In order to deal with these pollutants, many alternative strategies have been studied. One of the potential methods is to use the biological approach for hydrogen sulphide removal. This method has become more favourable and easier to accept by the industries because any issues due to toxicity of by-products from chemical treatment method can be ignored. For biological treatment in industry, it could be done in the application of biofilter. This system requires the use of optimized microorganisms capable of efficiently degrading malodorous gases for a long time while effectively tolerating by-products such as sulphate. Thus, it is important to identify microbial strains that are capable of tolerating high sulphate concentrations and low pH while maintaining the hydrogen sulphide removal efficiency. *Thiobacillus spp*. is a sulphur-oxidizing bacterium that utilizes hydrogen sulphide as a primary energy source without storing it in its cells. A few researches have spent their tremendous efforts to elucidate *Thiobacillus spp.* properties and abilities to reduce and use the inorganic sulphur compounds as a survival element. Current interest in *Thiobacillus spp.* particularly *Acidithiobacillus thiooxidans* (or *A. thiooxidans*) to be employed in wastewater treatment for conversion of hydrogen sulphide into sulphur has been elevated among the researchers. This is due to the major properties of *A. thiooxidans* such as capability of utilizing hydrogen sulphide as an energy source and growing at acidic pH while tolerating high sulphate conditions. The mode of action of *A. thiooxidans* on hydrogen sulphide assimilation is similar with bioleaching techniques as in the extraction of metals from their ores through the metabolism of microbes. In addition, *A. thiooxidans* possess the ability of oxidizing high levels of elemental sulphur under extremely acidic conditions. Therefore, in the present study, a locally isolated *A. thiooxidans* strain UniMAP-AIN01 from rubber latex processing mill will be assessed for its hydrogen sulphide removal efficiency from the hydrogen sulphide contaminated wastewater. The strain also was subjected to growth kinetic evaluation to understand the its growth performance.

2. Experimental

2.1 Media and Inoculum Preparation

The thiobacillus broth M789 (HiMedia, India) which comprised of 5 g/l of sodium thiosulphate ($Na_2S_2O_3$), 4 g/l monopotassium phosphate (KH_2PO_4), 0.5 g/l magnesium sulphate, 0.4 g/l ammonium sulphate ($(NH_4)_2SO_4$), 0.25 g/l calcium chloride ($CaCal_2$), and 0.01 g/l ferrous sulphate ($FeSO_4$) was used as a culture medium for the isolated *A. thiooxidans* strain UniMAP-AIN01 from latex wastewater treatment pond in Perak, Malaysia. The pH of the medium was adjusted to 4.2 using sulphuric acid (H_2SO_4) prior sterilization using an autoclave (Hirayama, Japan) at 121 °C for 15 minutes. The inoculum was prepared by culturing a loopful of *A. thiooxidans* strain UniMAP-AIN01 colonies into the 50 ml thiobacillus broth medium. The inoculated flask was incubated at 30°C with an agitation speed of 150 rpm in an incubator shaker (Fisher Scientific, USA) for 3 days [1].

2.2 Growth Profile and Kinetics of Acidithiobacillus thiooxidans strain UniMAP-AIN01

Growth profile of *A. thiooxidans* strain UniMAP-AIN01 was established by inoculating 1% (v/v) of *A. thiooxidans* strain UniMAP-AIN01 inoculum into 50 ml thiobacillus broth medium. A flask without bacterial inoculum was set as a control. the cultures were incubated for 7 days incubation at 30°C and 150 rpm. Growth samplings were done for 7 days with 24 hours interval. A 1 ml of culture was centrifuged (Eppendorf, Germany) at 10,000 rpm for 10 minutes. The resulted supernatant was discarded. The pellets were washed with sterile distilled water twice and resuspended in 1 ml of sterile

distilled water prior measuring the optical density of the sample at 600 nm against distilled water as blank using UV-Visible Spectrophotometer (Shimadzu, Japan). The growth profile of the *A. thiooxidans* strain UniMAP-AIN01 was established by plotting cell concentration based on optical density against incubation time. Based on the growth curve established, the maximum net specific growth rate, μ_{max}, and doubling time, t_d were calculated using the following equation (1) and equation (2), respectively [2]. A non-linear growth kinetic plot was then generated using POLYMATH software.

$$\mu_{max} = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1} \quad (1)$$

$$t_d = \frac{\ln 2}{\mu_{max}} \quad (2)$$

2.3 Hydrogen Sulphide Removal Efficiency Study

Wastewater was collected from rubber processing facility in Tapah (Perak) where the *A. thiooxidans* strain UniMAP-AIN01 was isolated. The wastewater was collected at a fixed depth of 30 cm from an anaerobic pond and stored at 4°C until further use. A 99 ml of wastewater sample was filled up in each seven units of 250 ml Erlenmeyer flasks. Each flask was inoculated with 1 ml of *A. thiooxidans* strain UniMAP-AIN01 which previously cultured in the thiobacillus broth medium. Meanwhile, a flask was filled with 100 ml wastewater sample which serves as a control. Initial hydrogen sulphide concentration in the wastewater was determined prior to incubation. The flasks were then incubated in an incubator shaker at 30°C by continuous shaking with an agitation speed of 150 rpm for 7 days. One flask is harvested each day where hydrogen sulphide concentration, pH and sulphate concentration were measured. The pH values were recorded using a pH meter (Mettler Toledo, USA) while Hydrogen Sulphide Test Kit (Hach, USA) was used to qualitatively determine the concentration of hydrogen sulphide in the wastewater samples.

2.4 Sulphate Formation Quantification

2.4.1 Preparation of Conditioning Reagent

A 250 ml of conditioning reagent was prepared by dissolving sodium chloride (NaCl) in 100 ml of distilled water prior adding 25 ml of glycerol, 15 ml of 37 % (v/v) hydrochloric acid (HCl), 50 ml of 95 % (v/v) isopropyl alcohol ($CH_3CHOHCH_3$) and topped up with distilled water.

2.4.2 Preparation of Sulphate Standard Solution

A 250 ml of sulphate standard stock solution were prepared by dissolving 0.3697 g of anhydrous sodium sulphate (Na_2SO_4) in 250 ml of distilled water. This solution is equivalent to 1.0 mg/ml of sulphate (SO_4^{2-}) stock concentration. The sulphate standards of varied concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were prepared by diluting the stock solution using distilled water.

2.4.3 Sulphate Standard Curve and Quantification of Sulphate Formation

A 10 ml of of each standard solution were added into the 2.5 ml of conditioning reagent in a beaker. The solution was diluted by adding 37.5 ml of sterile distilled water. Subsequently, 0.15 g of anhydrous barium chloride ($BaCl_2$) was added and stirred until it dissolved completely. The turbidity of the solution was measured by UV-Visible spectrophotometer at a wavelength of 420 nm. The sulphate standard curve was established by plotting the absorbance at 420 nm against sulphate concentration (mg/ml). These similar steps were done for quantitation of sulphate formation in the culture flask where the 10 ml of standard solution was replaced with 10 ml of culture sample.

3. Results and Discussions

3.1 Growth profile of A. thiooxidans strain UniMAP-AIN01

Figure 1 depicts the growth profile of *A. thiooxidans* strain UniMAP-AIN01 cultivated in thiobacillus broth medium. A typical growth pattern consists of lag, exponential, stationary and death phases. However, as can be seen in figure 1, there is slight to no visible lag phase is present in the growth profile. This phenomenon could be due to the inoculum preparation technique. The seed inoculum was cultivated in the broth medium with similar composition. This triggered the bacterial cell to actively proliferate. When the actively dividing inoculum culture was transferred into the fresh medium with similar composition, proliferation continues without any resistance. This explains why the growth pattern started with exponential phase.

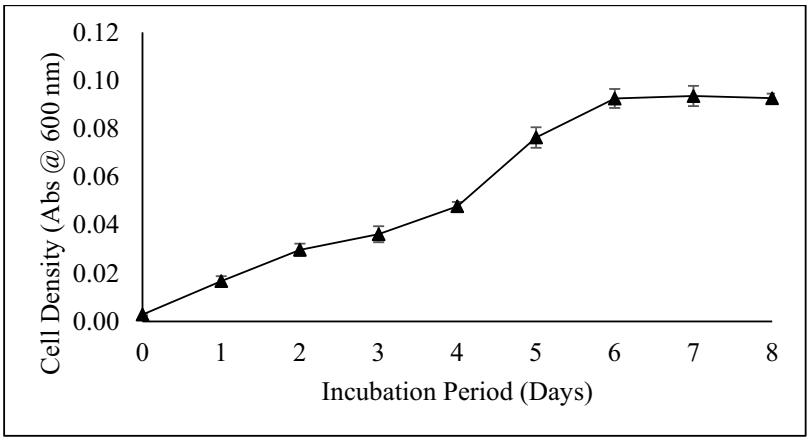


Figure 1. Growth profile of *Acidithiobacillus thiooxidans* strain UniMAP-AIN01.

According to this figure, the exponential phase lasted for 5 days starting day 1 to day 6 of the incubation period. In this period, the bacteria continuously multiplied rapidly and increased cell density. Then, from day 6 to day 7, the stationary phase of the culture was observed. This is where the size of the bacterial population remain constant as the number of cells to divide and dies are equal. Finally, on day 8 the cells enter the last phase which is the death phase. This is where the cells are dying at a rapid rate compared to newly dividing cells due to the depletion of one or more essential nutrients or the accumulation of toxic by-products of growth [3]. In this study, the study of bacterial growth profile is important in order to established a growth pattern of isolated *A. thiooxidans* strain UniMAP-AIN01 using thiobacillus medium supplemented with sulphate. This evaluation enlightens the capability of the isolated bacterial strain to grow despite the presence of toxic effect of sulphate to the bacteria. In addition, this result proves that our isolated *A. thiooxidans* strain UniMAP-AIN01 is a highly potential candidate for rubber latex processing wastewater treatment as the wastewater is high in sulphate content and low in pH. From the growth profile, the doubling time of this *A. thiooxidans* strain UniMAP-AIN01 can be determined. First, the specific growth rate, μ of the bacteria was calculated using the following equation (3) by substituting $X_0 = 0.0168$; $X_t = 0.0926$; and t = 5 days which was figured to be 0.3414 day^{-1}.

$$X_t = X_0 \cdot e^{\mu \cdot t} \quad (3)$$

X_t = microbial biomass concentration at specific time
X_0 = initial microbial biomass concentration
t = time
μ = specific growth rate

A non-linear growth kinetic plot was generated using POLYMATH software as in figure 2. X_t exp indicates the experimental values of microbial biomass concentration, meanwhile, the X_t calc represents the concentration of biomass at a specific time calculated by the model equation as in equation (3), using $\mu = 0.3414$ days^{-1} and $X_0 = 0.0168$.

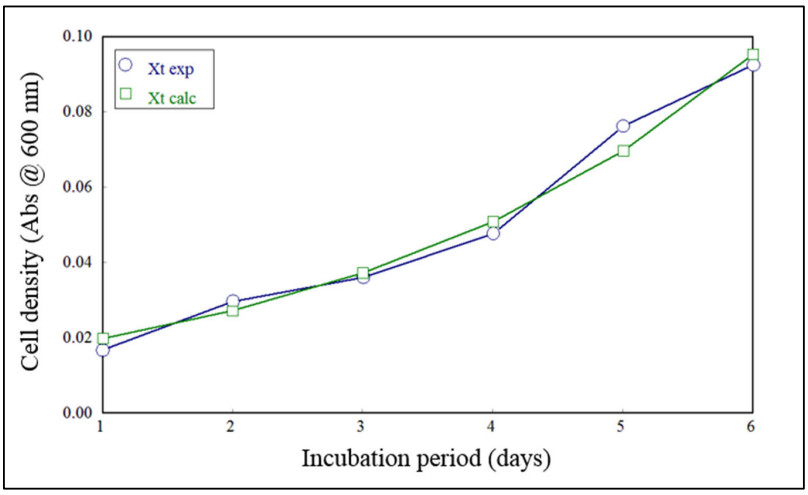


Figure 2. Growth kinetic plot of *A. thiooxidans* strain UniMAP-AIN01.

Regression analysis of the growth kinetic was also computed using POLYMATH software. A small margin between linear regression, R^2 (0.9811) and adjusted linear regression, Adj R^2 (0.9764) indicates the high correlation between experimental and adjusted values. Meanwhile, smaller root mean square deviation, RMSD (0.0015) and variance (1.992×10^{-5}) values indicate the error is insignificant for the data established [4]. This verifies the validity of the experimental data. Finally, the doubling time of the bacteria was calculated using equation (4) as follows;

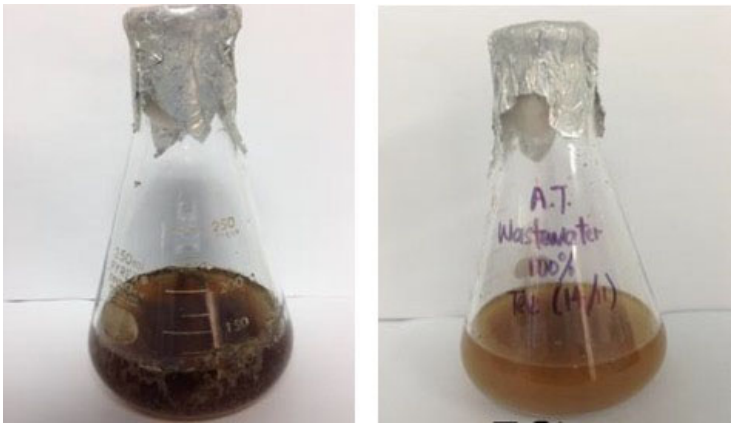
$$t_d = \frac{\ln 2}{\mu} \tag{4}$$

The doubling time of the bacterium was estimated to be 2 days which can be inferred that the *A. thiooxidans* strain UniMAP-AIN01 has a very long doubling time if compared to both *Thiobacillus thiooxidans* strain IFO 13724 of Konishi et al. [5] which was around 6.5 hours and *A. thiooxidans* strain ATCC 19377 (isolated from Kimmeridge Clay, Dorset, England, UK) which was around 8 hours [6]. This indicate that the *A. thiooxidans* strain UniMAP-AIN01 requires more time or slower to proliferate in sulphate rich toxic condition. According to Yousefi et al. [7], it was found that the *A. thiooxidans* exhibits more severe pH reduction and increased in bacterial density due to the supplementation of sulphur media compared to thiosulphate-containing media. This indicates that *A. thiooxidans* prefers elemental sulphur more than thiosulphate. In the present study, initially, we have done the cultivation of our isolated *A. thiooxidans* strain UniMAP-AIN01 in Thiobacillus broth medium supplemented with sulphur powder. However, the presence of this sulphur creates problems such as non-reproducibility and inaccurate measuring of biomass density (data were not shown here). Therefore, the evaluation of growth kinetics was performed using thiosulphate as a replacement to sulphur. Based on Liu et al. [8], while the entire metabolic pathway and the nature of the multi-enzyme systems involved in the degradation of sulphur and its derivatives are not clearly understood, the role of elemental sulphur in the growth of cells and the production of sulphuric acid as a result of primary metabolism of *A. thiooxidans* has been well recognized. Even if elemental sulphur was substituted by other alternative substrates in

the growth medium for the sake of cell separation and concentration measurement in other studies, the amount of sulphuric acid produced was much less than the medium containing elemental sulphur. This indicated that *A. thiooxidans* has the preference to utilize elemental sulphur as an energy source.

3.2 Removal of hydrogen sulphide by A. thiooxidans strain UniMAP-AIN01

Figure 3 below illustrates the comparison between the control (without *A. thiooxidans*) and treated (with *A. thiooxidans*) flasks of wastewater. The discoloration of treated flask in figure 3(b) compared to control flask in figure 3(a) indicates the capability of *A. thiooxidans* growing and surviving in the latex industry wastewater.



(a) **(b)**

Figure 3. Qualitative observations of undiluted latex wastewater before (a) and after 7 days treatment with *A. thiooxidans* strain UniMAP-AIN01 (b).

Further analyses of pH level, change in hydrogen sulphide and sulphate concentrations were tabulated and plotted as in figure 4 in order to compare among these three most important parameters that contribute to the growth of *A. thiooxidans* strain UniMAP-AIN01.

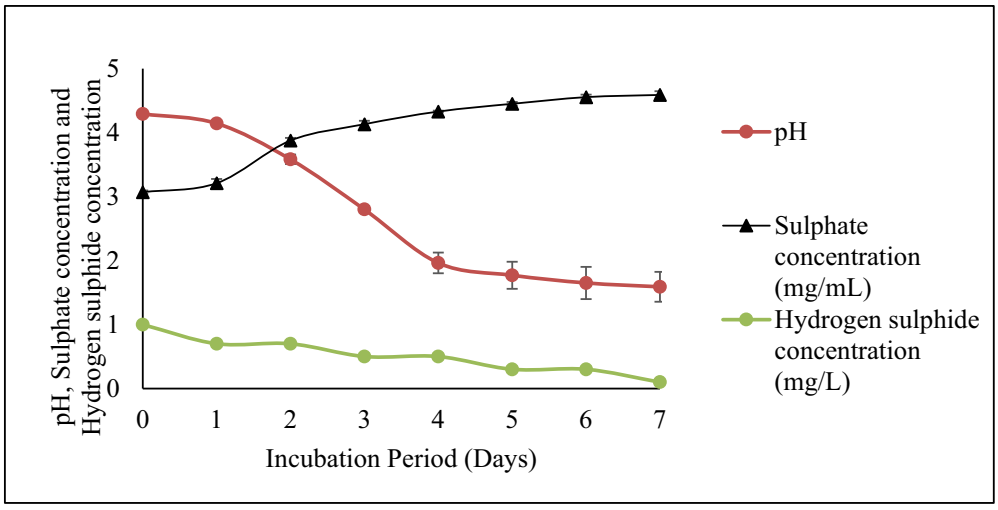


Figure 4. The content of sulphate and hydrogen sulphide concentrations as well as pH medium over incubation time during the treatment of wastewater by *A. thiooxidans* strain UniMAP-AIN01.

Based on figure 4, it can be observed that pH and sulphate concentration are inversely proportional to each other. The pH levels declined steadily from 4.29 to 1.59 over 7 days of incubation. Meanwhile, sulphate concentration was increased from 3.07 mg/ml to 4.59 mg/ml during the same period of time. This phenomenon was due to the sulphate ion formation from oxidation of sulphur into sulphuric acid. The following equations (5) to (7) elucidate the conversion process of sulphur into sulphuric acid that contributes to pH drop in latex wastewater. The sulphate ion formed as an intermediate of the sulphur oxidation by *A. thiooxidans*. The sulphate ions then react with water to form the acid that causes the pH to drop.

$$S^0 + \frac{3}{2}O_2 + H_2O \rightarrow H_2SO_4 \quad (5)$$

$$S^0 + 2O_2 \rightarrow SO_4^{2-} \quad (6)$$

$$SO_4^{2-} + H_2O \rightarrow H_2SO_4 \quad (7)$$

On the other hand, a comparison between the sulphate concentration and hydrogen sulphide concentration during 7 days of incubation verifies the conversion of hydrogen sulphide into sulphate. The hydrogen sulphide content in latex wastewater acts as a reactant which was converted into sulphate, a by-product of the oxidation by *A. thiooxidans*. According to the Environmental Quality (Industrial Effluent) Regulations 2009 (PU (A) 434) outlined by the Malaysia government, the discharge limit for sulphide in wastewater effluent should not exceed 0.5 mg/L. The untreated latex processing industrial effluent was tested to contain double the eligible discharge limit amount of hydrogen sulphide. Direct discharge of this wastewater could trigger severe environmental pollution and adversely affects marine life. Nevertheless, through the biological treatment of latex wastewater with *A. thiooxidans* strain UniMAP-AIN01, hydrogen sulphide removal by 50% was achieved on the third day of incubation, bringing the latex wastewater to the allowable 0.5 mg/L discharge limit of hydrogen sulphide. Meanwhile, up to 90% of hydrogen sulphide was successfully removed on the 7th day (last day) of incubation. Thus, this validates the potential of our isolated *A. thiooxidans* strain UniMAP-AIN01 to be used for the biological treatment of rubber latex processing wastewater.

4. Conclusions

The potentiality of the isolated sulphur-oxidizing *A. thiooxidans* strain UniMAP-AIN01 for removal of hydrogen sulphide from latex rubber wastewater was investigated in this study. As an acidophilic chemoautotrophic bacterium, the *A. thiooxidans* strain UniMAP-AIN01 cultivation is favoured in an acidic and sulphur-rich environment to grow. Efficiency of hydrogen sulphide removal was achieved after seven days of incubation with two days of doubling time. In future, more extensive researches such as process condition optimization, and scale-up study are required to be carried out to identify and apply the full potential of the strain.

Acknowledgement

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