

Study on Haloalkaliphilic Sulfur-Oxidizing Bacterium for Thiosulfate Removal in Treatment of Sulfidic Spent Caustic

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Keywords: Biotreatment, Sulfide-oxidizing bacteria, Sulfidic spent caustic

Abstract. Due to the disadvantages of physiochemical methods for sulfidic spent caustic treatment, attentions are drawn to the environmental-friendly biotreatments including sulfur-oxidizing haloalkaliphiles. *Thioalkalivibrio versutus* DSM 13738 was grown at alkaline (pH10) autotrophic medium with sodium carbonate/bicarbonate as the sole source of carbon and amended with sodium thiosulfate as the electron and energy source. The effect of various parameters including temperature (25-40 °C), pH (8-11), NaCl concentration (0.5-5 % w/v) and sodium thiosulfate concentrations (100-750 mM) was evaluated on bacterial growth and thiosulfate removal. This strain could eliminate sodium thiosulfate at very high concentrations up to 750 mM. The results showed that the highest specific growth rate was pH 9.5 and thiosulfate removal of *Thioalkalivibrio versutus* occurred at pH 10.5. The optimum salt concentration for thiosulfate removal was 2.5 % w/v and 5 % NaCl and specific growth rate elevated 2.5% w/v. It was also specified that this strain thrives occurred in 37 °C and at 35 and 37 °C higher removal of thiosulfate. Following chemical oxidation of sulfide to thiosulfate, application of *Thioalkalivibrio versutus* could be promising for spent caustic treatment. Since thiosulfate is utilized as an energy source, highest removal efficiency occurred at marginally different conditions compared to optimal growth.

INTRODUCTION

Releasing a large amount of toxic effluent produced in petroleum industry leads into environmental pollution of receiving bodies of water and soils and constitutes serious toxicity for living organisms and hence, reduces the capacity of contaminated habitat for supporting life [1-5]. Spent caustic is one of the most complicated and hazardous wastewaters produced in various gas and oil sweetening processes in refineries and petrochemical plants [3, 6-8]. Although characteristics of spent caustics may greatly vary from a refinery to another, these wastewaters are generally studied in three different categories namely sulfidic, phenolic and naphthenic [6, 9-12].

Diluted caustic solutions (NaOH, 5-10 % w/w) are usually used for scrubbing hydrocarbon streams to absorb sulfur compounds such as H₂S and thiols and finally this process results in sulfur-containing waste water known as sulfidic spent caustic (SSC). This harmful waste has alkalinity over 12, salinity of 5-12 % w/w and high sulfide (HS⁻ and S²⁻) level exceeding 2-3 % w/w [8, 13-15]. Sulfidic spent caustic may also contain toxic organo-sulfuric compounds such as methanethiol, and aromatic hydrocarbons like benzene [15, 16].

Discharge of these wastes without proper treatment may impose catastrophic environmental issues. In industrial applications, extreme alkaline wastes have been generally neutralized with sulfuric acid and subsequently released to the environment. Nowadays physicochemical methods such as wet air oxidation (WAO), advanced oxidation and the MEROX process, as well as biological methods are proposed for the treatment of sulfidic spent caustic [17-20], among which biological systems could be advantageous due to lower cost and higher safety [21]. The limited number of researches conducted on the biotreatment of sulfidic spent caustic mostly focused on the application of neutrophilic sulfur bacteria belonging to the genus *Thiobacillus*, where the stream needs to be diluted in order to decrease the pH and salinity levels [16, 22-24]. On the other hand, at pH levels below 9, most of the dissolved sulfide would be released as toxic hydrogen sulfide into the atmosphere [25, 26].

Recently, a specialized group of haloalkaliphile sulfide-oxidizing bacteria (HA-SOB) were obtained from soda lake sediments. These obligate autotrophic bacteria belong to gamma subdivision of the proteobacteria and are divided into *Thioalkalimicrobium*, *Thioalkalivibrio* and *Thioalkalispira* [27, 28]. To overcome acute sulfide toxicity towards bacteria, a two-step process was suggested by Marco de Graaff in 2012, in which sulfide was auto-oxidized to non-toxic thiosulfate in an aeration process and then remaining sulfide and thiosulfate was biologically oxidized to sulfate [14]. It has been shown that sulfide reduces the growth rates of alkaliphilic sulfur-oxidizing bacteria at concentrations of about 3mM, while at higher concentration of 6mM, is completely inhibitory [21]. Chen and Morris expressed that sulfide oxidation produces polysulfide, S_4^{2-} , S_5^{2-} , and thiosulfate [29]. The intermediates formed during chemical sulfide oxidation are less toxic to alkaliphilic sulfur-oxidizing bacteria and inhibits their activity at least six times less than sulfide. Gonzalez-Sánchez showed that during chemical oxidation, almost one-third of sulfide was converted to thiosulfate and sulfate [30]. Researchers also reported that sulfide oxidation increases at high pH values [29, 31].

Among haloalkaliphilic bacteria, the genus *Thioalkalivibrio* could thrive in extreme salt (0.6-4 M Na^+) and alkaline (pH 10.0-10.2) conditions [27, 28, 32]. All members of this genus could oxidize thiosulfate, sulfide and polysulfide in halo-alkaline conditions which makes them appropriate candidates for biotreatment of sulfidic spent caustic wastewater. In spite of the biotechnological importance of these extremophilic sulfur bacteria, little information is available regarding their thiosulfate utilization. In this study, optimal conditions of thiosulfate removal were investigated for the type strain of *Thioalkalivibrio versutus* DSM 13738 [27].

MATERIAL AND METHODS

Microorganism and Medium Composition

The bacterium, *Thioalkalivibrio versutus* DSM 13738, isolated from Hadyn Soda Lake in southeast Siberia by Sorokin, was used throughout this study [27, 33, 34]. The obtained standard strain was checked by 16S rRNA gene sequencing analysis. The basal mineral medium recommended by DSMZ for the growth of *Thioalkalivibrio versutus* was used in this study. This medium consisted of (g/L): Na_2CO_3 , 20; $NaHCO_3$, 10; $NaCl$, 5; K_2HPO_4 , 1; KNO_3 , 0.5; $MgCl_2 \cdot 6H_2O$, 0.1 and 2 ml/L trace element solution which contains (mg/L): EDTA, 5; $FeSO_4 \cdot 7H_2O$, 2; $ZnSO_4 \cdot 7H_2O$, 100; $MnCl_2 \cdot 4H_2O$, 30; $CoCl_2 \cdot 6H_2O$, 200; $NiCl_2 \cdot 6H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 30; $CuCl_2 \cdot 2H_2O$, 10; H_3BO_3 , 300. The mineral base medium was buffered at pH 10 with sodium carbonate/bicarbonate. Due to less toxicity, sodium thiosulfate was amended instead of sulfide as electron donor and energy source. Thiosulfate stock solution was sterilized separately and added at final concentration of 40 mM. For purity checking of the cultures, solid medium was prepared by mixing equal volumes of autoclaved 4 % (w/v) agar solution with the double-strength mineral base at 55-60 °C to prevent agar caramelization at high pH. Fresh cultures were prepared on liquid mineral medium with a two-month interval between transfers. For long-term preservation, concentrated bacterial biomass harvested by centrifugation, were suspended in glycerol (10 % v/v, final concentration) and kept at -70 °C until used in inoculum preparation [27].

Cultivation Experiments

In this study, parameters with the highest impact on the autotrophic bacterial growth and removal of thiosulfate were determined considering biotreatment of spent caustic waste and effect of important parameters for growth and thiosulfate removal was assessed. The experiments were performed by adjusting the temperature (ranging from 25 to 40 °C), pH (8-11), sodium chloride (0.5-5 % w/v), and sodium thiosulfate (100-750 mM). For the preparation of the mentioned pH range (8, 9, 9.5, 10, 10.5, 11), a set of carbonate buffers containing 2 M Na⁺ was used. The effect of key parameters for growth and removal of thiosulfate was surveyed. Growth experiments were performed in triplicate; the data represent the average value with standard deviation [35, 36].

For pre-culture preparation, *Thioalkalivibrio versutus* was inoculated in a 250 ml Erlenmeyer flask containing 50 mL basal mineral medium. The flask was incubated at 35 °C for 3 days to reach the cell turbidity of approximately 0.1 at 620 nm [37, 38]. The cultivation experiments were accomplished in 1000 ml Erlenmeyer flasks using 200 ml of culture medium with 5 % (v/v) of inoculum, prepared as described above. The flasks were incubated at 150 rpm to fulfill the oxygen and mixing requirement. For monitoring biomass production and thiosulfate removal, 1 ml samples were obtained from culture medium at three-hour intervals until the exhaustion of sodium thiosulfate. The rate of thiosulfate consumption by *Thioalkalivibrio versutus* was measured at different intervals to determine maximal thiosulfate removal rate [37, 39].

Analytical Techniques

Growth assay

Bacterial biomass was determined colorimetrically by measuring total protein content with Bradford method. Washed bacterial pellet of 1 ml withdrawn sample was suspended in 0.1 N NaOH and boiled for 1 hour to lyse bacterial cells. The cell debris along with elemental sulfur was removed by centrifugation at 11500 rpm for 10 minutes and the supernatant was used for total protein measurement by Bradford method (Bradford 1976). Bovine serum albumin (BSA) solutions, diluted in the mentioned mineral base medium, were used as a standard.

Chemical analysis

Thiosulfate consumption in batch culture was measured by standard iodometric titration. This method is based on the titration of thiosulfate with iodine in the presence of starch as indicator. To prevent sulfate formation, the reaction should be performed at acidic pH after neutralization of medium with acetic acid 50 % (v/v). Since thiosulfate would decompose at acidic pH, the titration should be done rapidly. For this purpose, 1 ml sample was centrifuged at 11500 rpm for 10 min to remove bacterial biomass and produced sulfur. Supernatant was titrated by iodine based on the formation of the blue starch complex as the endpoint [37, 40].

RESULTS

The harmful effect of hydrogen sulfide has triggered the application of a two-step treatment process for spent caustics in which the chemically formed thiosulfate is utilized by haloalkaliphilic sulfide-oxidizing bacteria. Important parameters affecting thiosulfate removal rate in *Thioalkalivibrio versutus* were elucidated in this study.

Effect of substrate concentration

Thioalkalivibrio versutus could grow at extremely high concentrations of thiosulfate up to 750 mM (that at this study was surveyed). Specific growth rate at a concentration of 100, 250, 500 and 750 was measured to be 0.069, 0.06, 0.05 and 0.06 h⁻¹. At a substrate concentration of 100 mM, complete removal of thiosulfate was achieved at 76th hour, whereas at a concentration of 250, 500 and 750 mM, 46 %, 43 % and 10 % of the substrate were respectively removed at 76th hour. On the other hand, at low concentration of thiosulfate (100 mM), produced elemental sulfur was further oxidized to sulfate to serve the energy requirements of bacterial cells after thiosulfate depletion, which resulted in the disappearance of sulfur particles from the media. As shown in Figure 1, increased substrate level neither imposed toxicity nor promoted the growth of the culture, but resulted in sulfur accumulation in the media.

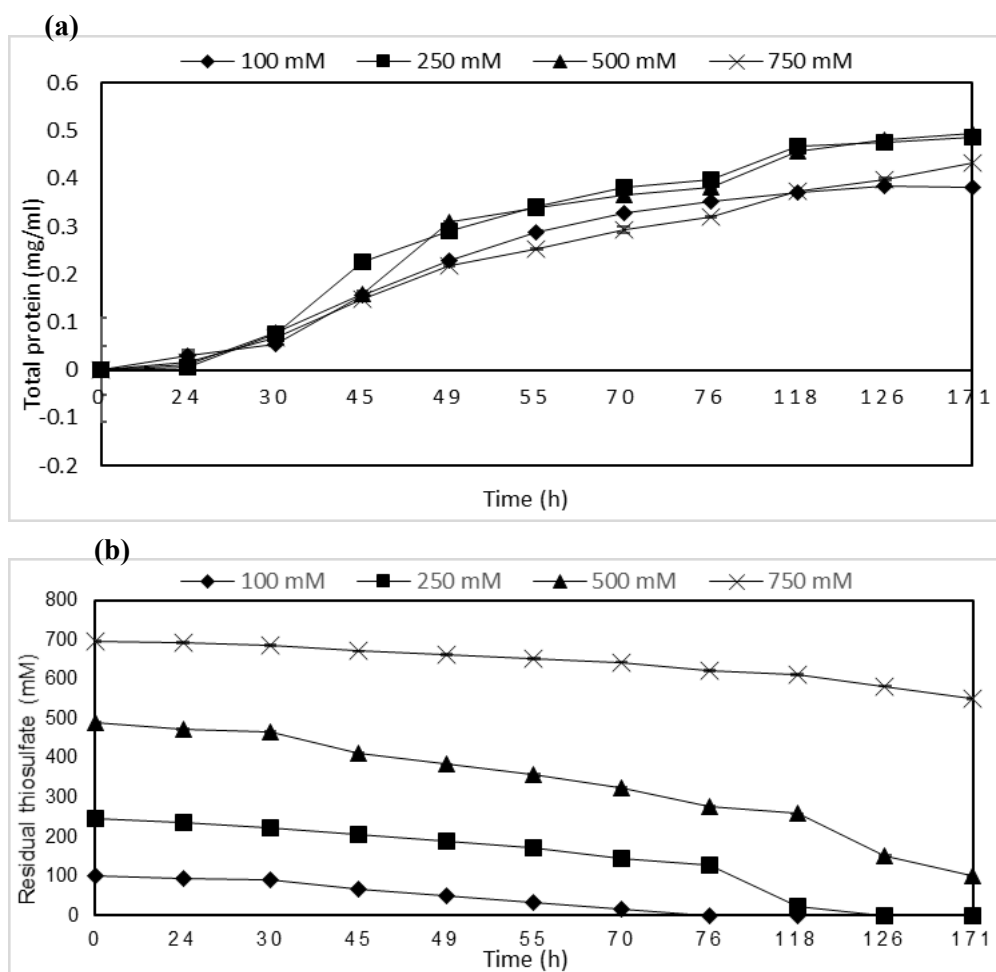


Figure 1: Comparison of (a) growth and (b) thiosulfate removal at different concentrations of sodium thiosulfate (100, 250, 500 and 750 mM).

Effect of pH

As depicted in Figure 2, no growth and thiosulfate removal were observed at extreme pH 11 after 70-hour incubation. The highest specific growth rate was measured at pH 9.5, 0.077 h^{-1} , in which thiosulfate was completely utilized in 50th hour. The specific growth rate at pH 8, 9, 10 and 10.5 was measured to be 0.034, 0.065, 0.076 and 0.064 h^{-1} respectively. The final biomass concentration at pH 9.5 reached to 0.28 mg/ml and highest biomass concentration was calculated at same pH. The rate of thiosulfate removal at pH 9, 9.5, 10 and 10.5 was calculated to be 0.76 mMh^{-1} while at pH 8, the rate of thiosulfate removal was 0.56 mMh^{-1} . pH changes during the period of growth were followed and was shown no significant change at different times of incubation that can result from low acid production or stop the process until the step of the production of sulfur.

Effect of temperature

Bacterial growth and thiosulfate consumption at various temperatures are plotted in Figure 3. During 70-hour incubation period, no growth and thiosulfate removal were observed at 40°C . At 35°C and 37°C the strain began to grow following a 20-hour lag phase total protein concentration reached to 0.29 and 0.27 mg/ml, respectively and sodium thiosulfate was completely consumed in both cultures at 41th hour. The lag phase extended up to 25 hours at lower temperatures of 25 and 30°C . However, the final biomass concentration reached to 0.28 mg/ml. Specific growth rates at 25, 30, 35 and 37°C based on Monod equation were calculated as 0.08, 0.082, 0.087 and 0.095 h^{-1} . The highest rates of thiosulfate removal at 35 and 37°C were calculated to be 1 mMh^{-1} . On the other hand, at 25 and 30°C thiosulfate was removed as 0.76 mMh^{-1} .

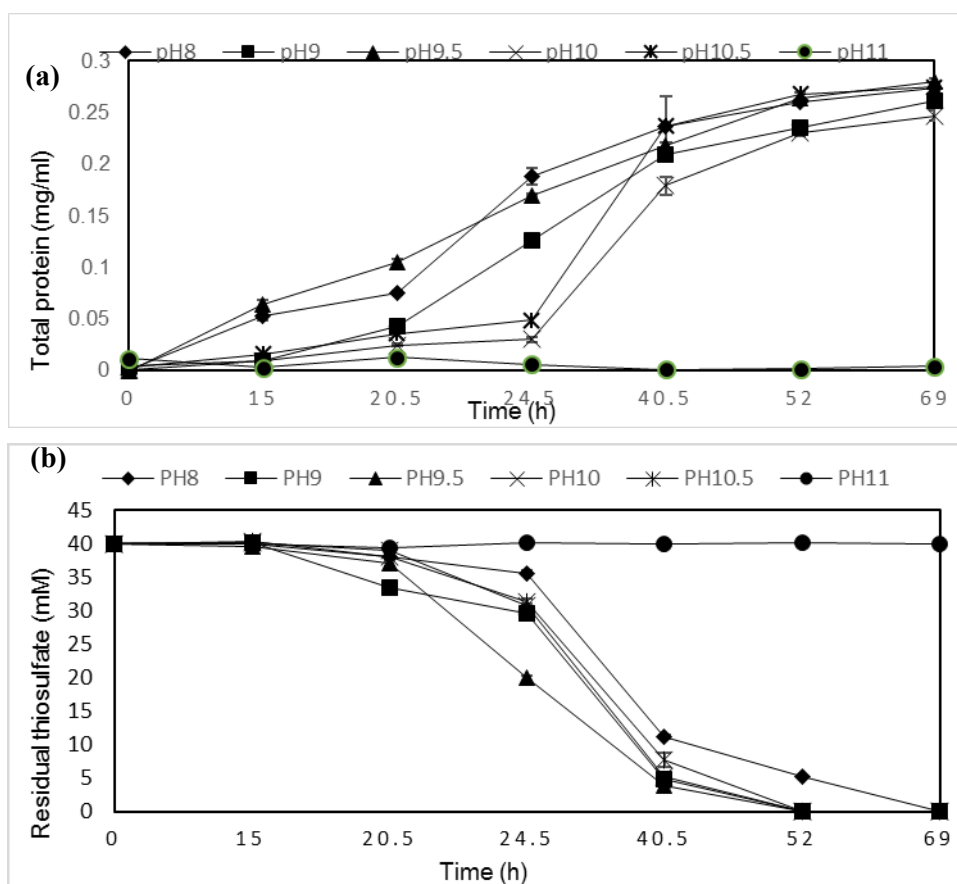


Figure 2: Comparison of (a) growth and (b) thiosulfate removal at different pH (8, 9, 9.5, 10, 10.5 and 11).

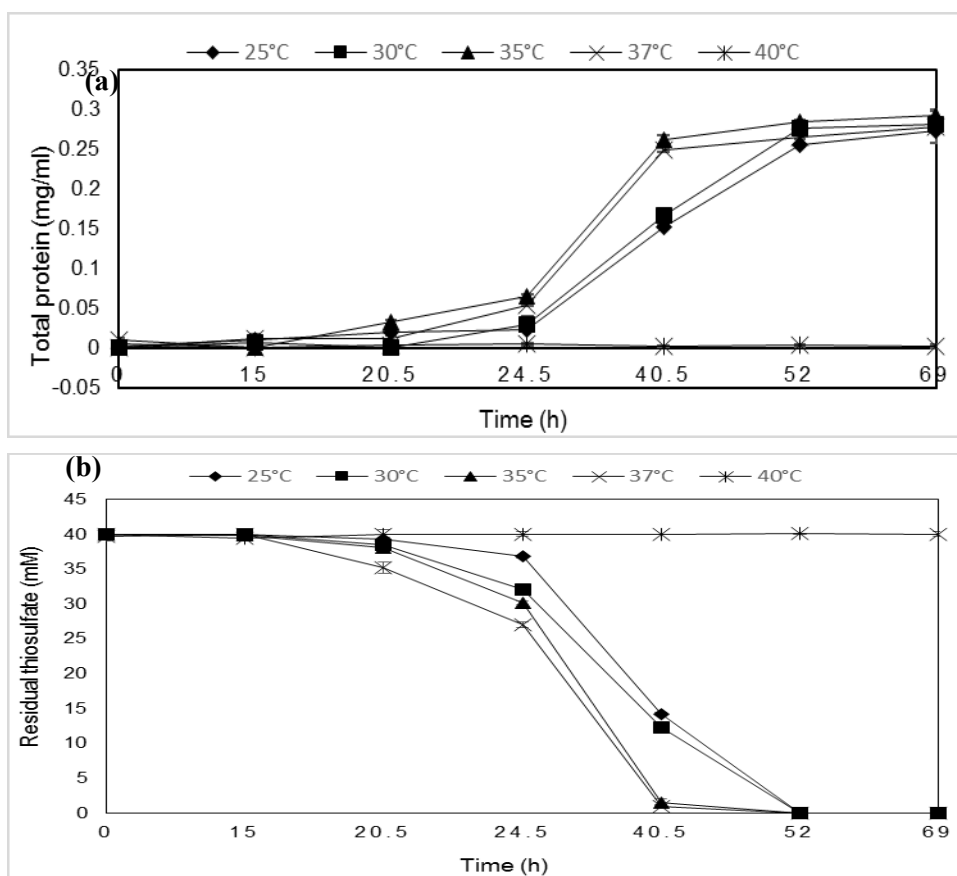


Figure 3: Comparison of (a) bacterial growth and (b) sodium thiosulfate removal at different temperatures (25, 30, 35, 37 and 40 °C)

Effect of salt concentration

Following a 24-hour lag phase, the highest growth rate was observed at NaCl concentrations of 2.5 % w/v with complete removal of sodium thiosulfate measured at 47th hour-growth. Specific growth rate of 0.5, 1, 2.5 and 5% of NaCl concentrations was calculated as 0.088, 0.124, 0.26 and 0.193 h⁻¹ respectively. The final biomass at NaCl concentration of 2.5% w/v reached to 0.31 mg/ml. At NaCl concentration of 5% w/v, sodium thiosulfate was completely removed after 47-hour incubation and final biomass concentration was measured 0.29 mg/ml. The rate of thiosulfate removal at NaCl concentration of 2.5 and 5 % w/v was 0.85 mM/h while at lower NaCl concentrations of 0.5 and 1 % w/v, the complete removal of thiosulfate was achieved after 69 and 52 hours and the rate of thiosulfate removal was measured as 0.57 and 76 mM/h, respectively (Figure 4). The strain showed high salt tolerance with the lowest lag phase at 2.5 and 5% NaCl concentration. However, the highest specific growth rate was showed at 2.5 % NaCl.

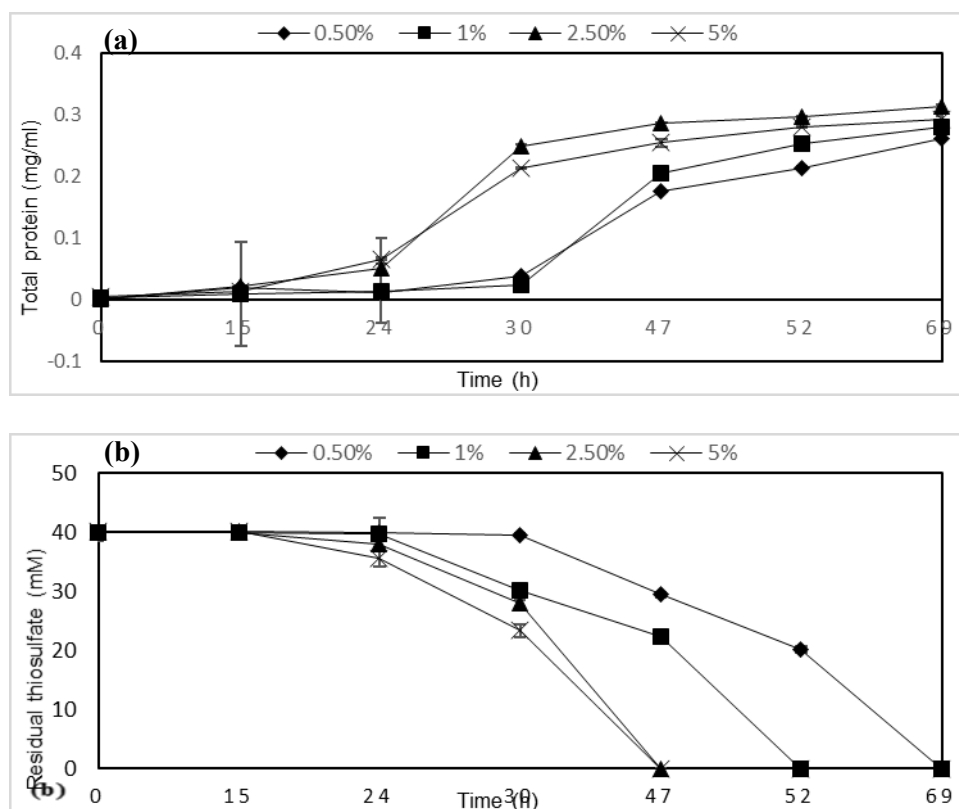


Figure 4: Comparison of (a) growth and (b) thiosulfate removal at different concentration of sodium chloride (0.5, 1, 2.5 and 5 %).

DISCUSSION

Sulfidic spent caustic is a harmful waste for which biological method could be advantageous due to lower cost and higher safety. Subramanian in 1998 used aerobic enrichment culture containing mixed Thiobacilli to stimulate the treatment of the inorganic and organic sulfur of a spent caustic. This enrichment could treat an actual waste by low rate due to existing inhibitory components. Jin Myung Cha isolated a strain of *Thiobacillus novellus* from sewage capable of oxidizing S₂O₃²⁻ to S₄O₆²⁻ and S₄O₆²⁻ to SO₄²⁻ [41]. In another investigation, where aerobic enrichment culture was immobilized in a novel support matrix, the efficiency depended on the maintenance of sulfide concentration below inhibitory levels [22].

Following the introduction of haloalkaliphilic sulfur-oxidizing bacteria by Sorokin in 2000 [28], the advantage of applying this microbial group for oxidation of sulfidic components in spent caustics was elucidated [25, 26].

In 2007, an alkaliphilic sulfur-oxidizing consortium was exploited for the treatment of sulfidic spent caustic without significant dilution in which the efficiency of sulfide oxidation reached about 100% [21]. Marco de Graff and colleagues also showed that sulfide could be completely removed at pH 9.5, Na^+ 0.8 M by applying a sulfur-oxidizing bacteria belonging to the genus *Thioalkalivibrio* [15].

In our study, optimal conditions for the growth and removal of thiosulfate was determined in batch cultures. The optimal temperature for growth was 37 °C and maximum rate of thiosulfate removal was concluded at 35 and 37 °C similarly, but no growth was observed at 40 °C. Marco de Graff used *Thioalkalivibrio* at 35° C, at alkaline condition by sulfide loading rates up to 27 mM day⁻¹ and sulfide was completely oxidized to sulfate [15]. Accordingly, highest thiosulfate depletion, as the sole energy source could be achieved at a temperature slightly above the optimal growth conditions, in which extra energy is required for cellular activities and maintenance.

Haloalkaliphile bacteria tolerate alkaline pH by K^+/H^+ pump, these bacteria make a gradient of pH that decreases cytoplasmic pH up to 2.3 unit. $\text{CO}_3^{2-}/\text{HCO}_3^-$ play an important role in producing alkaline pH in specific alkaline habitat. Similarly, the highest specific growth rate was observed at pH 9.5 and the highest rate of thiosulfate removal was at pH 9, 9.5, 10 and 10.5 with 0.76 mMh⁻¹. Sorokin at 2005 showed that optimal growth rate of *Thioalkalivibrio versutus* was at pH 9.5. Because there were no significant differences in specific growth rate and rate of thiosulfate removal between pH 9 to 10.5 and on the other hand higher pH have advantages due to reduced consumption of produced acid during oxidation of sodium thiosulfate. So it seems that pH 10.5 is more suitable for the oxidation of the sulfur substrate. It is being shown that Haloalkaliphile chemolithoautotroph bacteria for optimal activity of RuBisCo enzyme need alkaline pH. On the other hand, the optimal activity of enzymes existing at sulfur oxidation pathway in haloalkaliphilic sulfur oxidizing bacteria is in alkaline pH condition.

Results elucidated that higher specific growth rate was measured at NaCl concentration 2.5% (1.8 M Na^+). Whereas in a study conducted by Sorokin, the maximum growth rate was reported at less than 1 M Na^+ . The sodium salt is an osmotic compound that halophile microorganisms such as haloalkaliphiles need to resist to osmotic pressure. According to the results of Sorokin et al, Soda Lake makes approximately two times osmotic pressure than NaCl brines. In this study, by increasing concentration of Na^+ , specific growth rate increased up to 2.5 % but at higher than the concentration of NaCl-specific growth rate was diminished.

CONCLUSION

This bacterium was able to remove thiosulfate up to 750 mM. Although, at higher concentration of sodium thiosulfate, lower thiosulfate removal, could be explained by metabolic substrate saturation of the bacterial cells or presence of other limiting factors in the culture media. Haloalkaliphilic sulfur-oxidizing bacteria could tolerate harsh conditions of spent caustic and would be promising for biotreatment of this hazardous waste. Tolerance of *Thioalkalivibrio versutus* towards increased concentrations of thiosulfate is also an important criterion for biotechnological application, where the exploitation of a pretreatment stage for conversion of toxic sulfide to thiosulfate would pave the way for further oxidation. In addition, choosing proper conditions for thiosulfate oxidation activity could result in higher removal efficiency with minimal excess biomass production during wastewater treatment.

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