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Evaluation of Effective Strategies for Cultivation of *Acidithiobacillus Thiooxidans* as Cement-Degrading Bacteria

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Abstract
Acidithiobacillus thiooxidans is an acidophilic chemoautotrophic type of sulfur-oxidizing bacteria. This microorganism lowers the pH of the medium to less than 1, and intensifies the degradation rate of cement-based structures. Growth of acidophilic bacteria on an agar solid medium does not lead to formation of colonies and thus counting of living cells is impossible in the process of cement biodegradation. In this study, the growth of two strains of A. thiooxidans (PTCC 1692 and PTCC 1717) were investigated using some liquid and solid culture media containing elemental sulfur and thiosulfate ion, in different conditions. Thiosulfate liquid culture medium with no ions similar to cement was the most suitable medium for the study of cement biodegradation. Several solid culture media containing agar and agarose were examined and the bacterial colonies were observed only on solid medium containing calcium, thiosulfate ions and agarose. To further our studies, the bacterial growth rate was evaluated on a 4-liter polypropylene bioreactor at temperatures of 25 and 30°C by two methods of continuous and alternating aerations at different rates. The highest growth rate was obtained in the 4th day of cultivation under aeration rate of 1 vvm for A. thiooxidans PTCC 1717 type strain.

Keywords: *Acidithiobacillus Thiooxidans, Cement Biodegradation, Solid Culture Media, Aeration*

1. Introduction
Acidithiobacillus thiooxidans (*A. thiooxidans*) is a major type of microorganism, capable of oxidizing sulfur compounds and especially elemental sulfur present in the soil. This gram-negative bacteria [1, 2] is isolated from a degraded concrete structure containing sulfur and phosphate rocks. Most research works have indicated that *A. thiooxidans* plays a key role in the cement biodegradation process [3-6]. This bacterium thrives in acidic conditions with a pH less than 1 and the source of its energy is the oxidation of elemental and mineral sulfur. Even some species of *Thiobacillus* can produce sulfuric acid by oxidation of hydrogen sulfide [7]. *A. thiooxidans* coexists with the hydrogen sulfide oxidizing fungus. This fungus oxidizes hydrogen sulfide to thiosulfate ion

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and is used by *A. thiooxidans* bacteria as an energy source [8]. Thiosulfate is quite soluble in water, but it settles in acidic environment with pH less than 3.5 [9].

Sulfur-oxidizing bacteria can oxidize thiosulfate to sulfate ions through reactions (1) to (3), depending on the availability of oxygen [9]. Under conditions of more or less oxygen saturation, complete conversion of thiosulfate to sulfate ions takes place according to reaction (3) [10].

$$S_2O_3^{2-} \rightarrow S + SO_3^{2-} \rightarrow 2SO_4^{2-} \quad (1)$$

$$2S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow S + SO_4^{2-} + S_2O_3^{2-} \quad (2)$$

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \quad (3)$$

Low aeration of liquid medium of this bacterium facilitates the slow pathways (1) and (2) to their growth (by conversion of thiosulfate to sulfate ions) rather than the rapid oxidation pathway (3). The first two pathways lead to the production of elemental sulfur which, in later stages is converted to sulfate ion. So the amount of available air is very crucial for the growth rate of cement degrading microorganisms.

Because *A. thiooxidans* is a type of autotrophic bacteria, the necessary carbon source must be supplied by carbon dioxide in the air. Its nitrogen requirement is provided by ammonium or nitrate salts. This bacterium needs small amounts of potassium, magnesium, iron and phosphate ions for better growth [1, 2].

According to published literature, this microorganism easily grows in a variety of liquid media, but does not grow on common types of organic or inorganic solid media agar plate such as nutrient agar (NA), unlike the heterotrophic microorganisms which grow on solid media [2, 11]. On the other hand, the needed pH for this bacterial growth would be impossible for gelation of agar-based media and at low pH, a sterile agar does not create a stable gel.

Shortage of appropriate solid medium has prevented research on colonies of *A. thiooxidans*. It has been pointed out that to overcome these problems, solid media should be designed based on agarose or purified agar with an acid treatment [12]. To the best of our knowledge, no reports have been published on counting colonies of *A. thiooxidans* PTCC 1692 and PTCC 1717 on solid medium as it is not possible to follow the living bacterial population of degrading cement. In most research works, the "most probable number" (MPN) methods as well as microscopic studies are predominantly used [5, 13-24] for counting the number of *A. thiooxidans* bacteria. MPN method is a statistical method based on successive dilution and bacterial liquid cultures. The time length expended on this analysis is very important to its results. Microscopic method is based on counting a combination of live and dead microorganisms using Neobar Lam, which is not practical for effective removal of microorganisms from cement. Therefore, it is desirable to develop a solid medium for characterization and colony counting of the bacteria in the shortest time.

In this research, the composition of some liquid media without any components of cement and solid media for forming

perceptibility bacterial colony were investigated. Two strains of *A. thiooxidans* bacteria (PTCC 1692 and 1717) have been used to compare their growth and performance. In order to achieve the high required amount of biological sulfuric acid, which is the end-product of their metabolic activity in cement biodegradation process, various operating conditions such as temperature, method and rate of aeration were tested, and the rate of bacterial growth and reducing pH of the liquid culture were measured in a 4-liter Erlenmeyer flask and polypropylene bioreactor.

2. Materials and methods

2-1. Materials

The mineral salts for the preparation of culture media were purchased from Merck, Germany. Agar and agarose powders (solidifying agents for a liquid medium) were obtained from Liofilchem Co, Italy.

2-2. Microorganisms

A. thiooxidans PTCC 1692 and PTCC 1717 were purchased from Persian Type Culture Collection, Iranian Research Organization for Science and Technology (IROST). Optimum growth pH and temperature were between 3.5-4.5 and 25-30 °C, respectively. The microorganisms were cultivated in liquid medium containing 5 g/L of sodium thiosulfate (Y3 culture medium based on Table 1) and stored in the refrigerator (4 ^{o}C) and re-cultured every 2 to 3 weeks.

2-3. Liquid and solid culture media

In this research, 11 culture media were prepared for all the tests and their specifications are shown in Table 1. The first four media (Y1-Y4) were used in liquid medium, while all eleven culture media were prepared with 1.5% agar or 2% agarose as solid media. Each component of the medium was individually prepared and sterilized and the pH of medium was adjusted to about 4.5 after mixing the sterile materials.

Table 1. Components and mineral salts for preparation of culture media.

Chemical Materials (g/ L distilled water)	Culture Media Code										
	Y1	**Y2**	**Y3**	**Y4**	**Y5**	**Y6**	**Y7**	**Y8**	**Y9**	**Y10**	**Y11**
NH_4Cl	0	0.10	2.43	0	0	1	0.10	2.43	2.43	2.43	0
KCl	0.1	0	0	0	0	0	0	0	0	0	0
$MgCl_2.\ 6H_2O$	0	0.10	0.41	0	0	0.50	0.10	0.41	0.41	0.41	0
$FeSO_4$	0	0	0	0	0	0	0	0	0	0	0.01
$CaCl_2.\ 2H_2O$	0	0.14	0	0.25	0	0	0.25	0	0	0.25	0.25
$(NH_4)_2SO_4$	2	0	0	3	0.30	0	0	0	0	0	0.40
$MgSO_4.7H_2O$	0.25	0	0	0.50	0.50	0	0	0	0	0	0.50
K_2HPO_4	0.1	0	0	0	4	0.40	0	3	0	0	3
KH_2PO_4	0	3	3	3	1.50	0.60	3	3	3	3	0
$Na_2S_2O_3.5H_2O$	0	0	5	5	10	10	5	5	10	5	5
Sulfur powder	5	10	0	0	0	0	0	0	0	0	0
$FeCl_3$	0	0	0	0	0	0.02	0	0	0	0	0
Ref.	1717 PTCC	1692 PTCC	[19]		[23]	[6]	[2]	Current study			[1]

2-4. Microorganisms growth conditions in liquid cultivation medium

2-4-1. The preparation and amount of inoculum

A. thiooxidans strains were grown in Y3 media and used as inoculum in various liquid culture media. All liquid cultivations were inoculated with 2-day-old bacterial inocula at 1% v/v. Based on the fact that sodium thiosulfate produces sediments in acidic environment with pH < 3.5 [9], the acidity of the cultivation medium is considered to be very important as the amount of thiosulfate ions is high at the initial stage of the process. To avoid a sharp drop in the solution pH and sedimentation of sodium thiosulfate, the decreasing rate of the bacterial growth and its efficiency, the optimized volume of the inoculum was considered at 1% v/v. This went through thiosulfate quick path of reaction (3) instead of the pathways (1) or (2) which convert thiosulfate to tetrathionate and eventually sulfur and sulfate through which thiosulfate is formed again.

2-4-2. Growth curve experiments

The growth of bacteria was studied in a 100 mL-Erlenmeyer flask in a shaker incubator at 150 rpm and 30°C with the volume ratio of medium/flask of 1 to 5, with 1% v/v inoculum. These conditions were applied to determine the appropriate liquid medium and bacterial growth comparison in a variety of sulfur and thiosulfate media (Y1-Y4) using the experimental design given in Table 2. In order to compare the growth curves for each strain in the four different liquid media, several quantitative analyses were carried out.

Table 2. Test design and growth results of two bacteria strains in liquid medium containing sulfur and thiosulfate.

Sample Code	Culture Media	*A. thiooxidans* type strain	pH		OD_{600}
			Initial	Final	Final
1692-1	Y1	PTCC 1692	3.47	1.77	0.009
1692-2	Y2		4.30	1.60	0.233
1692-3	Y3		4.52	1.84	0.244
1692-4	Y4		4.58	1.91	0.290
1717-1	Y1	PTCC 1717	3.47	1.41	0.340
1717-2	Y2		4.30	1.34	0.494
1717-3	Y3		4.52	1.85	0.199
1717-4	Y4		4.58	1.96	0.158

2-4-3. Evaluation of growth rate of bacteria in different operating conditions

In order to have access to a large amount of biogenic sulfuric acid (2 L) in the shortest possible time for furtherfuture studies in cement biodegradation, the effect of temperature and various methods of aeration (alternately or continuously) on the growth rate were studied on *A. thiooxidans* PTCC 1717 with 1% v/v inoculated medium in the following manners:

a) A 3-L shake-flask and medium/flask volume ratio of 2 to 3 at 25°C (ambient temperature) by conventional mixing using a magnetic stirrer (sample code: E25), and a 4-L shake-flask with a medium/flask volume ratio of 1 to 2, in an orbital shaker incubator at 30°C, 150 rpm (sample code: S30)
b) A 4-L cubic polypropylene (PP) bioreactor (16 cm each side) with different operating conditions according to Table 3 with volume ratio of 1 to 2 for medium/bioreactor.

In order to determine the best condition for bacterial growth, the minimum required time was used as a benchmark to reach the pH of bacterial culture from 4.5 to 2 by variation in temperature, the inlet air rate and aeration method.

Table 3. The various operating conditions in bioreactor for growth of *A. thiooxidans* PTCC 1717.

Bioreactor Code	Temperature (°C)	Aeration Method	Aeration Rate (vvm)
A25	25	Alternate 1 min/day	1
A30	30		
C25	25	Continuous	0.5
C30	30		1

2-5. Methods of analysis

2-5-1. Quantitative methods

Methods of the optical density of the cell culture (OD_{600}), dry cell weight (DCW), colony counting of viable bacteria and the pH of the liquid culture were used for determining the bacterial growth curve.

OD_{600}: A UV–vis spectrophotometer (Metertech, SP8001model, Taiwan) was used at a wavelength of 600 nm to measure the turbidity of culture to correlate with the rate of bacterial growth.

DCW: Samples of bacterial cultivations were used for determination of dry cell weight. The samples were precipitated in a centrifuge (Megafuge1, Heraeus, Germany) with 4000 rpm for 15 min at 4°C. The wet cell mass was dried for 24 h at 70°C.

Bacterial colony counting: From each bacterial suspension sample, with a specific dilution, an amount of 100 µL was spread on a solid medium and incubated at 30°C. After a definite time, the number of colonies forming (CFU) per milliliter of sample (CFU/ml) were reported.

2-5-2. Qualitative method

Based on our observations, although *A. thiooxidans* grew on some solid medium, their colonies were invisible. One drop of reagent of bromophenol blue (0.01% w/v in distilled water) was used as an internal indicator for solid culture medium. This indicator is purple at pH > 4 and creamish at pH < 4, and it turns yellow with further lowered pH. The changes in the color of the culture medium with pH reduction are indications of bacterial growth, and were used to check the quality of bacterial growth.

3. Results and discussion

3-1. Determination of suitable liquid medium for biodegradation assessment of cement

The liquid culture media for bacterial growth such as *A. thiooxidans* PTCC 1692 and PTCC 1717 are proposed by IROST according to Table 1 (Y1, Y2), which contain sulfate, calcium ions and sulfur powder. In this study, the liquid medium for bacterial growth should have the following specifications:

- Due to the use of this type of bacteria for cement biodegradation tests, in future studies, it is necessary to avoid a liquid medium which contains those ions in common with the cement such as calcium, iron, aluminum and sulfate.
- The number of its salt components should be kept as low as possible to prepare a culture medium quickly and easily with proper bacterial growth.
- Good reproducibility to measure bacterial growth using OD_{600} method. The elemental sulfur should be removed from the culture medium due to its insolubility in aqueous solutions and adhesion to bacteria. Sodium thiosulfate is the accessible water-soluble sulfur compound and can provide the required energy for bacterial growth.
- Sufficient bacterial growth to study the

biodegradation of cement at pH below 2 as the final pH of the culture medium.

To maintain the above conditions for bacterial growth appropriate for *A. thiooxidans*, the culture medium Y3 is considered the most suitable among all others provided in Table 1. However, Y1, Y2 and Y4 media were also examined to compare the bacterial growth to be conducted in a sulfur and thiosulfate media.

The results of OD_{600} and pH of the tests after 10 days are given in Table 2. By comparing the results, it is found that bacteria exhibits more severe pH reduction and bacterial density increase in the proposed sulfur media (Y1: 1717-1 and Y2: 1692-2) compared with thiosulfate-containing cultures. Therefore *A. thiooxidans* bacteria consume elemental sulfur better than thiosulfate. However, the presence of sulfur creates problems such as non-reproducibility and inaccurate measuring of biomass density. On the other hand, in this study the lowering of the pH is more important than the bacterial biomass. As is evident in Table 2, the final pH of Y3 culture medium is comparable with Y1 or Y2 culture media for both strains of bacteria. Moreover, the thiosulphate concentration in Y3 (5 g/L) is equivalent to 21 mM and is the same as reported by Lors et al., [19] that the optimum concentration of thiosulphate ion is 20 mM in the culture medium for bacterial growth. On the whole and considering the above criteria, Y3 is the most suitable medium compared to other liquid media for the study of cement biodegradation by bacteria.

3-2. Determination of suitable solid medium for counting bacteria colonies

There are no limitations for growth of *A. thiooxidans* on solid medium, unlike liquid medium, which should be free of the ions in common with cement. The solid medium was only used to count the live bacteria colonies, as evaluation of the changes in live microorganisms is essential to trace cement biodegradation.

Based on literature review, the solid culture medium is not recommended for growth of *A. thiooxidans* and there is no expression to suggest employing such method in most published papers. Only a limited number of researchers [1, 3, 13] have reported on counting the bacterial colonies and therefore their recommended media (Y5, Y6, Y7) are evaluated in this study. Thus, the solid media were prepared from 11 sample salt culture media in Table 1 (Y1 to Y11) containing 1.5 % w/v agar (without chemical treatments).

After culturing the bacteria on all solid culture media, even after 30 days no visible colonies appeared. Contrary to the reported salt solid media, claiming to have bacterial colony [1, 3, 13], there was no result produced with untreated agar, which could be due to differences in the bacterial strains or impurities in the materials in different studies.

Although in some studies [12] agarose, as a solidifying agent, is recommended for its sulfur-containing compounds instead of agar for the same bacteria, in the present study Y3 and Y7 solid media containing 2% w/v agarose did not produce any promising result. Qualitative analysis of bacterial growth on Y3 and Y7 media, containing agar and agarose, was confirmed by bromophenol blue indicator which showed color changes in plates from purple to yellow after 20-30

days of bacterial culture (Fig. 1). This change of color implied acid production by the bacterial growth on agar plates but with no visible colony. Probably the amount of produced biomass was not sufficient for distinction of bacterial colonies. To confirm the growth of *A. thiooxidans* bacteria on agar plates, non-distinctive bacteria were grown in Y3 liquid medium and after 4 days showed good growth and the starting pH of 4.5 of the liquid culture dropped to 1.81.

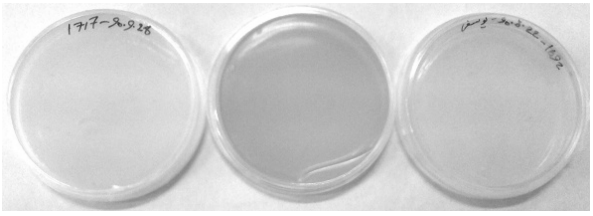

Figure 1. Change of solid media color containing bromophenol blue indicator with *A. thiooxidans* growth (from left to right plates containing: *A. thiooxidans* PTTC1717, control plate and PTTC 1692 strain).

Cream-colored colonies of two strains of bacteria were observed only on Y10 salt solid medium contain calcium chloride and 2% agarose. The colonies were visible on the 3rd day as bright spots, and on the 8th day it was possible to count the colonies (Fig. 2).

Culture medium compositions of Y10 are the same as Y3 but contain calcium ions. It should be noted that, the presence of calcium ions is essential for observation of *A. thiooxidans* colonies. Peng et al. reported that calcium ion is one of the components which leaves the bacterial cells during photocatalytic degradation of the cell [25]. This means that the calcium ion is one of the important components of bacterial cells and the presence of a significant amount of this substance is vital in the culture medium. Therefore, calcium chloride salt was used in the preparation of Y10 culture medium in which the bacterial colonies were detected in the presence of agarose. In addition, growth of *A. thiooxidans* PTCC 1717 in Y10 liquid medium resulted in pH drop (approx. 0.5 units) compared to Y3 liquid medium, and this result could be due to the presence of calcium ions and better growth of the bacteria.

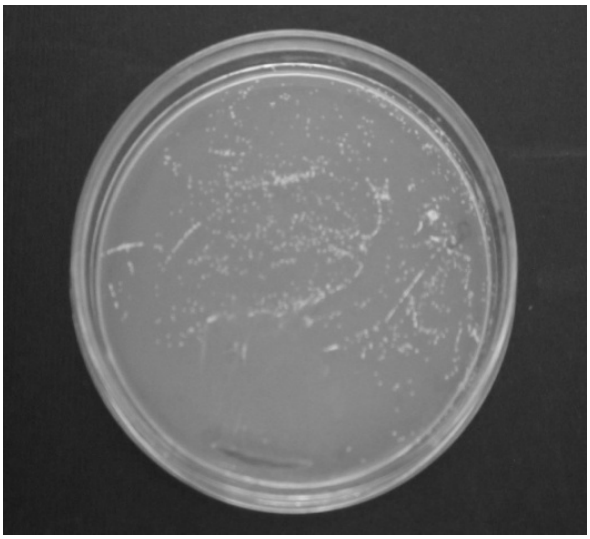

Figure 2. Visible colonies of *A. thiooxidans* PTCC 1717 on Y10 solid medium containing 2% w/v agarose.

3-3. Evaluation of bacterial growth in an optimized liquid medium

3-3-1. Bacterial growth curves

With selection of Y3 and agarose-containing Y10 culture media, as suitable liquid and solid respective media, the bacterial growth profile was studied in these culture media according to conditions provided in section 2-4-2. According to the results, the middle of log phase time for *A. thiooxidans* is 1.5 days. The growth curves of two different strains of *A. thiooxidans* are shown in Figs. (3) and (4).

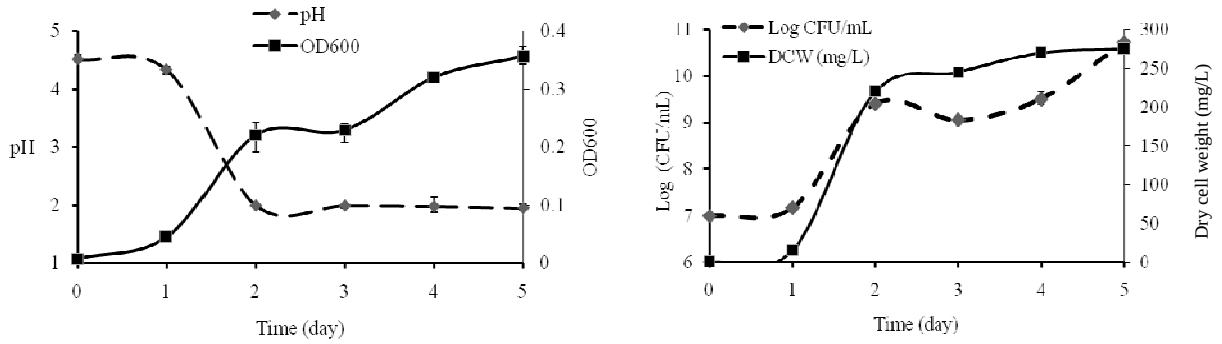

Figure 3. Growth curves of *A. thiooxidans* PTCC 1717 based on pH, OD_{600}, dry cell weight and colony counting.

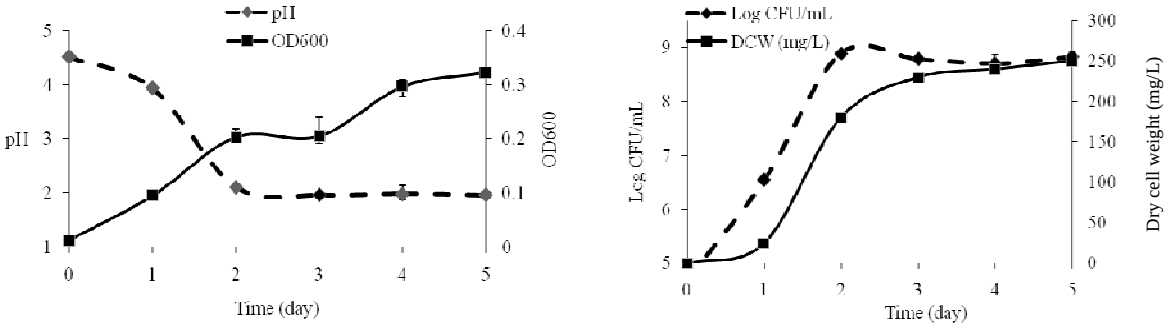

Figure 4. Growth curves of *A. thiooxidans* PTCC 1692 based on pH, OD_{600}, dry cell weight and colony counting.

By comparing these two Figures, it is clear that *A. thiooxidans* PTCC 1717 is slightly more active than PTCC 1692 strain, because the first strain has lower pH and more turbidity, greater biomass and higher numbers of colonies than the second strain under the same conditions at harvesting time. Therefore, in our later experiments, only *A. thiooxidans* PTCC 1717 growth was studied in different operating conditions.

3-3-2. Evaluation of bacterial growth in different processing conditions

A. thiooxidans PTCC 1717 growth was studied in 2 L of Y3 culture medium in each of two shake-flasks according to section 2-4-3-(a) and in the polypropylene bioreactor, under different conditions of temperature and aeration, according to section 2-4-3-(b) and Table 3. The following results are obtained:

a) Growth rate in shake-flask under variable temperature and aeration conditions

Due to the large amount of required bio-sulfuric acid (2 L), the microbial growth was tested in higher scale. Therefore, tests were carried out simultaneously in 3 L (E25) and 4 L (S30) flasks. Fig. 5 shows variations in pH and turbidity of bacteria growth versus time for these two flasks.

Comparing the growth curves in Figs. 3 and 5, it becomes evident that these methods display very slow growth rate. Due to increases in the scaled up culture medium in the flasks and different stirring methods for sample E25, the aeration rate and

consequently the dissolved amount of oxygen and carbon dioxide have changed in the culture medium for the aerobic autotrophic bacteria. Under these conditions, there is just a small quantity of oxygen available for the bacteria to oxidize thiosulfate and its further conversion to sulfuric acid. In E25 sample test the microbial culture loses its transparency over time and turns into white, cream and yellow color medium, and with time, yellow sulfur colloid particles begin to precipitate and accumulate at the bottom of the flask. These results are consistent with variations of turbidity displayed in Fig. 5. The maximum OD_{600} is 0.45, beyond which it appears to decrease.

At low aeration rate, the production of sulfuric acid does not directly emanate from thiosulfate and the reactions of (1) and (2) are taken by bacteria via a longer route of sulfur conversion into sulfuric acid. This would take 20 days, a longer time for pH of E25 sample to be reduced to 2 from the initial pH 4.5 of culture medium shown in Fig. 5.

In this figure, it is observed that in S30 test, the reduction of pH to 2 takes place by just over 6 days. On the whole, the S30 test is more efficient, due to higher temperature and higher aeration rate, but according to Fig. 3, there is still a long way to achieve higher speed and shorter time for conversion of thiosulfate to sulfate. So to speed up the conversion reaction of thiosulfate to sulfuric acid, a high oxygen concentration should be administered.

b) Growth under conditions of variable temperature and aeration rate in a 4-L cubic bioreactor

To attain high levels of dissolved oxygen, a polypropylene bioreactor by medium/ bioreactor volume ratio of 1:2, as given in section 2-4-3-b, was employed with various aeration speeds at two different temperatures (Table 3).

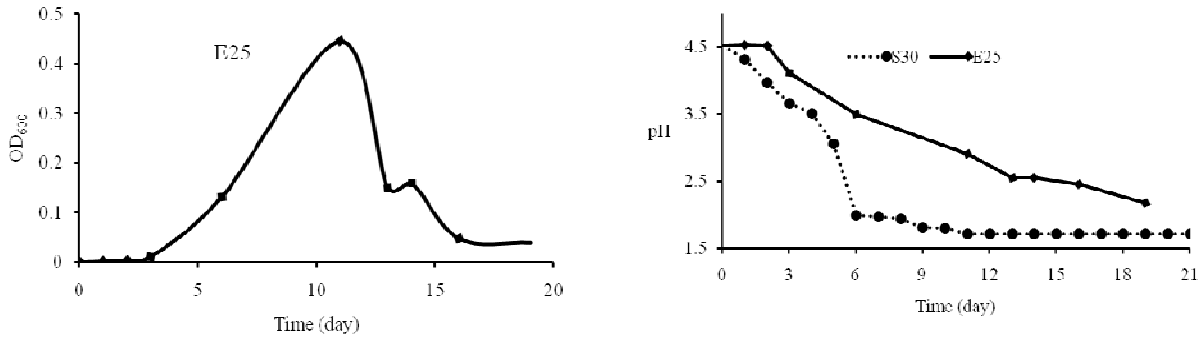

Figure 5. Growth curves of *A. thiooxidans* PTCC 1717 in two Erlenmeyer flasks, each containing 2 L culture medium (sample E25: medium/flask volume ratio: 2/3 at 25 °C (ambient temperature), mixed with magnetic stirrer, sample S30: medium/flask volume ratio: 1/2 at 30 °C in a shaker incubator and 150 rpm).

According to Fig. 6, it is evident that with a similar trend in alternate aeration, with increasing temperature from 25°C in A25 test to 30°C in A30 test, the time taken for the pH of the medium would take 12 days to reach below 2 from the 18 days of A25 test. Namely, there would be an enhancement of bacterial growth with increasing temperature to 30°C. Also, the growth rate is shown to be better in a 4-L bioreactor (A25 – Fig. 6), due to higher aeration rate and smaller medium content (E25 – Fig. 5). Daily alternate aeration i.e., one minute aeration intermittently is better than continuous stirring with a magnetic stirring. The microorganisms require more oxygen for growth to produce large quantities of sulfuric acid in a shorter time.

In continuation of the test, the mode of aeration from alternate A25 to continuous C25 tests and with increased aeration rate of 0.5 vvm for C25 to 1vvm for C30, an increase of specific growth rate was observed in the culture. As shown in Fig. 6, the shortest time to lower the pH to below 2 of a 2-L culture from its initial pH of 4.5 took 4 days for the C30 test (temperature 30°C with continuous aeration and speed 1 vvm).

The conditions are given in section 2-4-2 for a 20 mL culture where medium/flask volume ratio was 1:5 and it has taken 2 days to reduce the pH below 2. In C30 test the volume ratio of medium/flask was lowered to 1:2 for continuous aeration, for which the process was completed within 4 days. Therefore, the aeration of 1 vvm was not considered good enough. Because of limitations in increasing the inlet air velocity, it was not possible to change the aeration rate and to achieve a 2-L biogenic sulfuric acid. Therefore, the optimum aeration rate of 1 vvm, as the speed of inlet air, was considered acceptable.

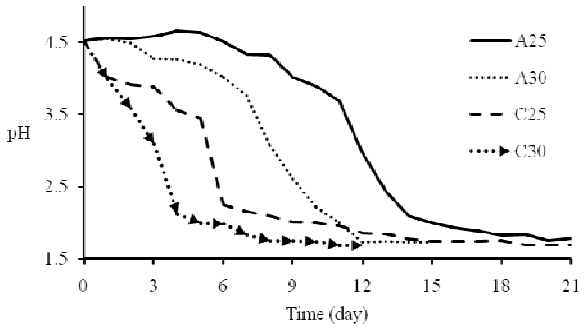

Figure 6. Growth curves of *A. thiooxidans* PTCC 1717 in operational conditions of Table 3.

4. Conclusions

Aerobic autotrophic *A. thiooxidans* bacteria grew slightly better in liquid medium containing elemental sulfur compared to a liquid medium containing thiosulfate, though the presence of sulfur in liquid medium caused reproducibility problems, turbidity and cell dry weight measurements. The liquid medium employed in this study, which did not contain ions in common with the cement, included thiosulfate ion in preference to sulfur powder. Both *A. thiooxidans* PTCC 1717 and 1692 strains of bacteria grew in this suitable medium with the reduction of pH to 2 after 2 days; a suitable pH for the study of biodegradation of the cement. Due to the large amount of needed bio-sulfuric acid for future studies on the biodegradation of cement specimens, scaling up of the tests was carried out in different environmental conditions and operations. Finally, the best conditions for the growth of *A. thiooxidans* bacteria in a 4-L cubic bioreactor were found to be a medium /bioreactor volume ratio of 1:2, aeration rate

of 1 vvm at 30°C. In this study, several salt solid media containing agar and agarose were tested to find a suitable medium for *A. thiooxidans* colony formation. Bacterial colonies were formed after 8 days on solid medium containing thiosulphate, calcium ions and agarose.

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