ORIGINAL ARTICLE

Isolation of *Thiobacillus* sp. for use in treatment of rubber sheet wastewater

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Abstract

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***Thiobacillus* spp. are extensively used worldwide for removal of both organic and inorganic sulphur compounds in wastewater; however, a little used in Thailand. Southern Thailand has numerous rubber factories producing wastewater containing high amounts of those compounds. Therefore, in this study, 4 *Thiobacillus* sp. were isolated from wastewaters of domestic and rubber factories. Each isolate grew both aerobically and anaerobically as both a chemolithotroph and chemoorganotroph. All isolates grew in a pH range from 2.0-7.0 with an optimum of 6.5 and in the temperature range of 25-45ºC with an optimum between 30-35ºC. After 14 days of incubation in rubber sheet wastewater *Thiobacillus* sp. WI 1 produced the highest removal of COD at 54%; but removal of BOD was only 33%. In contrast, strain WI 4 produced the highest BOD removal at 83% with removal of COD at only 46%.**

Key words : *Thiobacillus*, rubber wastewater, BOD, COD

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บทคัดย่อ

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การแยก *Thiobacillus* sp. เพื่อใช้บำบัดน้ำทิ้งจากการทำยางพาราแผ่น

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***Thiobacillus* spp. เป็นแบคทีเรียที่มีการนำมาใช้มากขึ้นเพื่อการบำบัดน้ำทิ้งจากแหล่งต่าง ๆ ที่อุดมด้วยสารอินทรีย์ซัลเฟอร์ และอนินทรีย์ซัลเฟอร์ แต่ในประเทศไทยการนำมาใช้ยังน้อยมาก ในภาคใต้ของประเทศมีโรงงานแปรรูปน้ำยางจำนวนมาก และน้ำเสียประกอบด้วยสารดังกล่าวอยู่สูง ดังนั้นในการศึกษานี้ได้แยกเชื้อดังกล่าว 4 ไอโซเลท จากน้ำเสียบ้านเรือนและโรงงานแปรรูปน้ำยางพารา ซึ่งสามารถเจริญได้ทั้งในสภาพที่มีอากาศและไร้อากาศ สามารถใช้แหล่งพลังงานและคาร์บอนทั้งจากสารอินทรีย์และอนินทรีย์ ทุกสายพันธุ์เจริญได้ดีในสภาวะเป็นกรดต่ำจนถึงเป็นกลาง (2.0-7.0) แต่ pH ที่เหมาะสมสำหรับทุกสายพันธุ์คือ 6.5 และเจริญได้ดีมากที่อุณหภูมิ 30-35°C โดยเชื้อสามารถเจริญได้พอสมควรที่ 25 และ 40 ถึง 45°C ส่วนผลการเจริญในน้ำทิ้งจากการทำยางแผ่นเป็นเวลา 14 วัน พบว่า *Thiobacillus* sp. WI 1 สามารถลดค่า COD ได้มากที่สุด (54%) แต่ลดค่า BOD ได้เพียง 33% ขณะที่เชื้อ WI 4 ลดค่า BOD ได้มากที่สุดคือ 83% แต่ลดค่า COD ได้เพียง 46%**

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Latex is processed into numerous forms of rubber materials such as sheet, block, skim-crape and concentrated latex prior to conversion into various rubber end products. These processes produce large amounts of wastewater that can contain large amounts of sulphur compounds such as sulphide (16.5 mg/l) and sulphate (375 mg/l) from rubber sheet factory. The amounts of these compounds depend on the processes used. Some use additives such as tetramethyl triuram disulphide (TMTD), sodium sulphide and sulphuric acid. Proteins are also present as components of the raw material (Gamgarn, 1996).

Anaerobic treatment is the most common method used in Thailand for treating rubber wastewater as it is an inexpensive treatment with high efficiency. However, this treatment results in the formation of hydrogen sulphide because sulphate-reducing bacteria use sulphate ion as an electron acceptor instead of oxygen. Hydrogen sulphide is toxic and gives rise to the objectional smell of rotten eggs. The gas also causes a big problem in biogas producing systems. Additional objectional odours arise from the degradation of rubber proteins (1-1.5% in natural latex rubber) producing organo-sulphur products such as dimethyl sulphide (DMS), dimethyl disulphide (DMDS), methyl mercaptan (MM) and hydrogen sulphide (H_2S).

In contrast, *Thiobacillus* sp. can use reduced inorganic sulfur compounds as an energy source (Kelly and Harrison, 1989) and are therefore used for removing sulphide from biogas systems and also from wastewater (Kim *et al.*, 2002; Kleerebezem and Mendez, 2002 and Martin *et al.*, 2002). Cha *et al.* (1999) have also studied the removal of organic sulphur compounds (ex. DMS, DMDS and MM) in wastewater using *T. novellus*. It is interesting that some species of *Thiobacillus* are obligately chemolithotrophic (microorganisms that obtain energy through chemical oxidation and use inorganic compounds as electron donors and cellular carbon from the reduction of carbon dioxide; also known as chemoautotrophs), while others are also able to grow chemoorganotrophic (organisms that obtain energy from the oxidation of organic compounds and cellular carbon from preformed organic compounds). To date, in Thailand, there has been little work on the use of *Thiobacillus* sp. in wastewater treatment and no reports on their microbial isolation from waste-water treatment facilities. Hence, the aims of this study were firstly to isolate *Thiobacillus* sp. from wastewater in local areas of Songkhla province and nearby and, secondly, to investigate their possibility for use in the treatment of rubber wastewater.

Materials and Methods

1. Isolation of *Thiobacillus* sp.

Twenty wastewater samples were collected from various sources of wastewater such as domestic and rubber factories (ex. Rubber sheet, rubber block and concentrated rubber) in Songkhla province and nearby.

Isolation medium was *Thiobacillus* mineral salts medium (MSM) which was composed of 2.0g KNO_3, 1.0g NH_4Cl, 2.0g KH_2PO_4, 2.0g $NaHCO_3$, 0.8 g $MgSO_4.7H_2O$, 5.0 g $Na_2S_2O_3.5H_2O$ and 1.0 ml trace element in 1000 ml distilled water and the pH was adjusted to 6 with 1N KOH. The composition of trace element as follows: 50g Na_2-EDTA, 7.34g $CaCl_2.2H_2O$, 5.0g $FeSO_4.7H_2O$, 2.5g $MnCl_2.4H_2O$, 2.2g $ZnSO_4.7H_2O$, 0.5g $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.2g $CaSO_4.5H_2O$ and 11.0g NaOH dissolved in 1000ml of distilled water (DSMZ, 2002). And agar of 15 g was added to solidly the medium.

To isolate *Thiobacillus*, 1ml of wastewater sample was inoculated into 10 ml of the isolation medium in a test tube using aseptic technique. These tubes were incubated at 35ºC without shaking for 14 days. The development of turbidity in the medium was assumed to be due to microbial growth. Turbid samples were streaked onto solid medium, incubated under the same conditions and single colonies further purified using two or three successive streak plate dilutions. The Gram stain was used to monitor the purification process and isolated strains were further assessed for their morphological and physiological properties as details in Table 1.

2. Chemoorganotrophic growth

The glucose *Thiobacillus* medium used to test for chemoorganotrophic growth contained no initial source of reduced sulphur compounds, 10g glucose, 3g $(NH_4)_2SO_4$, 0.50g KH_2PO_4, 1.00g $MgSO_4.7H_2O$, 0.10g KCl, 18.00mg $Ca(NO_3)_2.4H_2O$ and 0.01mg $FeSO_4.7H_2O$ in 1000 ml distilled water and the pH was adjusted to 6 with 1N H_2SO_4 (DSMZ, 2002). One loopful (2 mm diameter) from slant culture of each isolate was inoculated into 10 ml of the medium and incubated at 35ºC with static condition. In parallel tests, the isolated bacterial strains were also inoculated into the MSM medium and incubated under the same condition.

3. Effects of physico-chemical properties on bacterial growth

As growth of the isolated strains was best observed with Glucose *Thiobacillus* medium (DSMZ, 2002), this medium was chosen to study the effects on growth of the following changes to growth conditions:

a. Temperatures of 25, 30, 35, 40 and 45ºC

b. pH's of 2.0, 3.0, 4.0, 4.5, 5.5, 6.5 and 7.0 with the cultures incubated at 35ºC without shaking

c. Effect of oxygen and agitation in the medium at pH 4.0 and incubation at 35ºC as follows: Static conditions, agitation at 100 and 200 rpm with incubator shaker and anaerobic conditions, with a 1 cm layer of liquid paraffin added to the top of the growth medium held in tubes and with no shaking.

Bacterial growth was measured by either turbidity with a spectrophotometer wavelength at 600 nm or by dry weight determinations when cells clumped, such as when grown with high acidity (pH 2-4). For cell dry weights, culture medium was centrifuged at 6000 rpm, for 15 min to separate cells and sedimented material was heated at 105ºC in a hot air oven for 24 hr or until obtaining constant weight (Noparatnaraporn *et al*., 1980).

4. Biochemical tests for identification

The bacteria were characterised using morphology and biochemical tests according to Bergey's Manual of Systematic Bacteriology, vol. 3 (Kelly and Harrison, 1989). Biochemical tests used were: catalase, oxidase, urease, MR (methyl red), VP (Voges-Proskauer), H_2S production,

Table 1. Properties of strains of *Thiobacillus* sp. isolated from local sources of wastewater

Property	**WI1**	**WI2**	**WI3**	**WI4**
Source of isolation	Rubber wastewater	Domestic wastewater	Rubber wastewater	Rubber wastewater
Colony	Irregular, grey	Circular, raised, grey	Circular, raised, white	Circular, flat, grey
Gram stain	Negative	Negative	Negative	Negative
Cell (size)	Rod (0.5x1.0 µm)	Rod (0.5x1.0 µm)	Rod (0.5x1.0 µm)	Rod (0.5x1.0 µm)
Arrangement	Short chain	Single	Pair	Single
Aerobic growth	+++	+++	+++	+++
Anaerobic growth	++	++	++	++
Chemolithotroph (thiosulphate)	++	++	++	++
Chemoorganotroph (glucose)	+++	+++	+++	+++
Growth at pH 2.0	+	++	++	++
Growth at pH 3.0	++	++	++	++
Growth at pH 4.0	++	++	++	++
Growth at pH 4.5	++	++	++	++
Growth at pH 5.5	++	++	+++	++
Growth at pH 6.5	+++	+++	+++	+++
Growth at pH 7.0	++	++	++	++
Growth at 25ºC	++	++	++	++
Growth at 30ºC	+++	+++	+++	+++
Growth at 35ºC	+++	+++	+++	+++
Growth at 40ºC	++	++	++	++
Growth at 45ºC	++	+	++	++

+ = growth (Dry weight: 0.02-0.04 g/l or OD_{600}: <0.5); ++ = good growth (Dry weight: >0.04 g/l or OD_{600}: >0.5-1.0); +++ = very good growth (OD_{600}: >1.0)

glucose (acid-gas) and utilisation of thiosulphate, citrate, starch, arginine, ornithine, lysine, sucrose, lactose and maltose. The methods are those described in Principles of Biochemical tests in Diagnostic Microbiology (Blazevic and Ederer, 1975). The medium used was the glucose *Thiobacillus* medium except for situations when testing for carbon sources other than glucose.

5. Bacterial growth in wastewater from rubber-sheet manufacturing process

Bacterial strains used for this purpose were isolates *Thiobacillus* sp. WI1, WI2 and WI4. Isolate WI 3 was not used due to its poor growth (preliminary test). Preparation of inoculum: The selected strains were grown in glucose *Thiobacillus* medium under shaking at 100 rpm at 35ºC for 7 days. The wastewater was collected from a rubber sheet processing plant in Khlong Hoi Khong district, Songkhla province. Its relevant properties were: BOD of 3,360 mg/l, COD of 6018 mg/l, 448 mg/l phosphate, 180 mg/l nitrate, 95 mg/l ammonium and 0.42 mg/l hydrogen sulphide with a pH 5.32. Colorimetric method was used for determinations of phosphate, nitrate, ammonium and hydrogen sulphide. Prior to use the wastewater was centrifuged at 6,000 rpm for 15 min to separate suspended solids, and the supernatant was sterilised by autoclave, therefore the treated wastewater had a lower amount of BOD as 1628 mg/l due to removal of suspended solids but its COD received the same. The 100 ml amounts in 250 ml Erlenmeyer flasks were inoculated with 10 ml of each inoculum and all flasks, including a non-inoculated control, were incubated at 35ºC with shaking at 100 rpm. Parameters measured were: BOD, COD, and pH. Cell growth was monitored at the start of the experiment (t = 0 day) and at the end of the experiment (t =14 days). The pH was measured with a pH meter and bacterial growth was measured as dry weight. The supernatant from the culture medium centrifuged to separate cells for dry weight determinations was used to determine BOD and COD. BOD was measured using the Azide modification method and COD using the Dichromate reflux method following the procedures described in the APHA (1998).

Results

1. Isolation of *Thiobacillus* sp.

Only 4 isolates with distinct colony morphology were obtained from *Thiobacillus* mineral salts medium (MSM), each consisting of small rod-shaped cells 0.5-1.0 µm and Gram-negative (Table 1). The isolated strains were recognised as *Thiobacillus* sp. because of their ability to use thiosulphate as a sole source of energy. In addition, all isolates could use carbon dioxide as a sole source of carbon. Hence, the results show all isolates were capable of chemolithotrophic growth.

2. Chemoorganotrophic growth

After 14 days of incubation on solid glucose *Thiobacillus* medium, much larger colonies of all isolated strains were observed than with MSM after 21 days incubation (data not shown). Therefore, all isolated strains grew better with the glucose *Thiobacillus* medium (only glucose as a source of carbon) than with MSM. That means the isolates were also able to grow as chemoorganotroph (heterotroph). According to these properties the isolates exhibited both chemoorganotrophic and chemolithotrophic growth. Hence, the isolates could be classified as facultative chemolithotrophs.

3. Effects of physico-chemical properties on growth of isolated strains

a. Effect of temperature

All isolated strains grew at all temperatures investigated (25-45ºC), the optimal growth was between 30- 35ºC. Growth at 40ºC was a little higher than that at 45ºC, except for strain WI2 that showed poor growth at 45ºC (Table 1). These results indicate that the isolated strains were mesophilic bacteria.

b. Effect of pH

All isolated strains grew in the pH range of 2-7 with an optimum pH of 6.5 (Table 1). Based on these results the isolated strains were aciduric mesophilic bacteria.

c. Effect of oxygen and agitation

All isolated strains grew with both aerobic and anaerobic conditions. The results (Table 1) indicate that the isolated strains were facultative anaerobes. However, the bacteria grew better with aerobic conditions but the differences observed with different levels of aeration such as the use of static conditions or agitation at speeds of 100 or 200 rpm was not significant as shown in Table 1 (aerobic growth).

4. Biochemical test properties

All isolated strains were oxidase negative and catalase positive except for strain WI3 (Table 2). This, together with the finding that they were facultative chemolithotrophs, indicates that all isolated strains of *Thiobacillus* belonged to one of the following species: *novellas, versutus, acidophilus, intermedius, perometabolis* and *delicotus* (Kelly and Harrison, 1989). Based on biochemical test results, the strains WI1 and WI2 should be identical species, whilst the strains WI3 and WI4 were slightly different. For instance, glucose was fermented with gas production except that the strain WI3 produced only acid with no gas.

Table 2. Biochemical tests of strains of *Thiobacillus* sp. isolated from local wastewaters.

Test	WI 1	WI 2	WI 3	WI 4
Catalase	+	+	-	+
Oxidase	-	-	-	-
Thiosulphate	+	+	+	+
Glucose	AG	AG	A	AG
Hydrolysis of starch	+	+	+	+
Citrate	+	+	+	+
Indole	-	-	-	-
Methyl red	+	+	+	+
Voges-Proskauer	-	-	-	-
H_2S production	A	A	A	A(H_2S)
Urease	+	+	+	-
Arginine	-	-	-	-
Ornithine	-	-	+	+
Lysine	-	-	-	-
Maltose	+	+	+	+
Sucrose	+	+	+	+
Lactose	+	+	+	+

Based on information in both tables identification followed the classification of Kelly and Harrison (1989). All isolates grew under both aerobic and anaerobic conditions (facultative anaerobe), thereby they were not *T. novellus* because this species is obligate aerobe. Although the isolates were close to *T. versutus*, their growth compared to *T. versutus* was slow on thiosulphate as chemolithotroph and considerably stimulated by organic substrates such as glucose. Moreover, the isolates were also not *T. acidophilus* owing to this species being strictly aerobic and unable to grow at pH 7.0. With their slow growth on thiosulphate as chemolithotrophs the isolates could be *T. intermedius, T. perometabolis* and *T. delicatus*. However, *T. delicatus* is non-motile and cannot grow in media with single carbon substrate unless supplemented with thiosulphate. Consequently, the isolated strains could either be *T. intermedius* or *T. perometabolis*.

5. Growth in rubber-sheet wastewater

All isolated strains, except WI 3 due to its poor growth as early stated, incubated for 14 days in rubber-sheet wastewater medium, produced an increase in pH from the initial value of 5.32 to between 8.5-9.07. The uninoculated control showed no change (Figure 1). The bacterial growth based on dry weight determinations was almost identical for all strains (6.44-6.86 g/l) and the pattern of pH change was also similar (Figure 1). This might indicate that the factors limiting growth, whether organic, inorganic or physio-chemical, were the same for all cultures. However, according to the measurements of COD and BOD of the wastewater medium after 14 days incubation there were significant differences between strains. *Thiobacillus* sp. WI1 had the highest removal of COD (54%) following by WI4 and WI2 with 46 and 35%, respectively (Figure 2). In contrast strain WI4 achieved the best reduction of BOD (83%), with 40% removal by strain WI2 and 33% by strain WI1. No significant change in BOD and COD occurred during 14 days of incubation in the control set (uninoculated wastewater medium), thereby in Figure 2 only the set of treatments are shown.

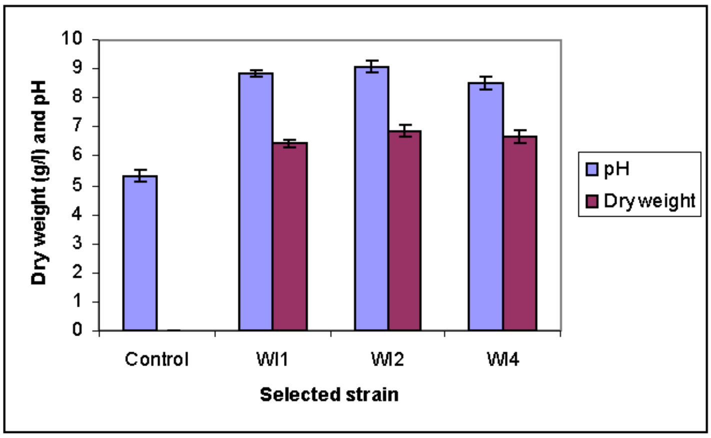

Figure 1. Growth of isolated strains of *Thiobacillus* sp. and changes of pH in rubber sheet wastewater medium during a 14 days incubation period.

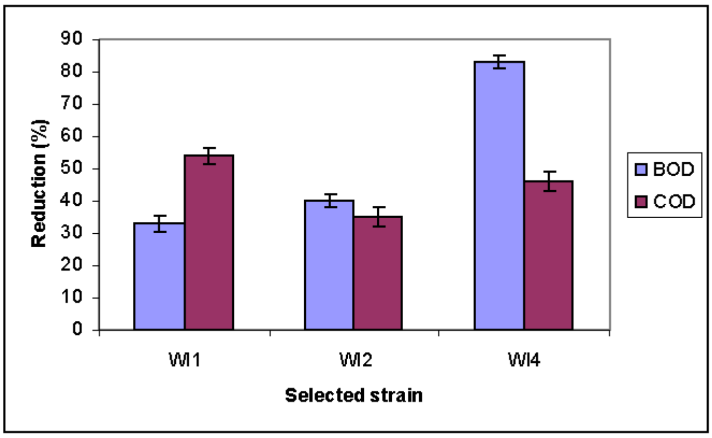

Figure 2. Reduction of BOD and COD after 14 days incubation of isolated *Thiobacillus* sp. in rubber sheet wastewater medium.

Discussion

The *Thiobacillus* MSM medium used for the isolation process consists of only mineral salts with thiosulphate ($S_2O_3^{2-}$) but no with addition of organic compounds. This means that the bacterial growth in this medium is chemolithotrophic using thiosulphate as the energy source and CO_2 as the sole source of carbon. Both ammonium (NH_4^+) and nitrate (NO_3^-) were present as a nitrogen source. Wastewater collected from a rubber sheet manufacturing process contained reduced inorganic sulphur compounds, organic compounds and both ammonium and nitrate. Therefore, the rubber sheet wastewater would be suitable for isolates of *Thiobacillus* sp.

All isolated strains also grew better with a medium containing no initial source of reduced sulphur compounds with organic compounds as a potential source of carbon and energy and were therefore chemoorganotrophic. They can be classified as facultative chemolithotrophic *Thiobacillus* species. The recognised *Thiobacillus* species with similar properties are *Thiobacillus intermedius*, and *T. perometabolis* (Kelly and Harrison, 1989). However, more investigations are required such as growth in mercury, tellurite, casein hydrolysate, L-glutamate, malate arabinose before species identification can be specific. The investigations should also include tests to identify the Coenzyme Q, fatty acids and rRNA gene composition. It is interesting that all isolated strains utilised starch and sugars such as glucose, maltose, sucrose and lactose including citrate as a sole source of carbon.

All isolated strains grew in the temperature range from 25 to 45ºC with an optimum of between 30-35ºC and a pH range from 2.0 to 7.0 with an optimum of pH 6.5. This is compatible with a potential for use in wastewater treatment. The bacteria were facultative anaerobes but increasing the availability of oxygen in the organic based medium by changing from static growth conditions to agitation up to 200 rpm had no significant effect on their growth. The oxygen levels in wastewater are likely to remain low. These results confirm that the bacteria had potential for use in a variety of sources and system of wastewater treatments, particularly those arising from various processes of rubber factories.

The rubber sheet wastewater used to test for the potential of the isolated strains had a pH of 5.32. This is probably due to the presence of nitrate, hydrogen sulphide and phosphate together with formic acid, which is used in the rubber sheet process (personal communication). Based on the observed growth characteristics the wastewater medium provided contains suitable nutrients to allow for growth of the isolated strains. The increase in pH observed is probably due to the removal of nitrate, phosphate and may be even formate. Previous work (Kelly and Harrison, 1989; Nugroho *et al*., 2002) with *Thiobacillus* sp. has shown a significant increase in pH when nitrate was a major component of the medium. Some *Thiobacillus* sp. are also known to cause denitrification using nitrate for anaerobic respiration. Hence one of our future studies will focus on the ability of our selected strains to remove high amounts of nitrate from wastewater. Further work is also required to investigate the processes involved in the removal of sulphide, phosphate and formate. Hydrogen sulphide can be used as a source of energy for *Thiobacillus* spp. (Kelly and Harrison, 1989; Holt *et al*., 1994). *Thiobacilus novellas* and *T. versutus* are known to utilize formate ((Kelly and Harrison, 1989).

The isolated strain WI4 had the greatest ability to use organic compounds in the rubber sheet wastewater medium catalysing a reduction of BOD of 83%. This strain was also able to utilise a wide range of organic compounds (Table 2) including ornithine and produced hydrogen sulphide in TSI medium. The other strains could not utilise ornithine and did not produce hydrogen sulphide. Strain WI4 is therefore the preferred strain for removing organic compounds from rubber sheet wastewater.

In contrast isolated strain WI1 tended to utilize inorganic compounds as this strain produced the highest COD reduction of 54%. The source of both these strains was rubber wastewater, so it is possible that they will be ideally suited for use in the treatment of rubber wastewater that has a rather different composition from other sources of wastewater. It will be interesting to investigate the possible benefits of using a mixed culture of both strains W14 and W11 in the treatment of wastewater from a rubber sheet process.

Conclusion

Our results indicate that *Thiobacillus* sp. isolated from a variety sources of wastewater show promise as microbial inoculants to treat wastewater from the rubber sheet process. Their ability to remove both organic and inorganic forms of sulphur needs to be confirmed. In addition, their use in the treatment of others source of wastewater from rubber factories such as rubber block need to be investigated.

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