

Ravindra Pogaku · Awang Bono
Christopher Chu *Editors*

Developments in Sustainable Chemical and Bioprocess Technology

 Springer

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Identification of Tetrathionate Hydrolase from *Thiobacillus Ferrooxidans*: An Enzyme Responsible for Enzymatic Devulcanization of Waste Rubber Products

M. D. Azratul and Y. Faridah

Abstract Malaysia is one of the largest producers of rubber and as a result produces million tonnes of vulcanized rubber waste from the rubber-based industries each year. Currently, less than 13 % of the waste tires generated annually are recycled by any means since the processes are costly and very inefficient. In the tire sector, many recycling methods have been adopted such as converting them into other useful rubber-based products, burnt as fuel or retreaded. Retreading is carried out via devulcanization process. The methods of devulcanizations adopted are chemical, mechanical, ultrasonic, microwave, and microbial or more specifically, enzymatic. However, none of the techniques previously developed have been proven to be commercially successful. Enzymatic devulcanization is one of the newest techniques to prepare waste rubber products for recycling. The process is deemed to be more efficient and less expensive compared to other devulcanization methods. Microbial devulcanization using *Thiobacillus ferrooxidans* provides some avenue of solving the problems associated with the other conventional processes. This microbe secretes an enzyme to utilize the elemental or organic sulfur together with iron available in the environment for its growth. The proposed enzyme, tetrathionate hydrolase, is responsible for degrading the sulfur cross-links in the devulcanized rubber rendering it to be more acceptable for recycling. The objective of the study is to identify secreted tetrathionate hydrolase which is responsible for devulcanization of rubber waste products. The condition needs to promote the growth of bacteria is by incubating the bacteria at 25 °C, initial pH buffer of 4, and at an agitation speed of 125 rpm,

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with salt concentration as follows: in (g/l) of $\text{KH}_2\text{PO}_4 = 4$ g, $(\text{NH}_4)_2\text{SO}_4 = 4$ g, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} = 0.3$ g. After preparing the initial sample, it was found that the specific activity of extracellular and intracellular tetrathionate hydrolase occurred on Day 1 and 2 is $0.070 \mu\text{mol min}^{-1}\text{mg}^{-1}(\text{U/mg})$ and $0.274 \mu\text{mol min}^{-1}\text{mg}^{-1}(\text{U/mg})$, respectively, at pH 4, 25 °C. Future work includes reaction of purified tetrathionate hydrolase with used rubber to observe the devulcanization process. This work is important mainly because it is an environmental friendly and safe method of devulcanization and with thorough study, it is hoped that the objective to recycle higher percentage of waste rubber can be achieved.

Keywords Enzymatic devulcanization • *Thiobacillus ferrooxidans* • Tetrathionate hydrolase

Introduction

After Thailand and Indonesia, Malaysia is the largest producer of rubber and has greatly contributed to the expansion of rubber technology in the present world. The technology of rubber creates a variety range of products such as rubber gloves, storage tanks, and insulation of electrical devices; however, the most important one is its usage in tire industries. The advancement of tire industries started in 1839 when Charles Goodyear discovered that by boiling crude rubber along with sulfur in solution produced an improved rubber material (Lewin 2004) which results in the production of vulcanized rubber tires. The advantages are good tensile strength, resistance to temperature changes and organic solvents as well as extreme elasticity. However, after these tires are no longer in use, they will be disposed to the environment. The biggest challenges are when the discarded vulcanized rubber tires are not biodegradable due to their sulfur cross-linking and have become one of the major sources of pollution. Disposal of waste tires and its recycling give a big task to mankind because the accumulation of discarded waste tires leads to environmental pollution (Gulzad 2011).

To date, there is no environmentally sound and economically viable method to recycle or dispose-off the vulcanized scrap tires. The only environmentally sound way to dispose vulcanized scrap tires in large quantity is to burn it in the cement kiln (E-Waste Management 2008). Vulcanized scrap tires virtually indestructible if left in the dump site as it cannot biodegrade even in 50 years. Environmental laws had forbidden the burning of scrap tires because burning tires emits a lot of black smoke and harmful chemicals. Dump site for scrap tire heaps is space consuming, fire hazard and health hazards due to mosquito breeding (U.S. Environmental Protection Agency 1993).

Therefore, there is an urgent need to search for a safe recycling method to reduce the scrap tire threats. One of the methods is retreading the tires which it is carried out via devulcanization process which comprises of chemical, mechanical,

ultrasonic, microwave, and microbial or enzymatic methods. Many researchers have attempted to discover the most effective way of recycling scrap tires in which they focus on the usage of bacteria to degrade rubber. This study is also known as microbial or enzymatic devulcanization. According to Romine et al. (1997), enzymatic devulcanization happened when a surface of a vulcanized rubber particle is exposed to at least one enzyme, maintaining the exposure for a time sufficient to convert sulfur to sulfoxide or sulfone, and halting conversion or preventing further degradation of the sulfoxide or sulfone. These two products are reactive, thus it is important to stop the reaction once sulfoxide and sulfone have been produced.

The manipulation of bacteria strains in devulcanization process will offer an environmentally and economically effective method of waste tires recycling. The main agents, namely the bacteria from the genus *Acidithiobacillus* species, have unique properties to catalyze aerobic oxidation of sulfide. These bacteria have been used in many decades in biomining industry for recovery of metals from sulfidic low grade ores and concentrates. *Thiobacillus ferrooxidans* are obligately aerobic, gram-negative, chemoautotrophic organism that generates its energy and reducing power for CO₂ fixation from the oxidation of inorganic iron and reduced sulfur compounds (Ingledew 1982). This microbe has the ability to secrete an enzyme known as tetrathionate hydrolase which is responsible to oxidize organic sulfur with iron that is available in the environment (Rawlings 2005). In addition, this enzyme is able to break the sulfur cross-link in vulcanized rubber which results in the tires being recyclable.

The objective of the study is to identify the secreted tetrathionate hydrolase by *T. ferrooxidans* which is responsible for devulcanization of rubber. This work is based on the previous work where the salt concentration as well as several process conditions such as temperature, initial pH of buffer, and agitation speed for the bacteria had been optimized by Yusof and Ahmad (2010) to promote higher growth of bacteria.

Methodology

Materials and Methods

Propagation of Bacteria

T. ferrooxidans (ATCC® 19859™) was secured from American type culture collection (ATCC), USA. These bacteria were first propagated to increase its amount using the method provided by ATCC, ATCC® MEDIA. The growths of the bacteria were observed within 1–2 weeks, when yellow–orange iron deposited.

Cultivation of Bacteria

T. ferrooxidans was cultivated in a sulfur-based media containing 4.0 g KH_2PO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.0 g $(\text{NH}_4)_2\text{SO}_4$, 0.25 g CaCl_2 , and 5.0 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in 1.0 L distilled water. The medium was prepared without thiosulphate. The pH was adjusted to 4 and autoclaved at 121 °C for 15 min. Thiosulphate was filter sterilized and aseptically added after autoclaving. After that 100 ml of sulfur medium was transferred into several shake flasks. 1.0 ml of propagated bacteria was aseptically withdrawn and added to the 100 ml of sulfur medium.

Finally, all the shake flasks were placed in incubator at 25 °C and shaken at a speed of 125 rpm. All the shake flasks were examined every day to monitor the qualitative tetrathionate hydrolase activity for 14 days. 1 ml of each sample were collected, everyday, during the 14 days and stored at −20 °C for further analysis.

Analytical Analysis

Optical Density

200 µl of each cultivation sample was transferred into microtiter plate for optical density (OD) by using microplate reader. All samples were analyzed at 440 nm every day from Day 1 to Day 14.

Protein Assay and Protein Content Determination

The protein assay was conducted based on Bradford method (1976). Bovine serum albumin (BSA) was used in estimating the protein concentration using the standard curve.

The analysis of protein content was done on extracellular and intracellular protein. A 1 ml of sample was transferred from shake flask into 2.0 ml centrifuge tube from Day 1 until Day 14. The sample was centrifuged at room temperature at 5,000 g for 30 min producing pellet and supernatant. Then, the supernatant was transferred into another 2.0 ml centrifuge tube. The first supernatant was analyzed as extracellular protein. 100 µl of protein sample was withdrawn and mixed with 1 ml of Bradford solution. OD value of the supernatant was measured at 595 nm (Bradford 1976). For control, sample will be replaced by media without inoculum along with 1 ml of Bradford solution.

For intracellular protein, the pellet was added with 1 ml culturing media. Next, this sample was sonicated at 50 % amplitude and 30 s break in 2 min. After that, 2–3 drops of ethanol 95 % was pipetted into the centrifuge tube to stop the reaction. Again, the sample was centrifuged at room temperature at 5,000 g for

30 min. The pellet was discarded and 100 μ l of protein supernatant was withdrawn and mixed with 1 ml Bradford solution. OD value of the supernatant was measured at 595 nm (Bradford 1976). For control, sample was replaced by media without inoculum along with 1 ml of Bradford solution. Lastly, protein concentrations were determined from the extrapolation of the standard curve of BSA.

Tetrathionate Hydrolase Assay

Tetrathionate analysis was done based on de Jong et al. (1997) method. This analysis was conducted on extracellular enzyme and intracellular enzyme. 1 ml of sample from shake flask was transferred into 2.0 ml centrifuge tube. The sample was centrifuged at room temperature at 5,000 g for 30 min. The first supernatant was transferred into another 2.0 ml centrifuge tube and analyzed as extracellular enzyme. For intracellular enzyme, the pellet was collected and added with 1 ml culturing media. The sample was sonicated at 50 % amplitude and 30 s break in 2 min. Next, about 2–3 drops of ethanol 95 % was added to stop the reaction at room temperature and finally, the sample was centrifuged at room temperature at 5,000 g for 30 min.

Enzyme activity was measured by addition of 40 μ l 50 mM potassium tetrathionate ($K_2S_4O_6$) into 1,300 μ l 1.5 M ammonium sulfate. 500 μ l of enzyme sample was added with 160 μ l distilled water. The sample was mixed thoroughly and incubated for 10 min at 30 °C. Then, 5 μ l 1 M NaOH was added to stop the reaction. The absorbance was measured using UV-spectrometer set at 290 nm. Control sample was prepared by replacing the enzyme sample with media without inoculation. The remaining samples were stored at –20 °C.

Results and Discussions

Absorbance at 440 nm for the Growth of T. ferrooxidans

The growth rate of *T. ferrooxidans* is influenced by several factors such as media composition, temperature, pH, oxygen supply, and the mixing times. The highest growth rate of *T. ferrooxidans* was on Day 1. The bacteria were well supplemented with the elemental sulfur in the medium that serves as the energy source. Ammonium sulfate serves as the nitrogen source while calcium, ferric chloride, and magnesium sulfate supply inorganic ions. Potassium dihydrogen phosphate buffers the medium against pH change. Even though *T. ferrooxidans* has a pH optimum of about 1.5, it also grew well at pH 4 (de Jong et al. 1997).

Protein Content Determination

Extracellular and intracellular protein secretion and accumulation were determined based on the extrapolation of BSA standard curve. All protein contents were calculated from Day 1 to Day 14. The highest extracellular protein content occurred on Day 11 was 9.814 µg/ml. For the intracellular protein content, Day 6 gave the highest value at 10.593 µg/ml.

Tetrathionate Hydrolase Activity

Tetrathionate hydrolase is a periplasmatic enzyme with pH optimum below 2.5. Based on the optimized condition by Yusof and Ahmad (2010), the pH was done at 4 and it was found in this work the highest production of extracellular and intracellular of Tetrathionate hydrolase was at Day 6 and 5, respectively. The enzyme activity had been measured by calculating the changes in absorbance reading divided by minute and total enzyme in ml at 25 °C.

For example,

$$\text{Activity of enzyme} = \frac{\Delta A}{\text{min. } 0.5 \text{ ml}}$$

The reading of time was taken at 1 min. The maximum activity of extracellular and intracellular enzyme was found to be 0.0920 µmol min⁻¹/ml (U/ml) and 0.268 µmol min⁻¹/ml (U/ml), respectively. It had also been proven by de Jong et al. (1997) in their work where maximum tetrathionate hydrolyzing activity by *T. ferrooxidans* occurred at pH 4.

Sugio et al. (2009) mentioned in their work that tetrathionate is a reduced sulfur compound soluble and stable in the water acidified with sulfuric acid that is easily used by *T. ferrooxidans* cells as energy source. In *T. ferrooxidans*, thiosulfate is oxidized through the S4-intermediary pathway (Pronk et al. 1990; Kelly et al. 1997; Ghosh and Dam 2009). During this reaction, two molecules of thiosulfate undergo condensative oxidation to produce four sulfur atoms intermediate tetrathionate catalyzed by thiosulfate dehydrogenase. In the second step of this pathway, tetrathionate was hydrolyzed by tetrathionate hydrolase to produce sulfate and disulfane monosulfonic acid. According to de Jong et al. (1997), tetrathionate hydrolase will generate sulfate, thiosulfate, and elemental sulfur at the end of the reaction due to the high activity of the hydrolysis products.

Fig. 1 Specific activity of extracellular tetrathionate hydrolase was measured during 14 days of incubation

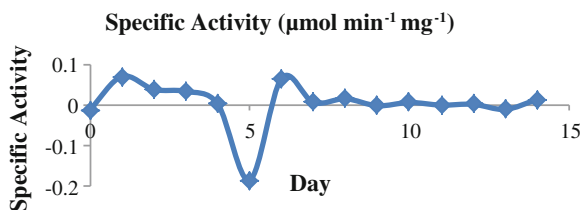
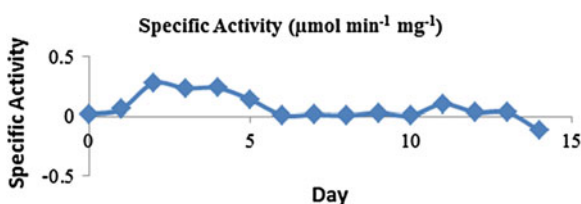


Fig. 2 Specific activity of intracellular tetrathionate hydrolase was measured during 14 days of incubation



Specific Activity of Tetrathionate Hydrolase

The specific activity of enzyme had also been studied. This measurement refers to the activity of enzyme per milligram of total protein ($\mu\text{mol min}^{-1}\text{mg}^{-1}$) at specific pH and temperature. The maximum specific activity of extracellular and intracellular enzyme occurred on Day 1 and 2 was $0.070 \mu\text{mol min}^{-1}\text{mg}^{-1}$ (U/mg) and $0.274 \mu\text{mol min}^{-1}\text{mg}^{-1}$ (U/mg), respectively, at pH 4, 25 °C (Figs. 1 and 2). This work is in accordance to the work done by Sugio et al. (2009), where they mentioned that the tetrathionate-grown *T. ferrooxidans* cells have high oxidizing activity at the early log phase where tetrathionate hydrolase produces Fe^{2+} from tetrathionate during growth on tetrathionate.

Conclusions

From this study, several findings could be concluded. Firstly, *T. ferrooxidans* was well grown by incubating the bacteria at 25 °C, initial pH buffer of 4 and at an agitation speed of 125 rpm, with salt concentration as follows: in (g/l) of $\text{KH}_2\text{PO}_4 = 4$ g, $(\text{NH}_4)_2\text{SO}_4 = 4$ g, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} = 0.3$ g. With these conditions, the activities of extracellular and intracellular enzyme are $0.0920 \mu\text{mol min}^{-1}$ and $0.268 \mu\text{mol min}^{-1}$, respectively. The specific activity of extracellular and intracellular tetrathionate hydrolase occurred on Day 1 and 2 is $0.070 \mu\text{mol min}^{-1}\text{mg}^{-1}$ (U/mg) and $0.274 \mu\text{mol min}^{-1}\text{mg}^{-1}$ (U/mg), respectively, at pH 4, 25 °C.

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