Enrichment, characterization and application of bacterial consortia for degrading 2-mercaptobenzothiazole in rubber processing wastewater

Miss Saowaluk Krainara

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การเพาะเลี้ยงเพิ่มจำนวน การศึกษาลักษณะสมบัติ และการประยุกต์ใช้กลุ่มแบคทีเรีย เพื่อย่อยสลายสาร 2-เมอร์แคปโตเบนโซไทอะโซลในน้ำเสียกระบวนการผลิตยาง

น.ส.เสาวลักษณ์ ไกรนรา

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By	Miss Saowaluk Krainara
Field of Study	Hazardous Substance and Environmental Management
Thesis Advisor	Associate Professor EKAWAN LUEPROMCHAI, Ph.D.
Thesis Co Advisor	Benjaphon Suraraksa, Ph.D.
	Peerada Prommeenate, Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the GRADUATE SCHOOL

(Associate Professor THUMNOON NHUJAK, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(Assistant Professor CHANTRA TONGCUMPOU, Ph.D.)

..... Thesis Advisor

(Associate Professor EKAWAN LUEPROMCHAI, Ph.D.)

..... Thesis Co-Advisor

(Benjaphon Suraraksa, Ph.D.)

..... Thesis Co-Advisor

(Peerada Prommeenate, Ph.D.)

..... Examiner

(Associate Professor ONRUTHAI PINYAKONG, Ph.D.)

..... Examiner

(Associate Professor TAWAN LIMPIYAKORN, Ph.D.)

..... External Examiner

(Parinda Thayanukul, Ph.D.)

เสาวลักษณ์ ไกรนรา : การเพาะเลี้ยงเพิ่มจำนวน การศึกษาลักษณะสมบัติ และการประยุกต์ใช้กลุ่มแบคทีเรีย เพื่อย่อยสลายสาร 2-เมอร์แคปโตเบนโซไทอะโซลในน้ำเสียกระบวนการผลิตยาง. (Enrichment, characterization and application of bacterial consortia for degrading 2-mercaptobenzothiazole in rubber processing wastewater) อ.ที่ปรึกษาหลัก : รศ. ดร.เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาร่วม : ดร.เบญจพร สุรารักษ์,ดร.พีรดา พรหมมีเนตร

สารเบนโซไทอะโซลถูกใช้เป็นสารเร่งปฏิกิริยายาวัลคาไนซ์ในอุตสาหกรรมยาง จากปริมาณการใช้งานที่สูง ส่งผลให้มีการปลดปล่อยสู่สิ่งแวดล้อมและอาจก่อให้เกิดผลกระทบต่อสุขภาพได้ การศึกษานี้มีวัตถุประสงค์เพื่อให้ได้แบคทีเรียที่ย่อยสลายสาร 2-เมอร์แคปโตเบนโซลไทอะโซลที่มีประสิทธิภาพสำหรับใช้บำบัดน้ำเสีย กลุ่มหัวเชื้อแบคทีเรียนี้ผ่านการเพาะเลี้ยงเพิ่มจำนวน โดยการบ่มกากตะกอนน้ำเสียยางพาราในอาหารที่มีการเติม สาร 2-เมอร์แคปโตเบนโซลไทอะโซล เป็นเวลา 28 วัน ตามด้วยกระบวนการปรับตัวให้ชิน โดยอาศัยการค่อยๆ เพิ่มความเข้มข้นของ 2-เมอร์แคปโตเบนโซลไทอะโซล ในอาหารเลี้ยงเชื้อที่มีแอมโมเนียมคลอไรด์ เป็นองค์ประกอบ เป็นเวลา 76 วัน กระบวนการนี้สามารถเพิ่มจำนวนแบคทีเรียที่ต้องการได้อย่างมีนัยสำคัญ และสามารถเปลี่ยนแปลงประชากรเด่นและยีนที่เกี่ยวข้องกับการย่อยสลายสารดังกล่าวได้ จากหัวเชื้อจุลินทรีย์ทั้งหมดที่ทำการทดสอบ กลุ่มหัวเชื้อแบคทีเรียชนิด EN มีอัตราการย่อยสลายทางชีวภาพจำเพาะของ 2-เมอร์แคปโตเบนโซลไทอะโซล สูงสุดที่ 5.2 ± 0.5 มิลลิกรัมต่อลิตรต่อวันต่อมิลลิกรัมโปรตีนในเซลล์ และสามารถย่อยสลายสาร 2-เมอร์แคปโตเบนโซลไทอะโซล ได้ที่ความเข้มข้นเท่ากับ 300 มิลลิกรัมต่อลิตร จากผลการหาลำดับเบสของชิ้นส่วน 16S rRNA พบว่า แบคทีเรียชนิด *Pseudomonas* เป็นประชากรเด่นที่สุด โดยมีประชากรมากที่สุด ประมาณ 70% ของประชากรทั้งหมด แบคทีเรียชนิด *Stenotrophomonas* เป็นประชากรเด่นลำดับที่สอง และกลุ่มแบคทีเรียนี้ไม่เคยมีรายงานเกี่ยวกับการย่อยสลายทางชีวภาพของ 2-เมอร์แคปโตเบนโซลไทอะโซลมาก่อน ปริมาณยีนที่เกี่ยวข้องกับกระบวนการย่อยสลาย และความทนทานสารมลพิษ ในหัวเชื้อแบคทีเรียที่ผ่านการปรับสภาพนี้ มีปริมาณเพิ่มสูงขึ้นเมื่อเทียบกับในกากตะกอนดั้งเดิม นอกจากนี้กลุ่มหัวเชื้อแบคทีเรียชนิด EN สามารถกำจัดสาร 2-เมอร์แคปโตเบนโซลไทอะโซลที่ปนเปื้อนในน้ำเสียยางพาราได้ 65-79% และสามารถกำจัดความสกปรกในน้ำเสียที่มีความเข้มข้นเริ่มต้นประมาณ 4,000 มิลลิกรัมซีโอดีต่อลิตร ได้ 90-93% ภายใต้ระบบการทดสอบแบบกะ ซึ่งค่าการบำบัดที่ได้สูงกว่าค่าการบำบัดตามธรรมชาติอย่างมีนัยสำคัญ งานวิจัยยังพัฒนากลุ่มหัวเชื้อแบคทีเรียที่พร้อมใช้งาน โดยตรึงกลุ่มหัวเชื้อแบคทีเรียชนิด EN ในวัสดุที่มีความพรุน พบว่าเซลล์ตรึงดังกล่าวสามารถใช้ซ้ำได้อย่างน้อย 4 รอบ ต่อมาได้ทดลองเติมหัวเชื้อแบคทีเรียชนิด EN แบบตรึงในระบบบำบัดน้ำเสียจากกระบวนการผลิตยางพารา พบว่าไม่เพียงแต่ลดปริมาณสาร 2-เมอร์แคปโตเบนโซลไทอะโซลได้ที่ระดับ 74-76% ของความเข้มข้นเริ่มต้น แต่ยังสามารถลดความสกปรกจากสารอินทรีย์ในน้ำเสียได้มากกว่า 80% ณ อัตราการรับสารอินทรีย์ที่ 3 กิโลกรัมซีโอดีต่อลูกบาสก์เมตรต่อวัน ดังนั้นการเติมจุลินทรีย์ที่ประกอบด้วยหัวเชื้อแบคทีเรียที่ตรึงในวัสดุที่มีความพรุน และกากตะกอนน้ำเสียยางพาราในระบบบำบัดที่อัตราส่วน 1:2 สามารถเพิ่มประสิทธิภาพการบำบัดน้ำเสียอุตสาหกรรมยางพาราภายใต้การทำงานแบบต่อเนื่องได้ จึงสรุปได้ว่าหัวเชื้อแบคทีเรียชนิด EN เป็นหัวเชื้อที่มีประสิทธิภาพ สามารถนำมาใช้สำหรับการบำบัดเบนโซไทอะโซลในขั้นสุดท้ายของกระบวนการบำบัดน้ำเสียจากโรงงานอุตสาหกรรมยาง

สาขาวิชา	การจัดการสารอันตรายและสิ่งแวดล้อม	ลายมือชื่อนิสิต .....
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		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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5787821320 : MAJOR HAZARDOUS SUBSTANCE AND ENVIRONMENTAL MANAGEMENT
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Saowaluk Krainara : Enrichment, characterization and application of bacterial consortia for degrading 2-mercaptobenzothiazole in rubber processing wastewater. Advisor: Assoc. Prof. EKAWAN LUEPROMCHAI, Ph.D. Co-advisor: Benjaphon Suraraksa, Ph.D., Peerada Prommeenate, Ph.D.

Benzothiazoles is widely used as vulcanized accelerator in rubber industry. The high volume of 2-MBT usages resulted in its release into the environment and can cause adverse health impacts. This study aimed to obtain efficient 2-MBT-degrading bacteria for wastewater application. The bacterial consortia were enriched by incubating rubber wastewater sludge in a medium containing 2-MBT for 28 days followed by a stepwise acclimation by gradually increasing 2-MBT concentrations in an NH_4Cl-containing medium for 76 days. The process significantly increased the bacterial number and changed the dominant populations and degradative genes. Among these consortia, the EN consortium had the highest specific 2-MBT biodegradation rate of 5.2 ± 0.5 mg L^{-1} day^{-1} mg $protein^{-1}$ and could degrade up to 300 mg L^{-1} 2-MBT. From 16S rRNA gene analysis, *Pseudomonas* was the dominant genus at approximately 70% of the total population. *Stenotrophomonas* was the second most abundant populations and have never been reported for 2-MBT biodegradation. The abundance of genes involved in xenobiotic substance biodegradation and tolerance mechanism in the EN consortium were higher than those in original sludge sample. In addition, the EN consortium removed 65-79% and 90-93% of 112 mg L^{-1} 2-MBT and ~4,000 mg L^{-1} COD in rubber wastewater under batch test, respectively. The values were significantly higher than that of natural attenuation. This research also developed a ready-to-use inoculum by immobilizing the EN consortium in porous carriers. The immobilized cells could retain their activities over 4 cycles of repeated uses. This study further carried out the bioaugmentation of immobilized EN consortium in rubber processing wastewater treatment system. These results suggested that the system not only reduced 2-MBT at approximately 74-76% of its initial concentration, it also exhibited greater than 80% of COD removal at an OLR 3 kg COD m^3 day^{-1}. Thus, the bioaugmentation of immobilized EN consortium and activated sludge in the bioreactor at the ratio of 1:2 could enhance wastewater treatment efficiency of rubber processing industry under a continuous operation. Hence, the EN consortium could be an efficient bacterial consortium to remove benzothiazoles by applying it in the post-treatment system of rubber industrial wastewater.

Field of Study:	Hazardous Substance and Environmental Management	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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INTRODUCTION

1.1 Statement of problem

The presence of organic pollutants in the environment is mainly attributed to the discharge of wastewater from treatment plant (Petrie, Barden, & Kasprzyk-Hordern, 2015). Thailand is currently the largest rubber producer in the world (Jawjit, Kroeze, & Rattanapan, 2010). The rubber industry consumes a large volume of raw water through their manufacturing process, thus one of the most serious problems confronting rubber industries is organic compounds in wastewater. Many recalcitrant compounds such as heterocyclic aromatic, aromatic, indole derivatives, phthalates, phenol, thiocarbamates, acids and petroleum hydrocarbons are often detected in rubber wastewater (Dsikowitzky & Schwarzbauer, 2013; Faber, 1979; G. Jungclaus, Avila, & Hites, 1978; Palma, Carvajal, Vásquez, & Contreras, 2011). This discharged effluent can cause serious environmental impact (Anumol, Vijayanandan, Park, Philip, & Snyder, 2016). The rubber wastewater also contains a high organic load and generates odor when discharged into receiving water bodies (Mukherjee et al., 2013). Currently, the industry uses chemical flocculent to remove some organic compounds before transfers the treated wastewater to the conventional biological treatment pond. The treatment process is considered an incomplete system.

One of the identified organic contaminants in rubber wastewater is heterocyclic aromatic group including 2-mercaptobenzothiazole (2-MBT) and their derivatives. 2-MBT is the most important members of the benzothiazole family, used as a vulcanization accelerator in rubber industry (Reddy & Quinn, 1997). Moreover, it also used as fungicide in leather (H. De Wever, Besse, & Verachtert, 2001) and pharmaceutical industries (Bujdakova, Kuchta, Sidoova, & Gvozdjakova, 1993) as well as anticorrosion (Yang et al., 2008). 2-MBT has been detected in various types of wastewater (H. De Wever et al., 2001; Reddy & Quinn, 1997; Stasinakis, 2012; Umamaheswari & Rajaram, 2017; Valdes, Zaror, & Jekel, 2003). It is known to be toxic

and poorly biodegradable compound, the half-life of 2-MBT ranges from 92 to 248 days (Kettrup, Maasfeld, Dubisch, & Kampschulze, 1982). In addition, it has been found to be refractory in activated sludge and anaerobic treatment systems (H. De Wever & Verachtert, 1994; M. A. Gaja & Knapp, 1998). The concentration of 2-MBT at 100 mg/L can inhibit the activity of microbes in biological wastewater treatment. It can solubilize cell membrane and lead to potassium leakage under bacteriostatic condition (Helene De Wever, Van den Neste, & Verachtert, 1997; Tölgyessy, Kollár, Vančo, & Piatrik, 1986). In addition, inhibitory effect of 2-MBT on aquatic organisms and human health was evaluated (Chen, Ortiz, Steele, & Stuckey, 2014; Liao, Kim, & Kannan, 2018; Whittaker, Gebhart, Miller, & Hammer, 2004). The 2-MBT has a potential allergenic and mutagenic effects to human (Geier, Uter, Schnuch, & Brasch, 2002; Gold, Slone, Stern, & Bernstein, 1993).

Physical and chemical treatments have been introduced to transform 2-MBT to readily degradable compounds, but they cannot completely destroy the target pollutants and are considered non-environmental friendly (Al-Ansari, Steevensz, Taylor, Bewtra, & Biswas, 2010; Derco, Kassai, Melicher, & Dudas, 2014; F. Li, Liu, Liang, Li, & Zhang, 2008; A. Martins, Teixeira, da Fonseca, & Yokoyama, 2017; redouane salah, Malouki, Badis, Santaballa, & Canle López, 2018). Biological treatment can be a feasible treatment solution using microbial cultures to transform toxic chemical to less toxic product or complete mineralization (Mamma, Papadopoulou, Petroutsos, Christakopoulos, & Kekos, 2006) . Currently, only a few strains of 2-mercaptobenzothiazole- degrading microorganisms have been reported in the literature (A. Drotar, G. A. Burton, Jr., J. E. Tavernier, & R. Fall, 1987a; El-Bassi, Iwasaki, Oku, Shinzato, & Matsui, 2010; Haroune et al., 2002; Umamaheswari & Rajaram, 2017; Wever, Cort, Noots, & Verachtert, 1997). Biodegradation pathways of 2-MBT have been partially elucidated in *Rhodococcus* species and *Alcaligenes* sp. (Haroune et al., 2004; Haroune et al., 2002). The biodegradation pathway in these bacterial strains have been presented in two main possible routes including oxidation and methylation reactions. The oxidation reaction of 2-MBT is further hydroxylated into catechol and entering to the Krebs cycle. Moreover, methylation reaction produces sulfite and ammonia, then

the sulfite is subsequently metabolized to sulfur and later to thiocyanate as the end product. *Alcaligenes* sp. MH146 strain CSMB1 exhibited *catechol 2, 3-dioxygenase* activity while *Rhodococcus rhodochrous OBT18* showed catechol 1, 2-dioxygenase activity. Moreover, *Pseudomonas putida* strain HKT554 is the first Gram-negative bacteria that can transform benzothiazole derivatives via *naphthalene dioxygenase* system (El-Bassi et al., 2010). From these results, they can be indicated that 2-MBT metabolism may related to various oxygenase reactions. Currently, the biotransformation of 2-MBT biodegradation in mixed cultures is largely unknown and inconclusive (H. De Wever et al., 2001; Kloepfer, Jekel, & Reemtsma, 2005; Reemtsma et al., 2006). Therefore, this study focused on elucidating the activities of various microorganisms on degrading 2-MBT and other benzothiazoles in rubber industrial wastewater.

Initially, 2-MBT degrading bacterial consortia were isolated from rubber processing wastewater sludges by enrichment and acclimatization processes. The use of complex microbial sources obtained from wastewater sludge, manure, paddy soil, freshwater sediment and compost has been proposed (Z. He et al., 2015; Simmons, Reddy, Simmons, Singer, & VanderGheynst, 2014; Zanaroli et al., 2010). These samples generally contain a high diversity of bacteria, because their environment can promote the activity of various microorganisms (Kachienga, Jitendra, & Momba, 2018; Wilkins, Rao, Lu, & Lee, 2015). This study was initially isolated the efficient bacterial consortia from rubber wastewater sludge through an enrichment and acclimatization process be increasing the concentration of 2-MBT in each subsequent transfer. The process is important in introducing native microorganisms to foreign pollutant by adaptation to the higher concentrations of the interested compound (Zawani, Guan, Fakhru'l-Razi, & Abdan, 2013). The subsequent transfers of enrichment culture have advantages for selecting the specialized microorganisms which have increasing number during the acclimation phase and can enhance the degradation rate of pollutants effectively (Zhou, Pan, Xu, Xu, & Liu, 2016).

2-MBT may be used by microorganisms as a sole carbon, nitrogen and sulfur sources as described previously (Umamaheswari & Rajaram, 2017; Wever et al., 1997).

However, some bacteria such as *P.* putida strain HKT554 do not grow on minimal medium with benzothiazole and 2-MBT as the sole nitrogen, and sulfur, it required the nitrogen and sulfur supplement in medium to enhance the biomass production (El-Bassi et al., 2010; Y.-S. Liu, Ying, Shareef, & Kookana, 2011). Alternative carbon sources such as acetate, benzoate, glucose and glutamic acid can serve as a primary growth substrates and also act as a stimulatory substrate for inducing catabolic enzymes involved in 2-MBT metabolism (Arora, Alok, & Singh, 2010; Haroune et al., 2002; Y. Li, Hu, & Gao, 2018; Lovanh & Alvarez, 2004). Consequently, this work investigated the influence of organic carbon and nitrogen sources in the enrichment medium by comparing the degradation rate and community structure of 2-MBT degrading bacterial consortia after acclimatization process.

An efficient 2-MBT degrading bacterial consortium was later selected and applied in bioreactors containing rubber processing wastewater. Little information is available about the feasibility of 2-MBT treatment in bioreactor systems and the relationship between 2-MBT treatment performance and microbial structure is limited. In general, they can be concluded that non-specific microorganisms or non-acclimatized cells in activated sludge have low 2-MBT degradation capacity. Previous work also indicated that benzothiazole derivatives had an adverse effect on acid accumulation and membrane fouling in fluidized-bed membrane bioreactor (Y. Li, Hu, Chen, Wang, & Gao, 2017). Since the performance of 2-MBT treatment in wastewater are low and the sustainable of microbial community in wastewater is difficult, cell immobilization is recommended for improving 2-MBT treatment in wastewater. The immobilized cells have several unique advantages over free cells such as considerable duration and prolong activity under stress environments (Aneez Ahamad & Mohammad Kunhi, 2011; Siripattanakul, Wirojanagud, McEvoy, & Khan, 2008).

Consequently, this study immobilized the efficient 2-MBT degrading bacterial consortium in a porous carrier and apply them for rubber wastewater treatment. The immobilization process was expected to effectively maintain active bacteria in the bioreactor and protect the bacterial cells from 2-MBT and benzothiazole derivatives. This study further employed NGS technique and metatranscriptomic analyses to

identify the diverse bacterial populations and their functional role in 2- MBT degradation and metabolism. The application of molecular approaches including next-generation sequencing (NGS) method, metagenomics, and metatranscriptomics allow the detection of overall microbial diversities and help linking the associated functions (Kachienga et al., 2018; D. P. Singh, Prabha, Gupta, & Verma, 2018; B. Wang et al., 2017). Finally, this work proposed an application of immobilized cells for 2- MBT biodegradation in rubber wastewater treatment system which could be extended to wastewater from other industries containing 2-MBT and benzothiazole derivatives.

1.2 Research hypotheses

1 The increasing of 2-MBT concentrations over time allows the bacterial consortia in wastewater sludge to acclimatize and consequently degrade 2-MBT at high degradation rates.

2 The efficient 2-MBT degrading bacterial consortia contain diverse populations and functional genes associated with the 2-MBT metabolism, while the bacterial community structures will depend on source of wastewater sludge.

3 Immobilization of 2- MBT degrading bacterial consortium can increase their activities and maintain the bacterial community structures in rubber processing wastewater.

1.3 Research objectives

Our ultimate goal is to develop an efficient 2-MBT degrading bacterial consortia in order to be used as a bioaugmented culture for treatment of 2-MBT in rubber processing wastewater.

1. To isolate 2- MBT degrading bacterial consortia from rubber processing wastewater sludge by acclimatization process
2. To characterize the bacterial community structures and functional genes in the 2-MBT degrading bacterial consortia by molecular analyses.
3. To develop an immobilized 2-MBT degrading bacterial consortium for applying in rubber processing wastewater.

1.4 Scope of the study

1. Rubber wastewater sludge was used as inoculum seeds for isolation process.
2. Rubber wastewater sludge was analyzed for bacterial community structures and presence of organic pollutants.
3. A sequential enrichment and acclimatization process by increasing the concentrations of 2-MBT in media with/without additional nitrogen or acetate was used to enrich and acclimatize the 2-MBT-degrading bacterial consortia.
4. Acclimatized bacterial consortia were investigated for 2-MBT biodegradation at varying initial concentrations in minimal salt medium.
5. Bacterial community structures in the efficient bacterial consortia were identified along with the factors influencing their development from the original rubber wastewater sludge.
6. Bacterial community structures were identified by 16s rRNA metagenomics.
7. The functional genes of 2-MBT degrading bacterial consortium were identified by metatranscriptome analysis.
8. A porous carrier was used as supporting material to immobilize bacterial consortium.
9. Immobilization of a selected 2-MBT degrading bacterial consortium was optimized to get the high number of attached cells.
10. 2-MBT degradation and stability of the immobilized 2-MBT degrading bacterial consortium and suspended cells were compared in a minimal salt medium.
11. The immobilized 2-MBT degrading bacterial consortium was applied in a continuous-batch reactor for treating rubber processing wastewater.
12. All the experiments were carried out in the laboratory.

1.5 Experimental framework

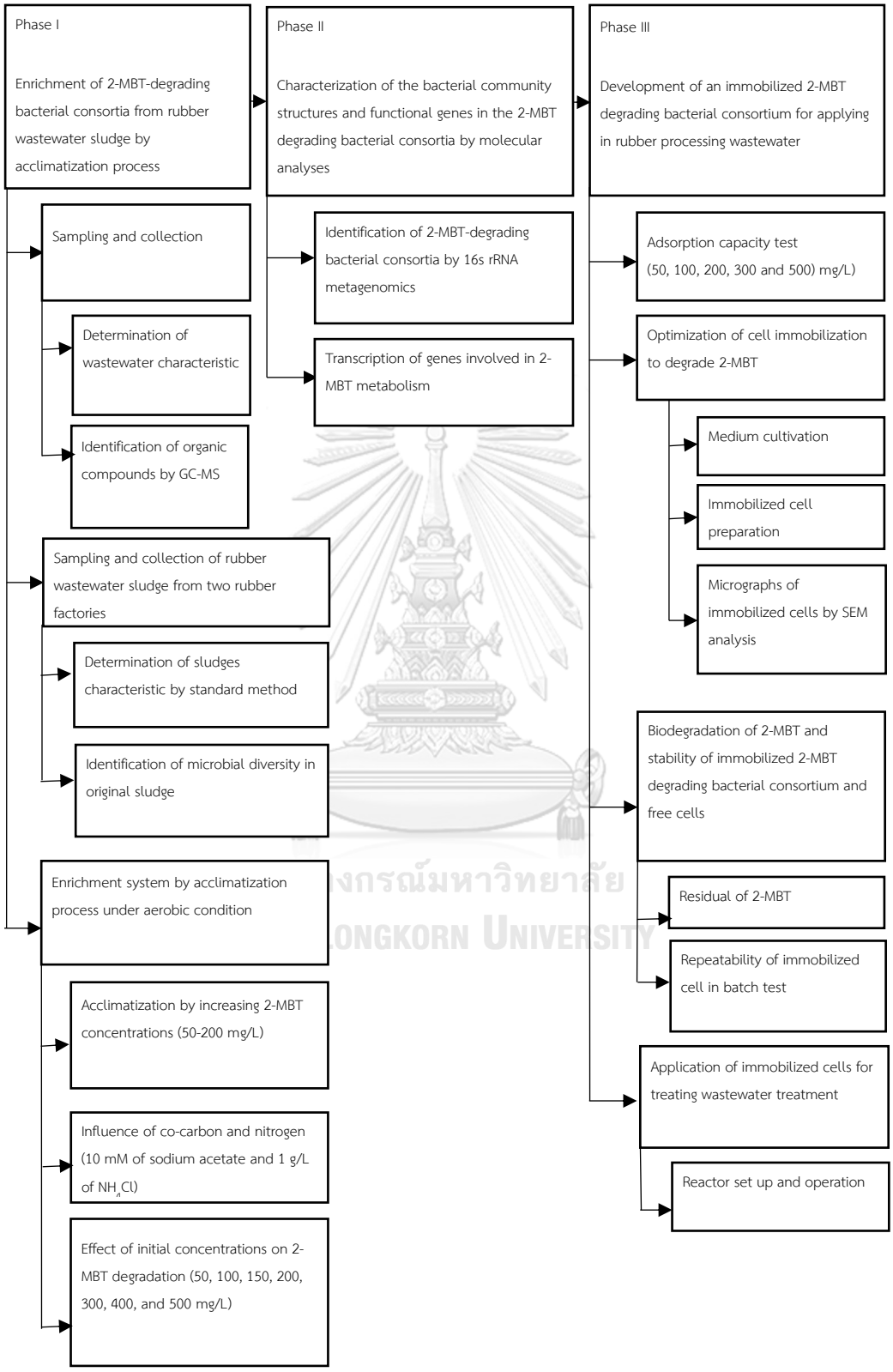


Figure 1.1 Experimental framework

CHAPTER 2
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Theoretical background

2.1.1 Benzothiazole family

Benzothiazoles are a class of five-membered nitrogen containing heterocyclic ring featuring sulfur as part of aromatic ring system (Patel & Patel, 2018). The benzothiazole group consisted of 2-mercaptobenzothiazole (2-MBT), benzothiazole (BT), 2-hydroxybenzothiazole (OHBT), benzothiazole-2-sulfonate (BTSO3), methylbenzothiazole-2-thione (MBTT), 2-(methylthio) benzothiazole (MTBT), methylbenzothiazole (MeBT) and 2-aminobenzothiazole (ABT). The general structure of these chemical substances is shown in Figure 2.1.

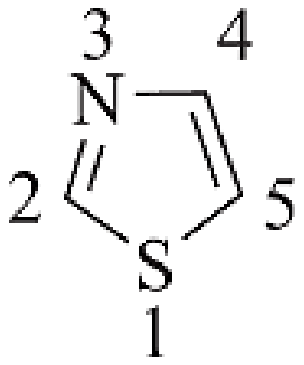
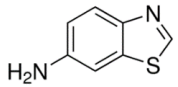
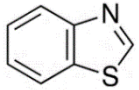
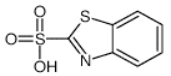
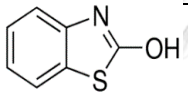
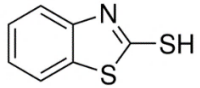
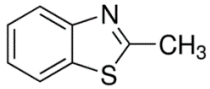
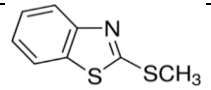


Figure 2.1 General structure of benzothiazoles (Patel & Patel, 2018).

Among benzothiazoles, BT is liquid, while other benzothiazoles are solids at room temperature. In addition, the polarity of the BT is higher than other benzothiazoles that led to its high solubility (Table 1). OHBT has also a good solubility of 2354 mg L^{-1} whereas 2-MBT, MTBT, and ABT are partial soluble. The physical and chemical characteristics of benzothiazoles are shown in Table 2.1.

Table 2.1 Physical and chemical properties of benzothiazoles.

CAS number	Chemical names	Formula	Structure	Abbreviation	Molecular weight (g $mole^{-1}$)	Water Solubility (mg L^{-1}) at 25°C
136-95-8	Aminobenzothiazole	$C_7H_6N_2S$		ABT	150	310.3
95-16-9	Benzothiazole	C_7H_5NS		BT	135	4300
941-57-1	Benzothiazole-2-sulfonate	$C_7H_5NO_3S_2$		$BTSO_3$	215.3	2.55e+005
934-34-9	Hydroxybenzothiazole	C_7H_5NOS		OHBT	151	2354
149-30-4	2-Mercaptobenzothiazole	$C_7H_5NS_2$		2-MBT	167	120
120-75-2	Methylbenzothiazole	C_7H_5NS		MeBT	149	366.3
615-22-5	Methylthio benzothiazole	$C_8H_7NS_2$		MTBT	181	125

2.1.2 Uses and application

Benzothiazoles represent an important chemical of various industries (Table 2.2). In addition, some benzothiazoles are present in several potent pharmacologically active molecules include antimicrobial drug, antiretroviral drug, and antifungal drug (Kumawat, 2018) . In rubber processing industry, 2-MBT is the most important member of the benzothiazole group, which is used as vulcanization accelerators in vulcanization process (Gu et al., 2018; Y. Wang, Shuai, & Chen, 2020).

Table 2.2 Industrial uses of benzothiazoles.

Industry	Purpose	Reference
Medical application	Anti-fungal drug	Bujdakova et al. (1993)
Cooling system	Corrosion inhibitors	
Pharmaceutical industry	Chemotherapeutic agents	
Leather industry	Fungicides	Reemtsma, Fiehn, Kalnowski, & Jekel (1995)
Rubber processing industry	Vulcanization accelerators	Kloepfer, Gnirss, Jekel, & Reemtsma (2004)
Flotation Agents	Collectors - Sulfide Ores	McDonald (1995)
Lubricants and Additives	Extreme Pressure Agents	
Plastics	Secondary Antioxidants	

Vulcanization is a cross linking process, in which individual polymer molecules of rubber are converted into a three dimensional network of interconnected chains through chemical additives (Lim, Park, & Kim, 2016). To improve the rubber quality and reduce time of vulcanization and used of vulcanizing chemical, 2-MBT is added into a rubber compound in order to increasing the speed of vulcanization (J. Zhao, Cheng,

Wang, Wang, & Song, 2017). For the BT and OHBT compounds, they are also generated as a byproduct in rubber processing product (Brownlee, Carey, MacInnis, & Pellizzari, 1992) and are the major compounds in both tire and road dust samples (J. Zhang et al., 2018). In addition, ABT is commonly used in rubber products to accelerate the vulcanization of rubbers and the manufacturing of azo dyes (Podsiadły et al., 2005).

2.1.3 Environmental occurrence of benzothiazoles

2-Mercaptobenzothiazole (MBT) exists in numerous industrial effluents, resulting in a large amount of release into the environment every year (Table 2.3). Benzothiazole (BT) is produced from a variety of sources such as the leaching of robber processing product, the 2-MBT production and use of 2-MBT in vulcanization process (G. A. Jungclaus, Games, & Hites, 1976). These compounds are frequently found in effluents of wastewater from rubber related applications (20 mg L^{-1}) (Puig, Ormad, Sarasa, Gimeno, & Ovelleiro, 1996; Valdés & Zaror, 2005), tannery wastewater (Reemtsma et al., 1995) and tap water (L. Wang, Zhang, Sun, & Zhou, 2016) at a concentration of 40.1-1310 ng L^{-1}. Methylbenzothiazole (MeBT) is identified in water originating from tire and rubber products at concentration of 0.534 μg L^{-1} (Liao et al., 2018). Methylthiobenzothiazole (MTBT) are also found in effluent of wastewater treatment plants (WWTPs) (Asimakopoulos, Ajibola, & Thomaidis, 2013). It is also detected in tannery wastewater samples at concentration of 39 μg L^{-1} (Fiehn, Wegener, Jochimsen, & Jekel, 1998).

Table 2.3 Occurrence of 2-MBT in wastewater

Industry	Region	Wastewater type	Concentration (mg L^{-1})	Reference
Rubber and tire production	USA	Effluent	0.03	G. A. Jungclaus et al. (1976)
	Spain	Partially treated wastewater	1.1	Puig, Ormad, Roche, et al. (1996)
	Thailand	Effluent	1.99	Worawit (2006)
	Japan	rubber thread wastewater	76	(Ghin Yeoh et al., 2002)
	Chile	Wastewater	200	Valdes et al. (2003)
	Iran	Wastewater	200	Habibi, Tangestaninejad, & Yadollahi (2001)
Tannery	Germany	Untreated	0.1	Reemtsma & Jekel (1997)
	India	Wastewater	50	Umamaheswari & Rajaram (2017)

2.1.4 Toxicity

Many researches have shown the toxicity of 2-MBT to microorganisms and aquatic life. Wastewater containing 2-MBT have toxicity effects on activated sludges (H. De Wever & Verachtert, 1997; M.A. Gaja & Knapp, 1997; Tölgyessy et al., 1986) and 2-MBT affected several bacterial species and yeast cells. Since 2-MBT can solubilize in membrane-bound system, it can caused potassium leakage under bacteriostatic condition (Helene De Wever et al., 1997). The potassium leakage is known to be the indicator of membrane damage (Fuller et al., 1985). Moreover, 2-MBT at 100 mg L^{-1}

inhibited the bacterial growth and respiration (H. De Wever, De Moor, & Verachtert, 1994). 2-MBT might interfere with oxido-reduction in membrane bound system and affect to the metabolic reaction (H. De Wever et al., 1994; Helene De Wever et al., 1997; Reemtsma, Zywicki, Stueber, Kloepfer, & Jekel, 2002). For aquatic toxicity test, 2-MBT poses toxicity effects to fish and zooplanktons. *Daphnia magna* is used as a planktonic crustacean model with the LC50 (48h) of 19 mg L^{-1} (Reemtsma, Fiehn, & Jekel, 1999). In addition, the aquatic toxicity of 2-MBT on *Vibrio fischeri* was also demonstrated with the luminescence inhibition and EC50 (20 h) at 0.75 and 0.12 mg L^{-1}, respectively (Reemtsma et al., 1995). Moreover, 2-MBT is toxic to several aquatic life (Gold et al., 1993). The fish toxicity test was carried out in Rainbow Trout, Bluegill Sunfish and Fingerling Trout with the LC50 toxicity value of 0.75, 1.5 and 0.73 mg L^{-1} respectively. Moreover, it has a potential allergenic and mutagenic effects to human (Whittaker et al., 2004).

2.1.5 Rubber processing industry

Rubber is cis-1,4-polyisoprene which is polymers naturally produced by Para rubber tree (*Hevea brasiliensis*). Indian and Central America were the first to use the natural rubber from latex releasing from the plant of *H. brasiliensis* (Simmonds, 1994). In addition, latex tapping and rubber preparation to produce the rubber products have been first discovered by Francois Fresneau (Jones & Allen, 1992). The growing demand of natural rubber product has been increased because of its performance to use as a raw material in more than 40,000 products which included more than 400 medical devices (Mooibroek & Cornish, 2000).

At present, Asia is considered as the crucial sources of natural rubber production. Thailand is the top three producers representing 35% of the global rubber output (Figure 2.2). The high quality of rubber and rubber processing products caused the country's important cash crops (Wangpimool, Pongput, Tangtham, Prachansri, & Gassman, 2016). Consequently, the government of Thailand is promoting the expansion of Para rubber plantation throughout the country and also pushing to export natural rubber (Ounsaneha, Suksaroj, & Chamondusit, 2012).

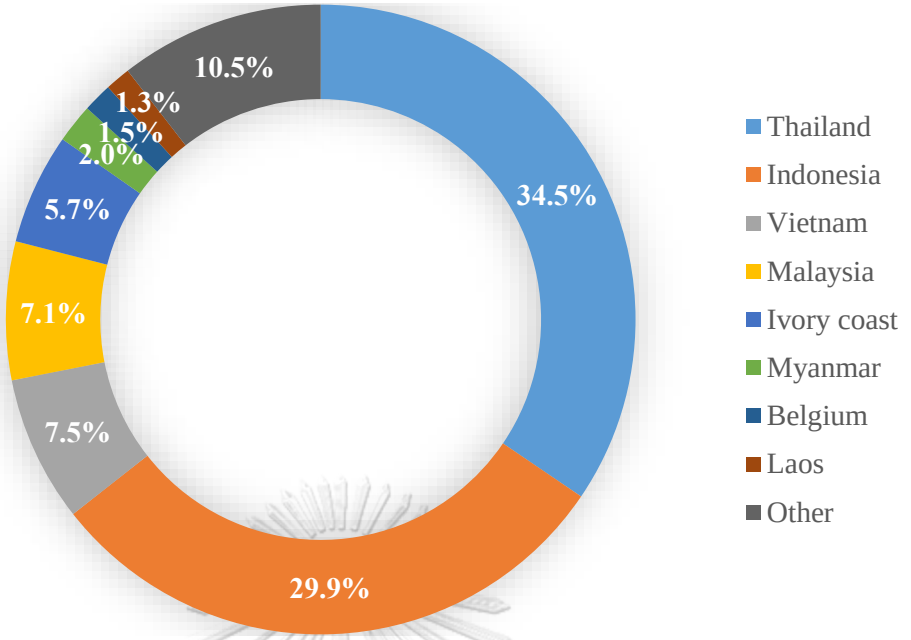


Figure 2.2 Natural rubber exports by various countries during 2018 (Modified from Trade Map, International Trade Centre. Accessed on May 7, 2019).

Value chain of rubber in Thailand consists of three major stages. The first stage is cultivated Para rubber tree, where is mostly located in southern of Thailand. The second stage is the production of various natural rubber products such as ribbed smoked sheet (RSS), block rubber (Standard Thai Rubber, STR), and concentrated latex. The final stage is development of natural rubber product such as medical gloves, condoms, tires, and industrial rubber parts (Jawjit et al., 2010; Musikavong & Gheewala, 2017). Since global rubber market is still growing consistently, the rubber industries have been continuously expanded and considered as the most importance economic sector and employment (Jawjit et al., 2010). Rubber entrepreneurs have challenged to seek for an appropriate tool to reduce several environmental problems such as global warming, acidification (water pollution), eutrophication (water pollution), malodorous problems (air pollution), combustion in drying process (air pollution) and toxicity of chemical uses (such as sulfuric acid and ammonia) (Jawjit, Pavasant, & Kroeze, 2015).

The rubber production process discharges a large amount of wastewater containing high concentration of COD, BOD, ammonia, and sulfate which caused a wide range impacts on human health and aquatic organisms. Without proper treatment, the industries have been complained from people who lived in vicinity area. In addition, these environmental effects could contribute long lasting consequences to undesirable eutrophication that lead to death of some aquatic life.

2.1.6 Characteristic of rubber processing wastewater

An understanding of the rubber wastewater characteristics from various region is necessary for developing appropriate environmental management approaches. Table 2.4 lists the chemical characteristics of rubber processing wastewater in different regions. The chemical oxygen demand (COD) of the rubber wastewater is varied in the range of 128-26,914 mg L^{-1} (Table 2.3). It is also contained high organic matter, nitrogen-containing pollutants and high sulfate content (Jing et al., 2018; Massoudinejad, Mehdipour Rabori, & Dehghani, 2015; Nhu Nguyen & Thanh Luong, 2012; D. Tanikawa et al., 2016). According to Table 2.5, there are high concentrations of organic contaminants in rubber processing wastewater. To compare the different rubber processing industries, the wastewater characteristics of concentrated latex, ribbed smoked sheets (RSS), rubber glove and the wastewater from the indirect process such as washing the container and transport rail (rubber slabs to the squeezing machine) are presented in Table 2.5.

Many hazardous organic substances in rubber wastewater have been characterized by Wongniramaikul (2006). The result was shown that seven organic groups in the rubber wastewater such as acids, indoles, phthalates, alcohols and phenols, benzothiazoles, thiocarbamates and the other groups. These organic compounds are recalcitrant to biodegrade and may potentially harmful to human health and aquatic life (Dsikowitzky & Schwarzbauer, 2013; Faber, 1979; G. Jungclaus et al., 1978; Palma et al., 2011). In addition, one of the identified organic contaminants in rubber wastewater is benzothiazole family, including 2-mercaptobenzothiazole (2-MBT), benzothiazole, hydroxyl-benzothiazole and their derivatives (Ghin Yeoh et al., 2002).

Table 2.4 Characteristics of rubber processing wastewater in different regions

Region	Parameter						Reference
	pH	BOD (mg L^{-1})	COD (mg L^{-1})	TSS (mg L^{-1})	Sulfate (mg L^{-1})	Total nitrogen (mg L^{-1})	
Thailand	5.54-7.84	502-8,670	128-9,700	261-1,780	4-1,430	229-1,370	D. Tanikawa et al. (2016)
China	6-9	1,025-3,561	1,760-6,884	450-500		70-128	Jing et al. (2018)
Vietnam	6.59-9.42	40-13,820	120-26,914	30-2,220		35. 3-1306	H. Nguyen & Luong (2012)
Malaysia	3. 7-5.5	1,500-7,000	3,500-14,000	200-700	500-2,000	200-1,800	Mohammadi, Man, Hassan, & Yee (2010)

Table 2.5 Characteristics of rubber wastewater in different rubber industries of Thailand

Type	Parameter						Reference
	pH	BOD (mg L^{-1})	COD (mg L^{-1})	TSS (mg L^{-1})	Sulfate (mg L^{-1})	Total nitrogen (mg L^{-1})	
Concentrated latex	5.54	8,670	9,700	1,780	1,430	1,370	D. Tanikawa et al. (2016)
Ribbed smoked sheets (RSS)	5.9	4,783-9,433	6,673-15,069	164-525	136-472.6	60.2-190.9	Tekasakul & Tekasakul (2006a)
Transport rail	5.3	3,433	5,371	93	225.8	79.5	
Washing process	5.8	1,391	1,928	525	136	60.2	

2.2 Literature review

2.2.1 Technologies for rubber wastewater treatment

Current technologies for treating wastewater from natural rubber latex and rubber industrial products (rubber gloves and toys hygienic products) factories have been developed with the aim to remove remaining latex and organic matter. A decantation tank or physical method is usually used to separate the remaining latex particles in wastewater before it enters to treatment stage. This process has been used in all the processing factories because of low construction and low operating cost (Jover-Smet, Martín-Pascual, & Trapote, 2017). There are many advanced technologies including electrochemical treatment (Abraham, Radhakrishnan Nair, & Madhu, 2009) and advanced oxidation processes utilized the hydroxyl radicals to oxidize organic compounds to the preferred end products of carbon dioxide and water (Amor,

Marchão, Lucas, & Peres, 2019). However, the disadvantages of chemical and physical technology are high operation costs, high electricity consumption and highly corrosive.

The additional technology of rubber wastewater treatment is conventional biological process. Several treatment methods, such as anaerobic digestion, activated sludge, oxidation ditch, pond process and lagoon systems have been developed for the rubber wastewater treatment (Table 2.6). Moreover, the treatment system of benzothiazoles contaminated wastewater have been also studied in previous work, as presented in Table 2.7. Most of researchers had investigated the benzothiazoles removal in synthetic wastewater. These might be due to the interference of organic compounds and biotic stresses contaminated in real wastewater, which could affect microbial activity in the bioreactor. Consequently, biological treatment of rubber wastewater can be effectively improved by specialized microorganisms for reducing the thiazole compounds and COD concentrations.

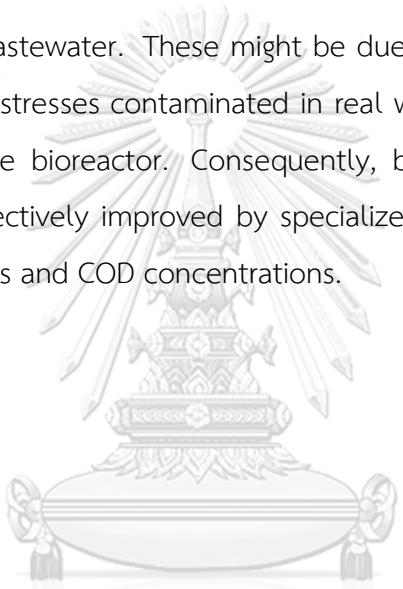


Table 2.6 Anaerobic and aerobic treatment of wastewater from rubber processing industry

Systems	Advantage	Disadvantage	Reference
Anaerobic baffled reactor (ABR) and down-flow hanging sponge (DHS) reactor	1. Easy construction 2. High sludge retention in supporting media of DHS	1. Difficult scale up 2. Expensive (DHS) 3. Potential of biomass sloughing off	Nurmiyanto & Ohashi (2019) Daisuke Tanikawa, Kataoka, Hirakata, Hatamoto, & Yamaguchi (2020)
Up flow anaerobic sludge blanket-Down flow hanging sponge	1. Simple construction and low operation 2. Less CO_2 emissions 3. Energy is generated as methane/hydrogen gas	1. Long startup time 2. Odor, toxicity, and corrosion problem 3. Expensive (DHS)	Watari et al. (2017) Nurmiyanto & Ohashi (2019)
Membrane bioreactor	1. Good quality filtered effluent 2. Low sludge bulking 3. Low energy consumption 4. Higher rate of nitrification and gentrification	1. High investment and operation cost 2. Membrane lifetime and replacement 3. Membrane fouling problem	Nik Sulaiman, Ibrahim, & Abdullah (2010) Kootenaei & Aminirad (2014)

Table 2.6 Anaerobic and aerobic treatment of wastewater from rubber processing industry (Cont.)

Systems	Advantage	Disadvantage	Reference
Sequential batch reactor and aerobic granular sludge	1. High effluent quality 2. Process is simplified 3. Operating flexibility and control. 4. Small footprint	1. Higher level of maintenance a 2. Excess Sludge 3. High energy consumption 4. To require post-treatment	Rosman et al. (2013) (Pal, 2017)
Oxidation pond	1. Easy to construct 2. Low maintenance costs 3. Handles varying wastewater types	1. Requires a large land area 2. High BOD and TSS with algae concentrations	Madhu, George, & Francis (2000) Butler et al. (2017)
Anaerobic filter	1. Low space requirements 2. Low space requirements 3. Low sludge production	1. High cost of the filter material. 2. To require post-treatment	Anotai, Tontisirin, & Churod (2007) Manariotis & Grigoropoulos (2006)

Table 2.7 Technology for treating benzothiazole-contaminated wastewater

Type of substance in wastewater	Country	Treatment technology	Reference
Benzothiazole	China	Integrated anaerobic fluidized-bed membrane bioreactor	Y. Li et al. (2018)
Hydroxybenzothiazole	Greece	Hybrid moving bed biofilm reactor	Mazioti, Stasinakis, Psoma, Thomaidis, & Andersen (2017)
Benzothiazole	China	Fluidized-bed membrane reactor	Y. Li et al. (2017)
Benzothiazole	Greece	Activated sludge and moving bed biofilm reactor systems	Mazioti, Stasinakis, Pantazi, & Andersen (2015)
Benzothiazole, Hydroxybenzothiazole Mercaptobenzothiazole and Methylthiobenzothiazole	Germany	Activated sludge in sequential batch reactor	Kirouani-Harani (2003)
Mercaptobenzothiazole	England	Activated sludge	(M. A. Gaja & Knapp, 1998)

2.2.2 Biotransformation of 2-mercaptobenzothiazole and derivatives

2.2.2.1 Biodegradation of 2-mercaptobenzothiazole and derivatives by single bacterial strain

Various bacterial strains have been isolated and evaluated for their 2-MBT biodegradation capability. Table 2.8 shows list of benzothiazoles-degrading bacteria such as Rhodococcus (H. De Wever, Vereecken, Stolz, & Verachtert, 1998; Wever et al., 1997) Pseudomonas (El-Bassi et al., 2010) and Alcaligenes (Umamaheswari & Rajaram, 2017). These strains have been investigated for potential 2-MBT biodegradation pathway (Haroune et al., 2004; Haroune et al., 2002).

As illustrated in Figure 2.3, the 2-MBT biodegradation pathway by Alcaligenes sp. MH146 strain CSMB1 has two main possible routes including oxidation and methylation reactions (Umamaheswari & Rajaram, 2017). Oxidation reaction of 2-MBT is further hydroxylated into catechol before entering to the Krebs cycle. On the other hand, methylation reaction leads to sulfite and ammonia production, then the sulfite is subsequently metabolized to sulfur and thiocyanate is obtained as end product (Umamaheswari & Rajaram, 2017). Moreover, Alcaligenes sp. MH146 strain CSMB1 exhibited catechol 2, 3-dioxygenase activity. In contrast, Rhodococcus rhodochrous OBT18 (Figure 2.4) showed several of enzyme reactions in different biodegradation pathway routes, including 2-mercaptobenzothiazole monooxygenase, 2-mercaptobenzothiazole dioxygenase and catechol 1, 2-dioxygenase (Haroune et al., 2004). Pseudomonas putida strain HKT554 is the first bacterial strain on the biotransformation of benzothiazole derivatives by naphthalene dioxygenase (El-Bassi et al., 2010). Thus, it can be indicated that 2-MBT metabolism may related to several oxygenase systems. Based on previous studies, single bacterial strains from three genera including Alcaligenes, Pseudomonas and Rhodococcus can completely remove 2-MBT. Although benzothiazoles-degrading bacteria have been isolated, they have low degradation activity under high concentrations of 2-MBT (Table 2.7). This might due to

the accumulation of 2-MBT and intermediates which are toxic towards these bacterial species during biodegradation period (Fazzini et al., 2010).

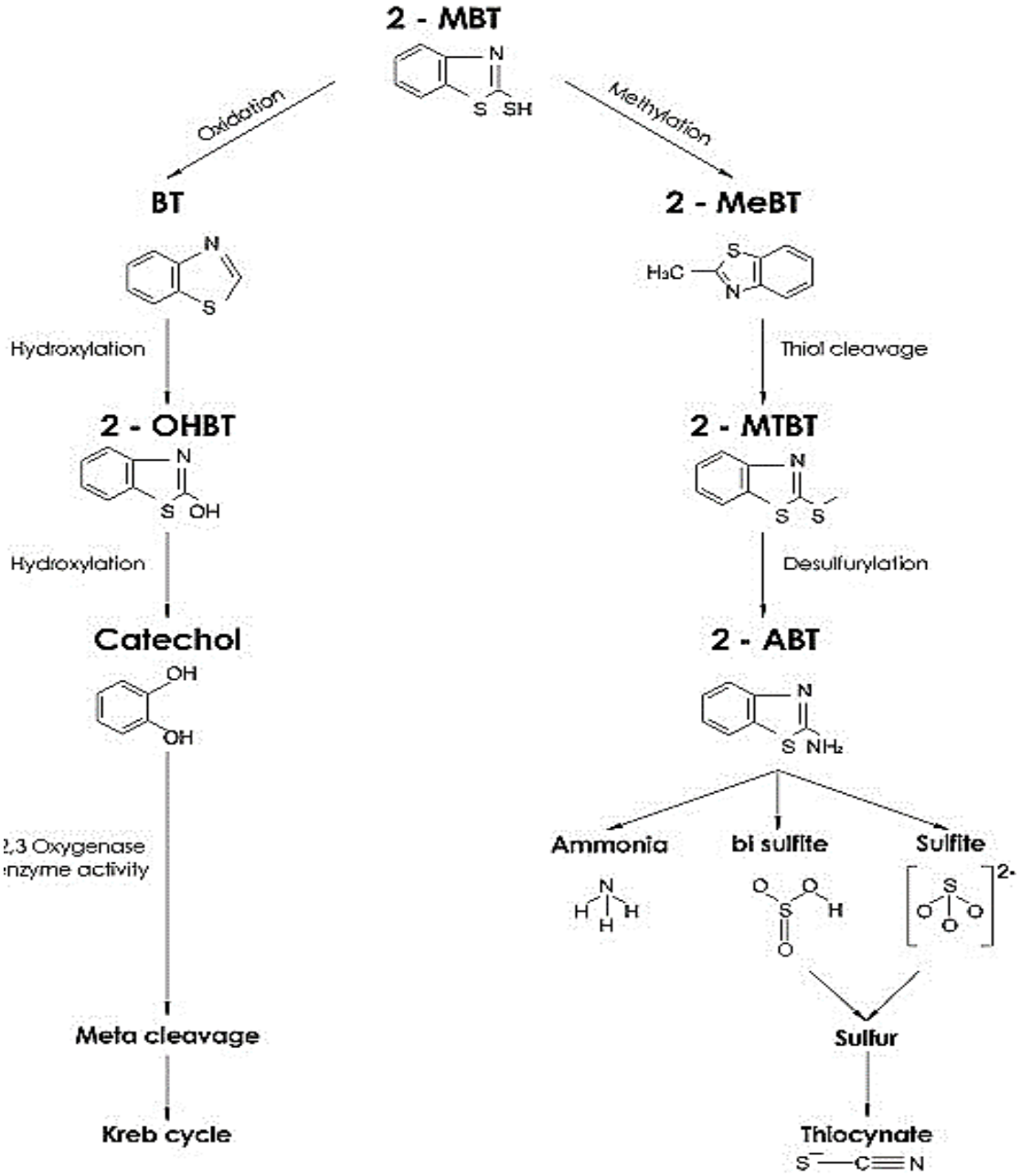


Figure 2.3 Biodegradation pathway of 2-MBT by *Alcaligenes* sp. (Umamaheswari & Rajaram, 2017).

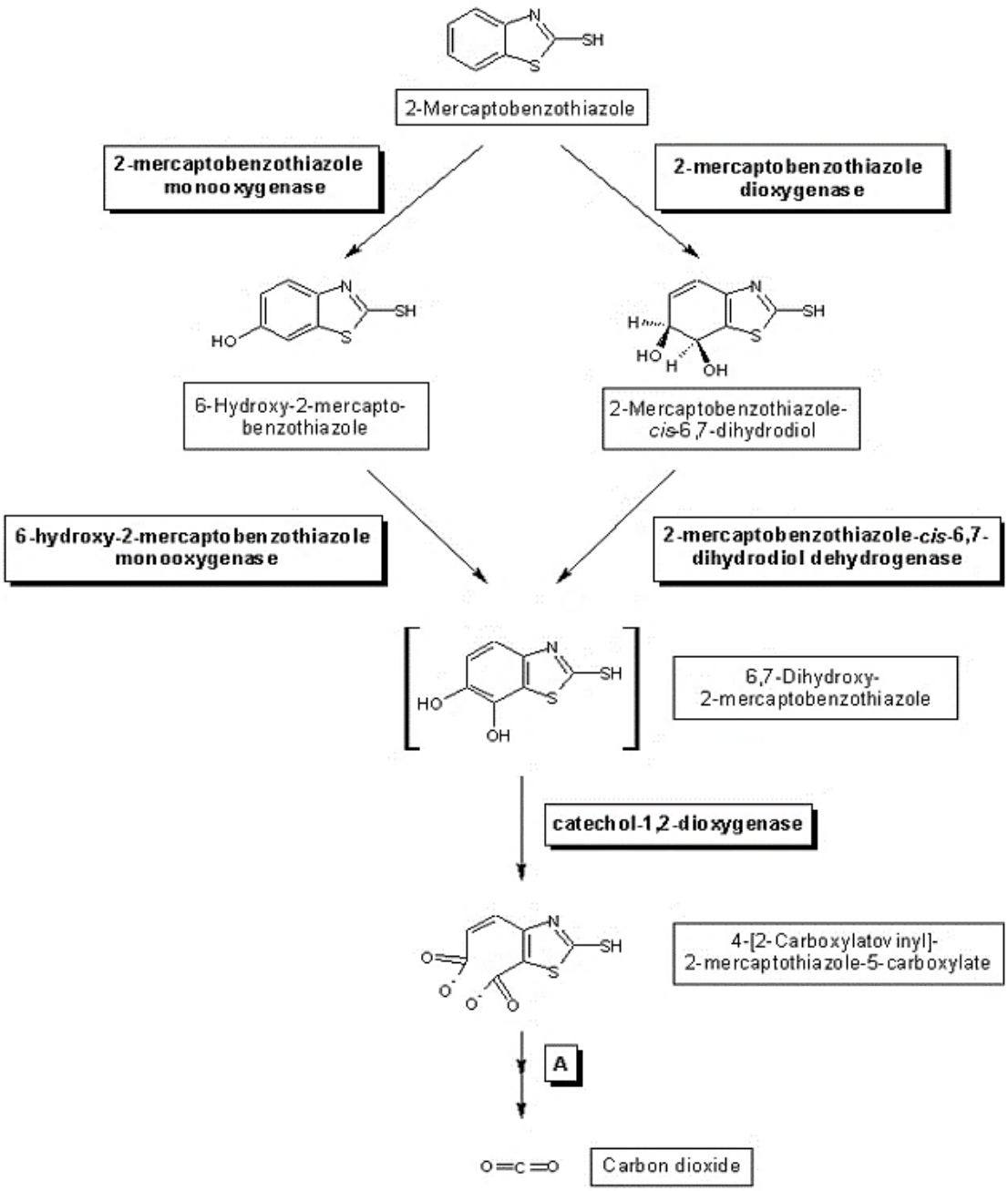


Figure 2.4 Biodegradation pathway and enzymes involved in 2-MBT metabolism by *Rhodococcus rhodochrous* OBT18 (Haroune et al., 2004).

Biodegradation of 2-mercaptobenzothiazole and derivatives by mixed culture

The benefits of using mixed microorganisms for bioaugmentation are the sharing of biochemical steps among community members in order to completely substrate biodegradation. There is few studies on the biodegradation of benzothiazoles and 2-MBT using mixed cultures (Table 2.8). In addition, activated sludge has low 2-MBT degradation capacity. At the initial concentration of 20 mg L^{-1}, only 10% of MBT were methylated by the bacteria in activated sludge systems to intermediate product within 28 d, whereas 87% remained unchanged (H. De Wever & Verachtert, 1994; Reemtsma et al., 1995). These studies used non-specific microorganisms in activated sludge or non-acclimatized cells to treat 2-MBT (H. De Wever et al., 2001; H. De Wever & Verachtert, 1994, 1997; M. A. Gaja & Knapp, 1998). Figure 2.5 shows bacterial consortium could transform the TCMTB to 2-MBT and further methylate to the MTBT, while the BT was completely degraded by aerobic treatment within 8 days.

Although, the efficiency of 2-MBT and BT biodegradation by mixed microorganisms is inconclusive, high complexity of biochemical steps naturally occurring among microorganisms can overcome the obstacles present in the environment with high concentration of the 2-MBT. Many studies have been done on adaptive response to stress condition for selecting target group of microorganisms (Kovárová-Kovar & Egli, 1998; Kurbatov, Albrecht, Herrmann, & Petruschka, 2006; Miran, Jang, Nawaz, Shahzad, & Lee, 2018). Moreover, the induction of several key enzymes in diverse population could be considered as an alternative source to enhance the recalcitrant compound biodegradation (Fazzini et al., 2010). Thus, the use of adapted bacterial consortia of 2-MBT-degrading bacteria is interested in removing 2-MBT in higher concentration as compared with the single strains.

Table 2.8 Biodegradation of benzothiazoles by mixed cultures and single strains

Microorganisms	**Type of benzothiazoles**	**Initial concentration (mg L^{-1})**	**% removal**	**Operation period (days)**	**Reference**
Sludge from a rubber chemicals waste-water treatment plant	2-MBT	200	100 %	95	H. De Wever & Verachtert (1994)
Activated sludge	2-MBT	167	85%	25	M. A. Gaja & Knapp (1998)
Rhodococcus rhodochrous OBT18	2-MBT	251 (1.5 mM)	30%	128	Haroune et al. (2004)
Pseudomonas putida strain HKT554	Mixture compounds	2-MBT, 20 (120 μM) BT, 19 (140 μM) MTBT, 18 (100 μM)	87%	1.6	El-Bassi et al. (2010)
Alcaligenes sp. MH146 strain CSMB1	2-MBT	50	86% (as TOC)	3	(Umamaheswari & Rajaram, 2017)

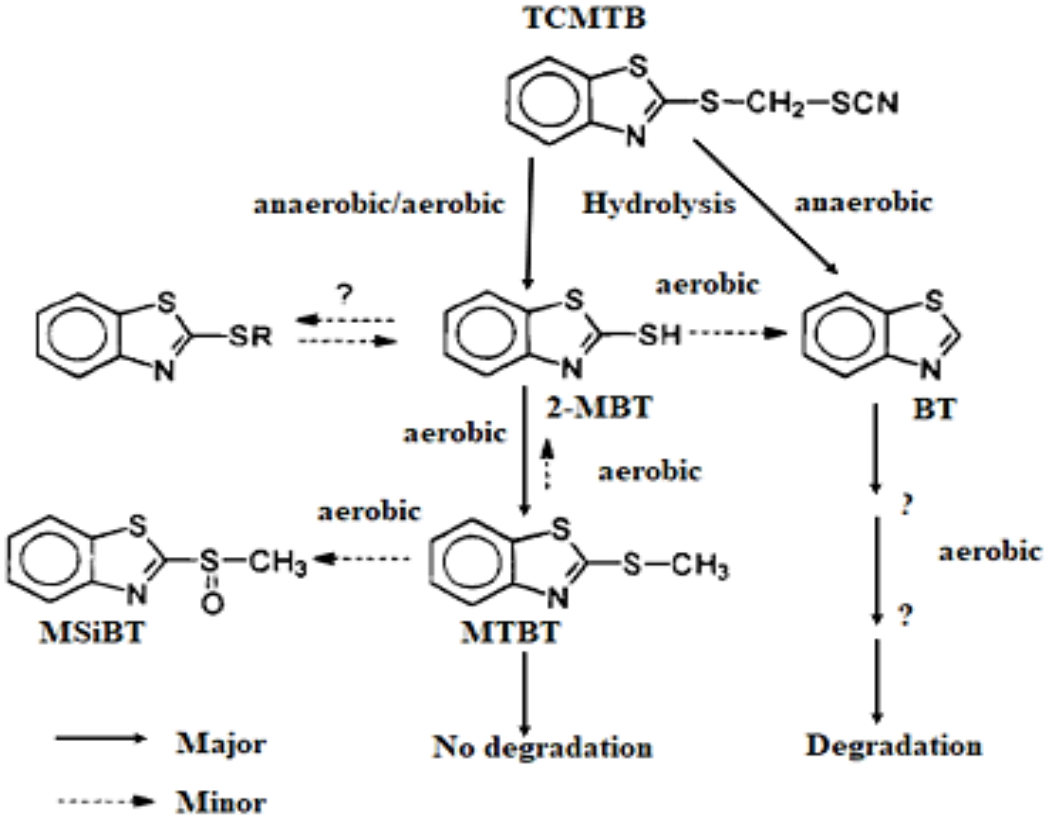


Figure 2.5 Biodegradation pathway of benzothiazoles by mixed cultures (Reemtsma et al., 1995).

2.2.3 Isolation of efficient bacterial consortia

Application of isolated microbes has been a promising strategy to raise the efficiency of biological processes when dealing with hazardous substances that are refractory by natural conditions. Microbial consortia owning to their diverse bacterial community and composition had more functional stability and efficiency than single strains (Kang et al., 2020). However, microorganisms within the large diversity of the consortium are comprised of non-active and active microorganisms. Thus, the biodegradation efficiency of the target compounds could be improved by selecting specific microorganisms. The following review describes two main criteria for a proper bacterial consortia selection.

2.2.3.1 Source of microorganisms

Features of proper cultures are fast growing, survival under high concentration of organic compounds and tolerant to a wide range of environmental changes (Mrozik & Piotrowska-Seget, 2010). Mixed cultures from contaminated soil (Poddar, Sarkar, & Sarkar, 2019), wastewater (Panigrahy, Barik, Sahoo, & Sahoo, 2020) and sludge (Pattanasuttichonlakul, Sombatsompop, & Prapagdee, 2018) have been widely used as inoculum starter for development of microbial culture for the treatment of recalcitrant organic pollutants. Thus, many researches have been proposed that the origin of the sludge (Prado, Ochoa, & Amrane, 2009) and history of soil or sludge contamination showed a significant impact on the biodegradation potential of hazardous compounds by bacterial cultures (Cerqueira et al., 2011).

2.2.3.1 Artificial selection approaches

There are several selection approaches that allow the selection of effective single strain and microbial consortium for introducing as bioaugmented cultures. Enrichment process is one of the most efficient technique to select microbial consortia (Puentes-Téllez & Falcao Salles, 2018). This technique enriched the proportion of specialized bacteria by inoculating sample collected from contaminated sites in the culture media under controllable conditions in order to reinoculated back to the contaminated sites. Another approach is acclimation, which allow the adaptation of bacteria under a stepwise increasing pollutant concentration. The adaptation will

select enzymes for degrading a compound to which it is exposed (Knapp & Bromley-Challoner, 2003). The acclimation process also increases the bacterial resistant to high concentration of chemical compounds (Chattaraj, Johnson, & Madamwar, 2016; H. Wang et al., 2018). Consequently, the enrichment and acclimatization of microbial consortia are mainly recommended to select the cultures for degrading organic compounds (Bessa, Moreira, Tiritan, & Castro, 2017; Pattanasuttichonlakul et al., 2018; Poddar et al., 2019; Y. Wang et al., 2020).

2.2.4 Bioaugmentation strategy

2.2.4.1 Bioaugmentation for removal of recalcitrant compounds in wastewater

Bioaugmentation is the inoculation of specific and efficient pollutant-biodegrading microorganisms harboring the functional genes into contaminated sites to enhance the degradation of contaminant (Garbisu, Garaiyurrebaso, Epelde, Grohmann, & Alkorta, 2017). This strategy has been developed to enhance the performance of biological process in wastewater treatment (Guo et al., 2009; Nzila, Razzak, & Zhu, 2016). The selection of a suitable bacterial strain is essential to the success of bioaugmentation. Many researches have investigated the study of bioaugmentation to remove recalcitrant molecules in wastewater. Example of using bioaugmentation for the pollutant removal from wastewater are presented in Table 2.9.

The inoculant of pure bacterial strains and mixed culture is attractive for addressing the emerging pollutants and pollutants that are present at high concentrations in industrial wastewater. However, the selection of bacteria suitable for introduction into the existing bioreactor have been concerned. The number of exogenous bacteria can be decreased under stress environment, including abiotic and biotic stresses. The abiotic stresses are depended on physical and chemical characteristic of chemical compounds, insufficient substrate, temperature, pH, high pollutant load, and nutrient limitation. For the biotic stresses, including competition between exogenous microbes and indigenous microbes is the main factor of treatment failure due to carbon sources limitation (Bouchez et al., 2000).

Table 2.9 Use of bioaugmentation for removal of recalcitrant compounds in wastewater

Inoculum	Pollutant	Set up	Bioaugmented bacteria	Reference
Mixed culture	Phenolic compounds	Electrocoagulation/bio-active fixed-bed absorber	Actinomycete consortium	Abdulla et al. (2019)
	Electroplating wastewater	Membrane bioreactor	Mixed consortium	Wen et al. (2018)
	Thiabendazole	Batch experiment	Thiabendazole-degrading bacterial consortium	Papadopoulou et al. (2018)
	p-nitrophenol	Sequence batch biofilm reactors	Three bacterial consortia	Yue, Chen, Cheng, Xie, & Li (2018)
	Petroleum hydrocarbons	Biocluster bioreactor	Microbial consortium (A) with 22 microorganisms	Poi, Shahsavari, Aburto Medina, Chum Mok, & Ball (2016)

Table 2.9 Use of bioaugmentation for removal of recalcitrant compounds in wastewater (Cont.)

Inoculum	Pollutant	Set up	Bioaugmented bacteria	Reference
	2,4-dichlorophenol	Continuous flow complete-mixed reactors	2,4-DCP degrading mixed culture	Quan, Shi, Liu, Lv, & Qian (2004)
Single strain	Sulfamethoxazole	Membrane bioreactors	*Achromobacter denitrificans* strain PR1	P. Y. Nguyen et al. (2019)
	Alcohol ethoxylates	Activated sludge reactor	*Pseudomonas* sp. LZ-B	Ji et al. (2019)
	Tricyclazole	Batch reactors	*Sphingomonas* sp. NJUST37	H. Wu et al. (2018)
	Crude oil, PAHs	Batch reactors	*Comamonas testosteroni* BR60	X. Zhao, Yang, Bai, Ma, & Wang (2016)
	4-Fluoroaniline-	Batch reactor	*Acinetobacter* sp. TW	M. Wang et al. (2013)

2.2.4.2 Potential solutions for improvement of bioaugmentation

Potential strategies have been introduced to overcome the limitations of bioaugmentation for recalcitrant compound contaminated wastewater treatment. The possible solutions can be separated into 3 main objectives to increase bacterial

growth/population, biochemical ability and efficiency of bioaugmentation (Nzila et al., 2016).

The use of high inoculant at 10^6– 10^7 CFU mL^{-1} can be addressed for increasing cell growth and enhancing survival of bioaugmented bacterial cells (Lyon & Vogel, 2013). Moreover, quorum sensing inducer could contribute to increase the colonization of bacteria that lead to bacterial cooperation and biofilm formation and thus to an increase in the bacterial population within bioaugmentation (Valle, Bailey, Whiteley, & Manefield, 2004; Yeon et al., 2009).

The use of plasmids encoding function genes is attractive to increase the biodegradation (Nusslein, Maris, Timmis, & Dwyer, 1992; J. S. Singh, Abhilash, Singh, Singh, & Singh, 2011). However this approach still has some limitations that include the high cost of enzyme production, stability of enzyme, the reusability and recovery of enzyme for large scale use (Ba, Arsenault, Hassani, Jones, & Cabana, 2013).

Bioaugmentation- based cell immobilization which embedded the cells within a gel or carrier materials can be used to enhance biodegradation of organic compounds in wastewater. Several advantages of immobilized cells have been provide over freely suspended cultures (Zhu, 2007). Biological water treatment systems containing immobilized cells have been used for the degradation of various wastewater contaminants (Z. Bouabidi, El-Naas, & Zhang, 2018).

2.2.5 Bacterial cell immobilization

2.2.5.1 Cell immobilization methods

There are many ways to immobilize cells in the materials. They can be classified into two major groups: carrier binding and entrapment.

2.2.5.1.1 Cell attachment onto surfaces through adsorption

This method is performed directly in bioreactor by introducing the inoculum and the supporting matrix to the medium (F. W. Bai, Zhao, & Xu, 2011). The adsorption of cells (Figure 2.6) can be attached on surface matrix by van der Waals forces and ionic and hydrogen bonds. Thus, the adsorption of cells is depended on chemical and physical characteristics of the support surface. After cells are attached

to the support, it can release waste products, synthesize, and secrete exo-polymers. It is the so-called biofilm formation (Roig, 2000).

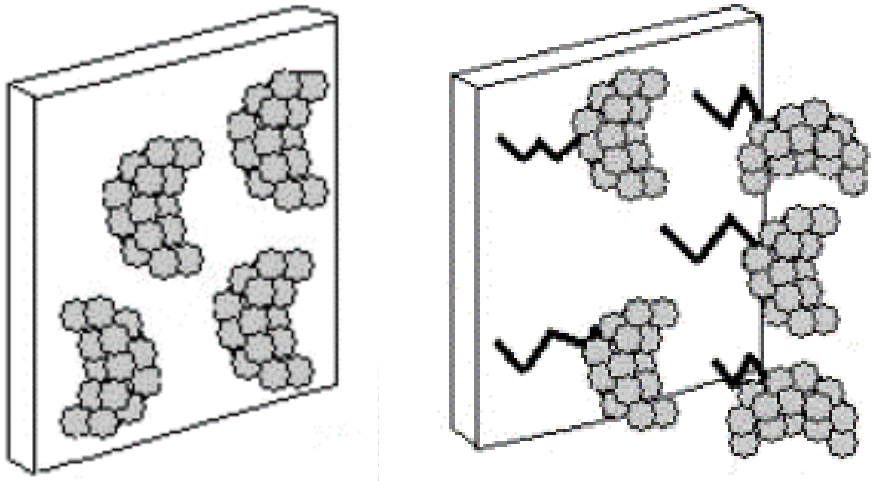


Figure 2.6 Adsorption of cells on carrier; left and covalent bond between biocatalysts and a carrier; right (Z. B. Bouabidi, El-Naas, & Zhang, 2019).

2.2.5.1.2 Cell entrapment in gel matrix

Cell entrapment is widely used in the field of cell immobilization and has several advantages including providing inexpensive and mild conditions for the reaction process. The immobilization technique in which particles or cells are captured within a support matrix (Bayat, Hassanshahian, & Cappello, 2015) , as illustrated in Figure 2.7.

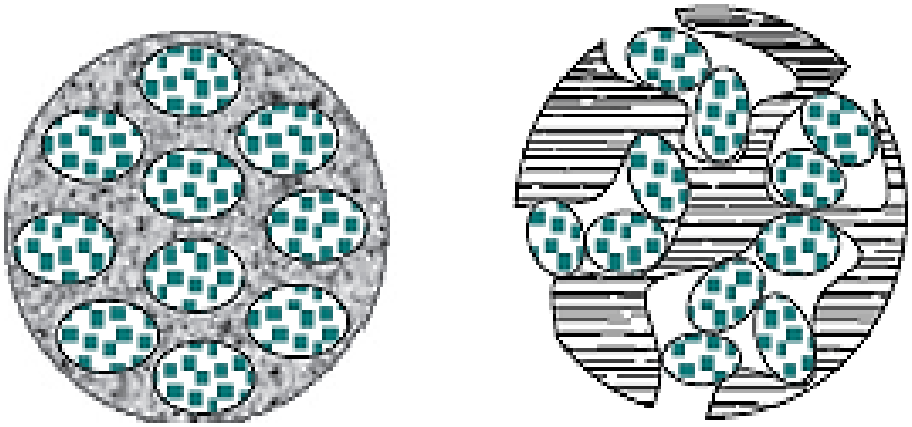


Figure 2.7 Entrapment of cells in gels and occlusion of cells within porous materials (F. W. Bai et al., 2011).

2.2.5.1.3 Cell immobilization in porous media

Porous carrier is defined as solid containing pores. The porous media are required for various industrial wastewater treatments (Vayenas, Michalopoulou, Constantinides, Pavlou, & Payatakes, 2002) which used as a carrier for microorganisms in bioreactors. The use of this kind of immobilization material can generate beneficial properties including mass transfer and permeability; however, its functionalities of this carrier depend on the structural characteristics such as pore size, pore geometries and framework materials (Doonan, Tranchemontagne, Glover, Hunt, & Yaghi, 2010) (Figure 2.8).

A numerous of pore can stimulate the biodegradation process for dealing with recalcitrant organic chemicals. Cell immobilization within porous matrices could allow the diffusion of substrate and product of metabolisms. In addition, it can be used as sorbent has been widely used in biodegradation of petroleum hydrocarbon (Quek, Ting, & Tan, 2006).

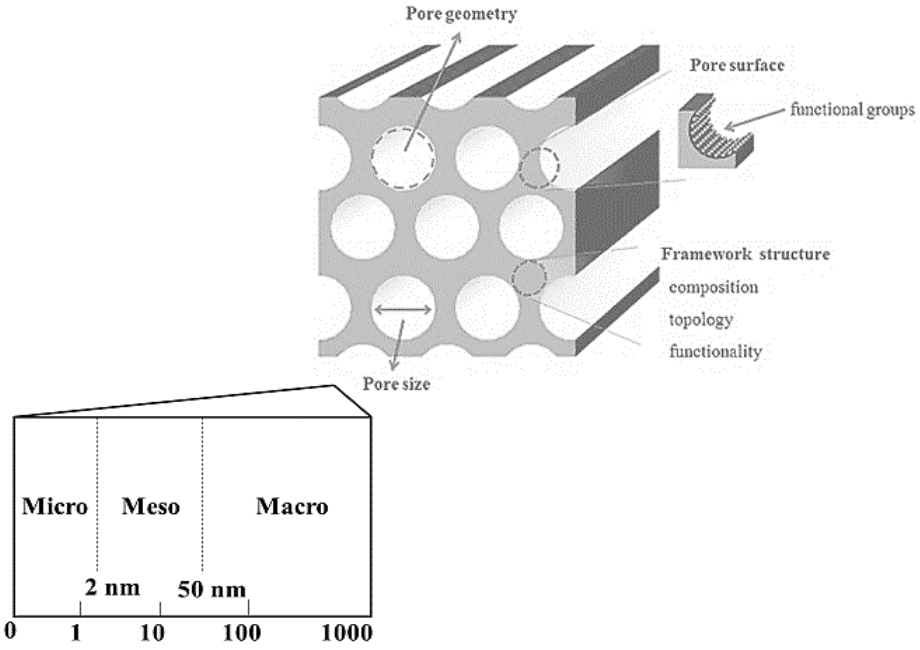


Figure 2.8 Pore surface, pore size, pore geometry, and framework structure of porous media. Modified from previous work (D. Wu et al., 2012).

2.2.5.2 Selection of supporting material for cell immobilization

A supporting material selection is an important step in an immobilization process, because the carrier affects the cell viability and cell physiology that lead to the efficiency of wastewater treatment process (Siripattanakul et al., 2008; Zhu, 2007). The carrier must be biocompatible, insoluble, long shelf life, non-toxic, high cell mass loading capacity, inexpensive, easily to handle and reusability (Bayat et al., 2015). Three types of carriers have been developed for cell immobilization, including organic material, inorganic material and composite carriers (Z. B. Bouabidi et al., 2019) (Figure 2.9). Natural carrier provides higher diffusion rates and are more environmentally friendly, whereas synthetic carrier are more stable and high mechanical strength as well as high oxygen transfer (Hu, Tang, & Wang, 2014; L.-s. Zhang, Wu, & Wang, 2007). Many immobilized cells were used with both natural and synthetic materials (C. Chorao et al., 2009; Nopcharoenkul, Netsakulnee, & Pinyakong, 2013; Zain, Suhaimi, & Idris, 2011; Zia, Zia, Zuber, Rehman, & Ahmad, 2015). Moreover, the material for increasing the ability of cell entrapment is a porogen to make the porosity such as $CaCO_3$ (Y. Zhang & Ye, 2011) and polyethylene glycol (X. Bai, Ye, Li, Zhou, & Yang, 2010; Xiangli, Zhe, Zhiwei, Yinglin, & Zhengjia, 2010). They were used to form a more porous matrix, providing channels for microbial metabolism.

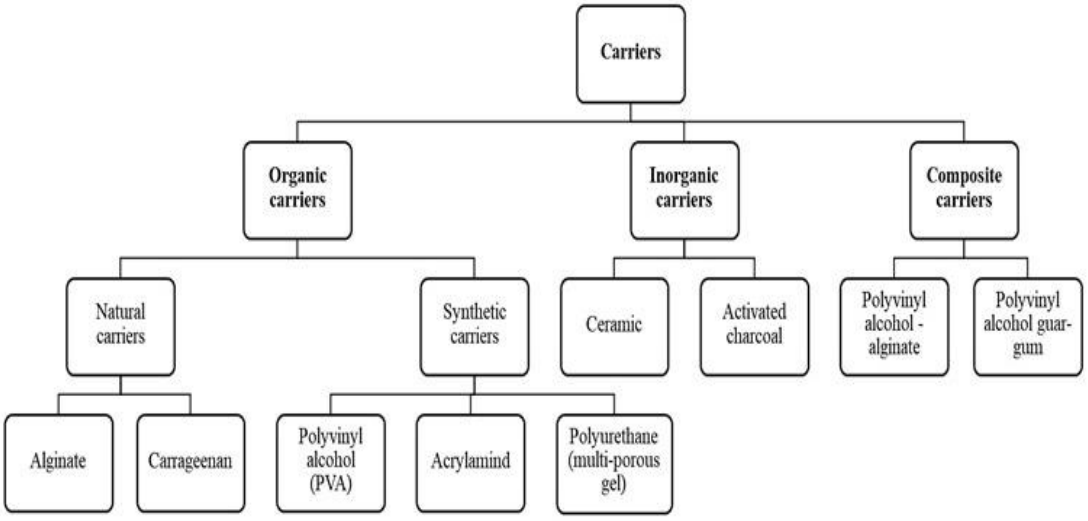


Figure 2.9 Types of immobilizing organic carrier, inorganic carriers, and composite carriers. Modified from Bouabidi et al (2019).

2.2.5.3 Application of immobilized cells for treating benzothiazoles and other recalcitrant compounds in wastewater

Immobilization technology is an effective and promising technique to enhance biological wastewater treatment processes (Abdulla et al., 2019). Recently, the immobilized cells have been developed in bioreactor for treating wastewater. The immobilized cell- based bioreactor can delay the suspended biomass, bound extracellular polymeric substances and soluble microbial products during operation system (C. Juntawang, C. Rongsayamanont, & E. Khan, 2017; Juntawang, Rongsayamanont, & Khan, 2019; Kim et al., 2014; Meshram et al., 2016). A feasibility studies of immobilized cell for removing benzothiazole family and the organic compounds in various wastewater were investigated in different material matrixes as presented in Table 2.10.

Table 2.10 Application of immobilized cells for treating benzothiazoles and other recalcitrant compounds in wastewater.

Type of wastewater	Pollutant	Material carrier	Microorganisms	Reference
Synthetic wastewater	Benzothiazole	Alginate, sand, diatomite, and activated carbon	*Rhodococcus rhodochrous*	Cincilei et al. (2011)
	Amino-benzothiazole	Ca-alginate	*Rhodococcus rhodochrous*	Charlène Chorao et al. (2009)
Textile industry	Synthetic dyes	Polyurethane foam	*Coriolus versicolor* RC3	Kasamsuk & Khanongnuch (2019)
Ammonia-rich wastewater	Ammonia	A phosphorylated-polyvinyl alcohol	Nitrite- Oxidizing Bacteria	Kunapongkiti et al. (2019)
Petroleum refinery wastewater	Phenol and m-cresols	Polyvinyl alcohol (PVA)	*Pseudomonas putida*	El-Naas, Surkatti, & Al-Zuhair (2016)

2.2.6 Identification of bacterial diversity and functional genes in bacterial consortium

Many researches have reported most persistent organic compounds especially benzothiazoles that were resistance to remove in conventional WWTPs. Activated sludge (AS) (M. A. Gaja & Knapp, 1998) , aerobic granular sludge and anaerobic digestion (Y. Li et al., 2017) have been used to remove these pollutants in the wastewater with the complex microbial diversity in wastewater sludge. There are the key drivers of

toxicant treatment that comprised the numerous biodegradation genes. Consequently, the utilization of microbial consortia is an increasing interest in removing or detoxifying organic pollutants. Many culture-dependent conventionally analyses with isolating benzothiazoles-degrading bacteria was observed in the bacterial community are enriched in the artificial selection approach. These observations suggest that *Rhodococcus* sp. (Wever et al., 1997), *Pseudomonas* sp. (El-Bassi et al., 2010), and *Alcaligenase* sp. (Umamaheswari & Rajaram, 2017) jointly contribute to the benzothiazole biodegradation. In addition, the enzyme involved in benzothiazoles metabolism also determined in the above-mentioned species. Owing to the unfavorable condition of rubber wastewater habitat, a microbial consortium with different microorganisms endowed with diverse metabolic capacities had often shown greater in functional stability and efficiency than a pure culture (Cydzik-Kwiatkowska & Zieliński, 2016). Therefore, the utilization of the consortia is important process in the aerobic biodegradation of benzothiazoles.

Traditionally, the culture-dependent tools were carried out to isolate the specific microbes by the enrichment procedure. However, this method has not been established all community within enrichment cultures (Phulpoto, Maitlo, & Kanhar, 2020). Currently, omics are comprehensive approaches based on meta-genetic material directly isolated from environmental samples in order to be used for sequencing and data analysis (Datta, Rajnish, Samuel, Pugazlendhi, & Selvarajan, 2020). This technology consisted of metagenomics, metatranscriptomics, proteomics and metabolomics (Figure 2.10). These effective tools provide excellent opportunities for finding new bacterial strains and functional genes involved in bioremediation of xenobiotic compounds and also understanding how contaminants and treatments affect the complex communities (Bhardwaj, Singh, Jadeja, Phale, & Kapley, 2020; Breton-Deval et al., 2020; Datta et al., 2020).

To date, a few researches on biodegradation of benzothiazoles by mixed microorganisms have been investigated, there are shown low capacity of biodegradation and inconclusive knowledge of functional genes. Consequently, the selection of keys microorganisms and construction of new consortium capable of

degrading recalcitrant organic compounds was investigated in order to be applied for reducing potential toxic of benzothiazoles in the rubber wastewater. Microbial community involved benzothiazoles biodegradation process is available in anaerobic systems, as presented in Table 2.11. Thus, the keys players in 2-MBT biodegradation under aerobic system are necessary to be investigated by metagenomics approach for determining the microbial diversity in the consortia.

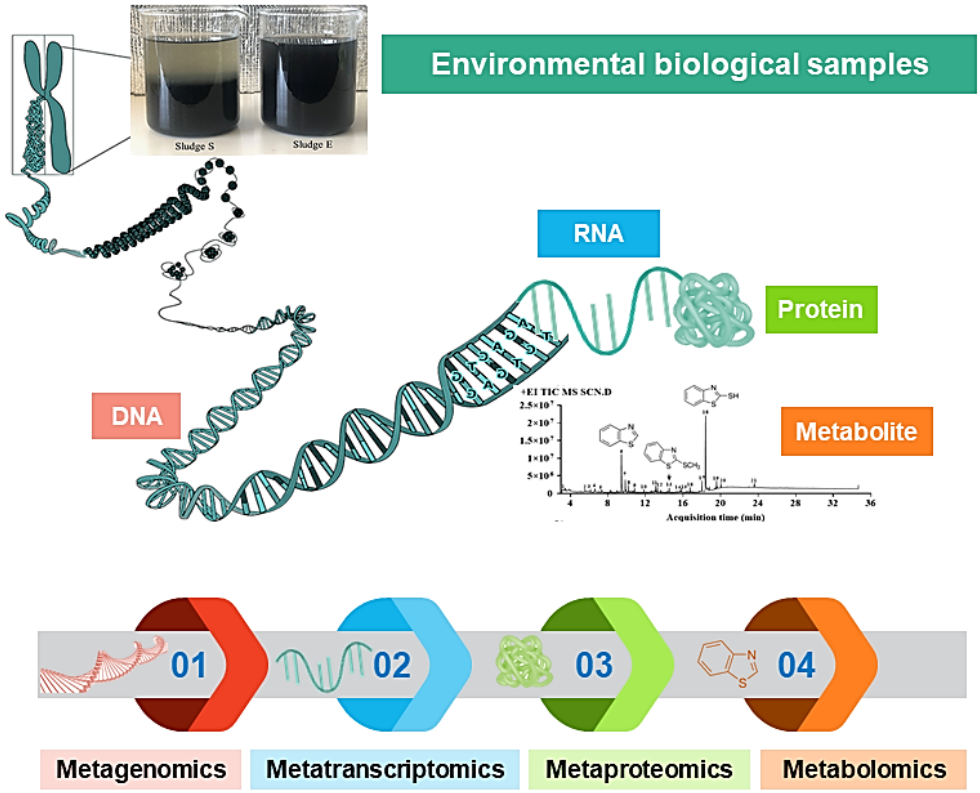


Figure 2.10 Overview of biotechnological tools for microbial community analysis.

In addition, the knowledge of catabolic genes involved in benzothiazoles metabolism is inconclusive in mixed microorganisms (Table 2.12). There is not much knowledge based on meta RNA sequencing approach for a better understanding of the microbial enzymatic degradation of benzothiazoles in diverse community.

Table 2.11 Bacterial community of sludge samples for benzothiazole treatment

Microbial inoculum	Test system/ duration	Type of benzothiazoles	Reference
Anaerobic granular sludge with *Trichococcus* and *Clostridium sensu stricto* as the dominant populations	Anaerobic batch with mixed liquor suspended solids in synthetic wastewater/ 84 h	Benzothiazole	Y. Li et al. (2017)
Diluted river mud with *Geobacter*, *Bacterioides* and *Rhodococcus rhodochrous* as the dominant populations	Microbial electrolysis cells (MECs) conducting in fed-batch mode with mineral medium/ 30 h	2-MBT	San-Martin, Escapa, Alonso, Canle, & Moran (2020)

Table 2.12 Enzymes involved in benzothiazoles metabolism

Microorganisms	Pathway I	Pathway 2	Reference
Rhodococcus rhodochrous OBT18	2-mercaptobenzothiazole monooxygenase	2-mercaptobenzothiazole dioxygenases	Haroune et al. (2004)
	6-hydroxy-2-mercaptobenzothiazole monooxygenase	2-mercaptobenzothiazole-cis-6,7-dihydrodiol dehydrogenase	
	catechol 1,2-dioxygenase	catechol 1,2-dioxygenase	
Escherichia coli HB101	thiol S-methyltransferase		A. Drotar, G. A. Burton, J. Tavernier, & R. Fall (1987b) Reemtsma et al. (1995)
Corynebacterium sp. strain SO1A			
Pseudomonas sp. Strains PF4, PF12, FB1024			
Microbial consortium	2-mercaptobenzothiazole desulfurase	thiol oxidase or sulfhydryl oxidase	Reemtsma et al. (1995)
Alcaligenes sp. MH146	catechol 2,3-dioxygenase	thiol S-methyltransferase	Umamaheswari B. et al (2017)
Pseudomonas putida HKT554	naphthalene dioxygenase.		El-Bassi et al. (2010)

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and analytical methods

3.1.1 Materials

3.1.1.1 Chemical and media

2-Mercaptobenzothiazole (2-MBT) was purchased from TCI Company (Japan). The nitrogen-free minimal salt medium (NF medium) containing (g L^{-1}): KH_2PO_4 1; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.02; $FeCl_3$ 0.004 were used in this study based on Haroune et al. (2002). The nitrogen-containing MSM (N medium) included 1 g L^{-1} of NH_4Cl and yeast extract to NF medium composition. When sodium acetate (AC) was applied at a concentration of 0.82 g L^{-1} (10 mM) for use as a co-carbon source (Haroune et al., 2002) in NF and N media, the media were designated NF-AC^+ and N-AC^+, respectively. The composition was dissolved in 1 liter of distilled water and adjust pH to 7.20 by 1.0 M NaOH. Luria-Bertani (LB) medium was used for quantification of cell number as colony forming unit (CFU). All other chemicals were of analytical grades. All media were autoclaved at 121°C for 15 min.

3.1.1.2 Porous carriers

A commercial porous carrier, aquaporousgel (Nisshinbo Chemical Inc. Tokyo, Japan), was used as supporting media for immobilizing bacterial cells because of its high porosity, highly compatible with microorganisms and good mechanical properties. This material is composed of polyurethane resin, polyethylene polypropylene glycol (porogen) and barium sulfate. The feature of this material is high water absorption and large surface area for bacterial affinity. The amounts of attached bacteria on material are approximately 10 to 12 gSS $carrier^{-1}$ L^{-1}, as described by Nisshinbo Chemical Inc.

Table 3.1 Characteristic of porous carrier used in this study

Model	 CC-10B
Material	> 80% of Polyurethane < 10% barium sulfate < 3% polyethylene polypropylene glycol
Appearance	Cube
Size	0.7 × 0.7 cm
Density	30 dry-Kg wet $^{-1}$ m^{-3}
Amounts of attached bacteria	10-12 g SS carrier^{-1} L^{-1}

3.1.2 Analytical methods

3.1.2.1 2-MBT analysis

Residual of 2- MBT in cultured medium was analyzed via UV– Vis spectrophotometer (Spectroquant Pharo 100 Merck) at OD_{320} (Redouane-Salah, Malouki, Khennaoui, Santaballa, & Canle, 2018). The 2- MBT concentration was calculated from a standard curve plotting the absorbance of pure chemical of 2-MBT versus its concentration in MSM (R^2, 0.9978). The detection limit was 1 mg L^{-1}.

3.1.2.2 Benzothiazoles analysis

GC-MS in scan and selected ion monitoring (SIM) mode was employed to analyze the presence of benzothiazoles and 2-MBT in rubber processing wastewater. For sample preparation, the raw wastewater was filtered through 0.7 µm glass fiber paper (Whatman GF/F) and extracted twice with ethyl acetate at a 1:1 ratio as described in Umamaheswari and Rajaram (2017). The extractant was further dried with Na_2SO_4 and dissolved in methanol before being subjected to GC-MS analysis (Umamaheswari & Rajaram, 2017). GC-MS analysis was performed by using a model 7890B Triple Quad MS (Agilent, USA) equipped with an HP-5ms column (30 m × 0.25 mm, 0.25 µm). The column temperature started at 70°C, was raised at a speed of 10°C min^{-1} to 180°C with a holding time of 1 min, and was increased further to 250°C at 15 min with a final hold time of 15 min. The injector temperature was set at 270°C. The optimized MS conditions were as follows: ionization energy, 70 eV; scan mode, full scan; and 50-550 amu.

3.1.2.3 Wastewater analytical methods

The analytical methods pertaining to parameters of wastewater characteristics and degradation, namely, COD (5220 D), total Kjeldahl nitrogen (TKN) (4500-N_{org} B.), and sulfate (4500-SO4-E), were estimated as per standard methods (APHA, 2005). pH was measured using a benchtop pH meter (WTW Inolab 7110, Germany).

3.1.2.4 Biomass determination

3.1.2.4.1 Suspended cells

The volatile suspended solid (VSS) content was analyzed using the standard method (USEPA, 2001). Briefly, the bacterial culture was harvested and filtered through a glass fiber paper (Whatman GF/F). Filter paper with the samples were dried at 105 °C until a constant weight is obtained. The dried filter papers with the sample were then heated at 550 °C, at this stage the organic matter was volatized. For cell turbidity, the bacterial culture was determined as cell optical density at 600

nm using a spectrophotometer. Biomass concentration was measured by a UV-visible spectrophotometer (Spectroquant Pharo 100 Merck, USA) at OD600.

3.1.2.4.2 Bacterial numbers in porous carriers

To quantify the bacterial numbers in the porous carriers, 1 g of porous carrier were cut into small pieces, resuspended in 10 mL of 0.85% of NaCl in a test tube, sonicated by ultrasonicate bath for 10 min, and subjected to vigorous vortex mixing for 2 min, which designated as cell suspensions. The dilution series of cell suspension were plated on LB agar by drop plate technique and incubated before calculating the bacterial numbers as CFU ml^{-1}. Since the bioreactor had high turbidity provided by the seed inoculum, the cell suspensions of immobilized activated sludge were measured as volatile suspended solids (VSS).

3.1.2.5 Protein concentration determination

The samples (1 mL) were collected and centrifuged at 10,000 rpm, 5 min. Then, the cell pellets were washed twice and suspended in 0.85% (w/v) NaCl. The protein in cells were extracted by heating at 100°C for 10 min and centrifuged at 10,000 rpm for 5 min. The cell-free supernatant (100 μL) was used to examine protein concentration. Protein determination was determined according to the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard protein. For the sample, the 30 μL extracted protein from cells was used and added 1.5 mL of Bradford reagent (F. He, 2011) into each tube. The mixture was mixed and incubated at room temperature (RT) for at least 5 min. The sample protein reaction was measured absorbance at 595 nm. The protein concentration of sample was calculated from the equation that provided by the standard curve of BSA.

3.1.2.6 Quantification of bacterial survival by qPCR analysis

Quantitative PCR of *Pseudomonas* genera was used to monitor the number of dominant populations in the bacterial consortium using an Agilent Mx3005PQPCR System (Agilent Technologies). A pure culture of *Pseudomonas aeruginosa* CDRS2 (Appendix C. 1) was used to make a standard curve, which was calculated as previously described (Park & Crowley, 2005). Standards, unknown

samples, and negative controls were simultaneously quantified in triplicate using a 20 μL reaction volume containing 10 μL of SYBR Green PCR Kit (Qiagen, USA), 0.7 μL each of 10 μM F and R primers, 1 μL of DNA, and 7.6 μL of dH_2O (Millipore, USA). Total genomic DNA was quantified using the primer set Pse435F/Pse686R (Bergmark et al., 2012) with pre-denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 60°C for 20 s and 72°C for 25 s. The linear correlation coefficient for the standard curves was 0.997, and q-PCR was analyzed with an efficiency of 100 ± 10.

3.1.2.7 Scanning electron microscopic (SEM) analysis

The morphology and microstructure of porous carrier-immobilized cells were observed by a scanning electron microscope (SEM). The immobilized cells were prepared at Scientific and technology research equipment center, Chulalongkorn University, Thailand. Each sample was examined using a JEOL scanning electron microscope; model JSM-6610LV (Jeol, Tokyo, Japan) at 15 kV. The results of this experiment were used to confirm that the bacterial cells completely adhered and distributed on the surface and inside the carrier. In addition, the SEM observation was conducted after reusability test. The result was useful for the investigation of cell damage or cell abundance after the application of immobilized cells in biodegradation process.

3.2 Experimental procedure

The experiments were divided into four major sections including 1) wastewater collection and characterization, 2) enrichment and characterization of 2-MBT-degrading bacterial consortia from rubber wastewater sludge, 3) characterization of the bacterial community structures and functional genes of 2-MBT degrading bacterial consortia by molecular analyses and 4) development of an immobilized 2-MBT degrading bacterial consortium for applying in rubber processing wastewater.

3.2.1 Wastewater collection and characterization

3.2.1.1 Sample collection

Two rubber wastewater sludge samples were collected from a block rubber production factory, E in Eastern part of Thailand and a latex concentrate production factory, S in Southern, Thailand. For factory S, the wastewater is discharged to the integrated wastewater pond, this wastewater derived from the skim crepe production at the rubber trap pond and concentrated latex wastewater. This industry generated wastewater at approximately 500 m^3 day^{-1} depending on the manufacturing practices. In addition, the major products of factory E are Standard Thai Rubber (STR) and compound rubber. This factory produces these products reach to 12,000 and 4,500 metric tons per month, respectively. The wastewater released from this factory is at approximately 800 m^3 day [1]. Rubber industrial wastewater treatment diagram of both S and E factories were presented in Figures 3.1 and 3.2.

The samples were collected at the integrated wastewater ponds of both factories S and E representing the raw wastewater samples (influent), while the sample collected at the pond after releasing from activated sludge process in wastewater treatment of factory E representing treated wastewater sample (effluent). The samples were stored in PE bottles and sealed with Para-film to reduce the oxidation and further transferred to the laboratory. All the samples were refrigerated at 4°C. Before analysis, all wastewater samples were filtered through a 0.7 µ glass filter paper (Whatman GF/C) to remove the suspended solids. The wastewater quality was tested as in topic 3.1.2.

The original sludge samples were collected at the same period with wastewater collection, as presented in Figure 3.3. The initial biomass concentrations of the sludge E and S samples were measured by VSS determination. The biomass concentrations of both original sludge E and S samples were 10.52 and 13.08 g VSS L^{-1}, respectively. Before using the original sludge, the genomic DNA in all the sludge samples were extracted for bacterial community analysis.

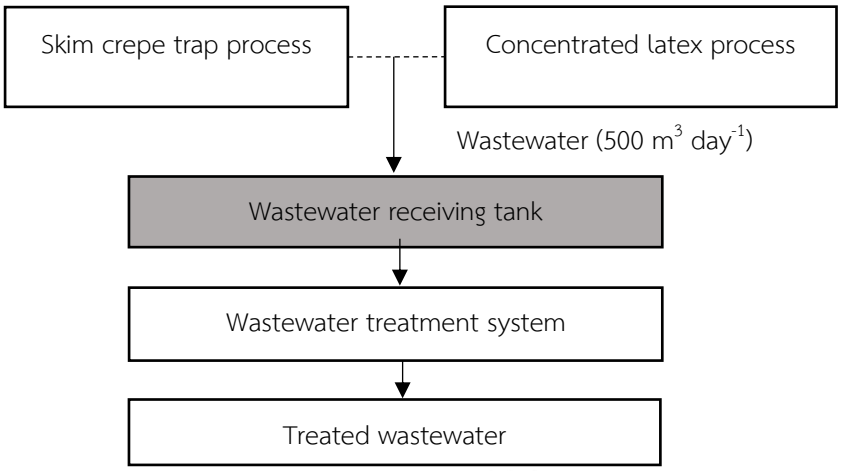


Figure 3.1 Rubber industrial wastewater treatment diagram of factory S. Influent rubber wastewater was collected from the wastewater receiving tank (grey).

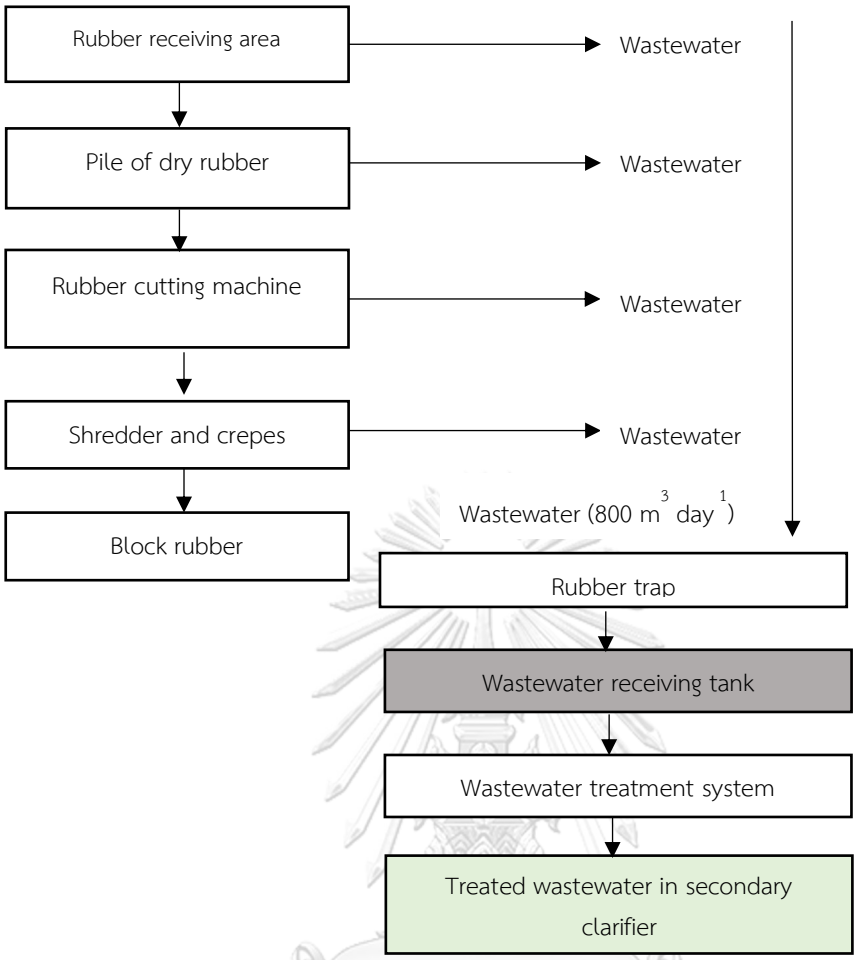


Figure 3.2 Rubber industrial wastewater treatment diagram of factory E. Influent and effluent of rubber wastewater were collected from wastewater receiving tank (grey) and secondary clarifier (green), respectively.

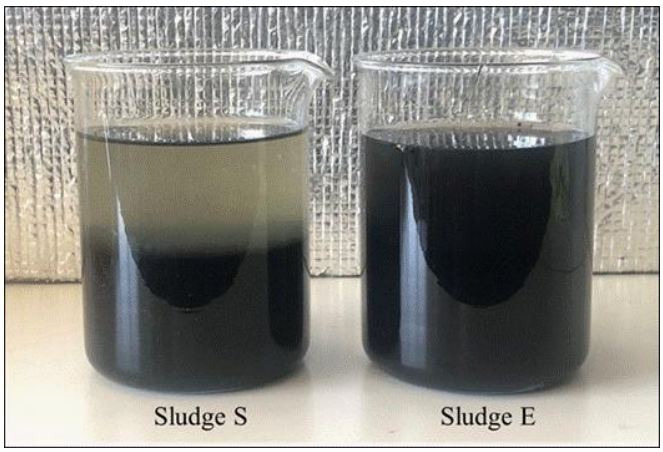


Figure 3.3 Original sludge samples i.e. sludge S and sludge E from full scale digesters in WWTPs of factories S and E, respectively.

3.2.1.2 Characterization of organic compounds in rubber processing wastewater

This work characterized organic compounds from the rubber wastewater using gas chromatography-mass spectrometry (GC-MS) analysis. Scan and selected ion monitoring (SIM) modes were employed to analyze the presence of benzothiazoles and 2-MBT in rubber processing wastewater. For preparing the samples before determining by GC-MS, the liquid-liquid extraction (LLE) was selected to extract the wastewater samples. The LLE was based on the partition of organic compounds between aqueous sample phase and organic solvent. Ethyl acetate was selected to use as organic solvent which had the polarity index of 4.4 (Wells, 2003). The use of ethyl acetate 1:1 (v/v) with the sample was according to the previous work (Umamaheswari & Rajaram, 2017). In addition, the shaking time of samples with ethyl acetate was 15 min as described in previous work (Wongniramaikul, 2006).

3.2.2 Enrichment and characterization of 2-MBT-degrading bacterial consortia from rubber wastewater sludge

3.2.2.1 Enrichment process

The enrichment process was used to enrich active 2-MBT-degrading bacterial consortia and increase the number of 2-MBT-tolerant in rubber wastewater sludge from wastewater sludge collected from two rubber factories. The effect of wastewater sludge sources on 2-MBT biodegrading consortia was compared. This research hypothesized that the different characteristics of rubber wastewater from various rubber processing industries could affect the diverse and adaptation of microbial communities in wastewater sludge.

Briefly, 2 g VSS L^{-1} of original sludge was applied to 100 mL of nitrogen-containing medium (N medium) which supplemented 25 mg L^{-1} of 2-MBT in 250-mL flasks and incubated for 14 days at 30 ± 2°C on a rotary shaker operated at 150 rpm. In this work, the 250-mL flasks are used to enrich the efficient 2-MBT degraders (Figure 3.4).

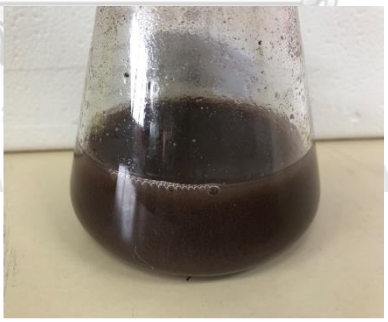


Figure 3.4 Photograph of the 250-mL flask containing sludge for enrichment process

The enriched sludge was transferred at 10% (v/v) to freshly medium containing 50 mg L^{-1} of 2-MBT and incubated for another 14 days. After 28 days of incubation, the enriched sludge samples were analyzed for VSS concentration, and 2 g VSS L^{-1} sludge was applied to a new medium containing 50 mg L^{-1} for the 2-MBT biodegradation test. The study compared four media, including N, NF, N-AC^{+} and NF-

AC^+, to identify the necessary nutrients for further acclimatization of 2-MBT-degrading bacteria. After 28 days, the enriched sludge samples were analyzed for VSS concentration, and 2 g VSS L^{-1} sludge was applied to a new medium containing 50 mg L^{-1} for the 2-MBT biodegradation test.

The media conditions where nitrogen-free medium (nitrogen in the substrate; NF medium), nitrogen-containing medium (1 g L^{-1} of NH_4Cl; N medium) and co-substrate addition (10 mM of sodium acetate; AC as co-carbon) at 30°C and 150 rpm. Sodium acetate have been used as co-carbon source for bacteria cultivation (H. De Wever et al., 2001; Y. Li et al., 2018), while 2-MBT is known to be toxic and rarely used as a primary growth substrate for cultures derived from activated sludge (H. De Wever et al., 1994; M.A. Gaja & Knapp, 1997). In this study, sodium acetate was therefore selected as co-carbon source for increasing population of bacterial cells obtaining from rubber wastewater sludge.

There were sixteen of batch test that consisted of enriched sludges compared with the original sludge in the same media conditions (SN, SNF, SN-AC^+, SNF-AC^+, EN, ENF, EN-AC^+, and ENF-AC^+) as shown in Figure 3.5. All tests were incubated at 30 ± 2°C with constant shaking at 150 rpm and carried out in triplicate.

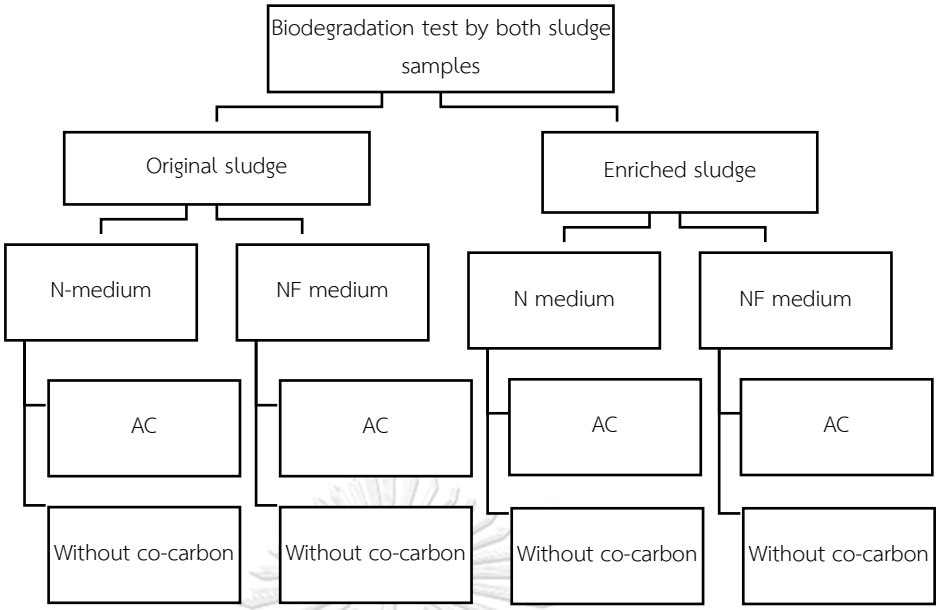


Figure 3.5 Scheme of biodegradation tests of enriched sludge and original sludge samples in various media conditions

The samples were collected every 24 h to determine the residual concentration of 2-MBT and calculated by the following equation 1.

$$\text{Biodegradation efficiency } (\%) = \frac{\text{Initial 2-MBT concentration} - \text{Final 2-MBT concentration}}{\text{Initial 2-MBT concentration}} \times 100 \quad \text{(eq. 1)}$$

where the initial concentration is the original added concentration of 2-MBT, and the final concentration is the remaining concentration of 2-MBT in the supernatant after removing the sludge by centrifugation. The enriched sludges and media showing high 2-MBT biodegradation efficiency were selected for the following acclimatization process. All data were statistically analyzed via Tukey's multiple comparisons following a two-way ANOVA with GraphPad Prism 8.0.1 software (CA, USA). The enriched sludges and media showing high 2-MBT biodegradation efficiency were selected for the following acclimatization process.

3.2.2.2 Acclimatization process

Since 2-MBT concentrations at higher 100 mg L^{-1} can interfere with membrane-bound system of bacterial cells, the condition of gradually increasing 2-MBT concentrations in artificial bacterial selection system was studied for reducing the toxicity effect of 2-MBT and increasing the ability of bacterial cells to adapt in 2-MBT containing system.

All acclimatization experiments started by adding 2 g VSS L^{-1} enriched sludge to 250-mL flasks containing 100 mL medium and incubating the flasks at 30 ± 2°C with constant shaking at 150 rpm. The acclimatization process was divided into three stages, I, II and III, based on the 2-MBT concentrations in the medium, which were 50, 100 and 200 mg L^{-1}, respectively. The 2-MBT concentrations in this work corresponded to the ranges existed in rubber processing wastewater (Derco et al., 2014). In each stage, dilution-to-extinction approaches was employed, there were three rounds of cultivation. When the 2-MBT concentration was reduced by 80%, 10% (v/v) of the cell suspension was transferred to freshly medium supplemented with 100 mg L^{-1}. After three rounds, the 10% (v/v) of cell suspension further transferred to a new flask with 100 mL of MSM supplemented with 200 mg L^{-1} of 2-MBT. All enrichment cultures were cultured at 30°C and 150 rpm and performed in triplicates. All acclimatized cultures were performed in triplicate and sampled at 24-h intervals to determine the residual 2-MBT, soluble chemical oxygen demand (CODs) and bacterial growth. Autoclaved cells were also used as abiotic control to account for 2-MBT absorption. The 2-MBT biodegradation efficiencies of the bacterial consortia at the end of each acclimatization stage were determined as described and using equation 1.

All the data were statistically analyzed via two-way ANOVA followed by Tukey's multiple comparison test with GraphPad Prism 8.0.1 software (CA, USA). The bacterial consortia obtained from acclimatization after stage III were preserved in MSM containing 20% glycerol and 50 mg L-1 2-MBT and stored at -80°C until use. The bacterial consortia obtained from acclimatization after stage III were preserved in MSM containing 20% glycerol and 50 mg L-1 2-MBT and stored at -80°C until use. The

acclimatized bacterial consortia were then characterized for biodegradation of 2-MBT in both MSM and rubber wastewater.

3.2.2.3 Efficiency of bacterial consortia on degrading 2-MBT at varying concentrations

The bacterial consortia (SN, SN-AC^{+}, EN and EN-AC^{+}) were achieved by gradually increasing the 2-MBT concentrations from 50 mg L^{-1} until it reached 200 mg L^{-1}. In this study, their biodegradation ability at different 2-MBT concentrations (50, 100, 150, 200, 300, and 500 mg L^{-1}) were examined to comparative analysis of the specific degradation rate under resting cell experiments. This result was used for selecting an efficient consortium in order to be applied in benzothiazoles-contaminated rubber wastewater. Resting cells condition has been carried out for batch biodegradation tests to minimize changes in physiological and biochemical properties of cells (Chudoba, Capdeville, & Chudoba, 1992).

To prepare the resting cells, bacterial consortia were individually cultured in LB broth with shaking condition at 150 rpm and 30 ℃ for overnight in order to increase their cell number, designed as cell starter. The active starter culture at 5% was transferred into 100 mL of minimal medium containing 50 mg L^{-1} of 2-MBT as activator substrate and incubated for 16-18 h at 30 ℃ and 150 rpm. The bacterial cultures were centrifugal collected at 10,000 rpm, for 10 min and washed twice with sterile 0.85% NaCl. After adjusting the cell density to 2.0 OD at 600 nm, 1 mL of the active cells corresponded with an initial protein content of approximately 1.6 mg cell protein was added into 9 mL of N medium supplemented 2-MBT in different concentrations.

All batch experiments were incubated at 30 ± 2℃ with constant shaking at 150 rpm. The duplicate samples were collected at 24 h intervals to determine the residual of 2-MBT occurred in the liquid medium and protein concentration. The specific biodegradation rates were calculated by a plot of the 2-MBT concentration versus time based on the protein content of the biomass at each sampling time.

3.2.2.4 Efficiency of the bacterial consortium on degrading 2-MBT in rubber wastewater

The efficient bacterial consortium from acclimatization process stage III was selected by the result of highest specific degradation rate to evaluate its the potential application with wastewater collected from an activated sludge plant at the E factory. Prior to characterizing bacterial consortium on 2- MBT removal, the wastewater sample was analyzed by GC-MS in SIM/scan mode.

To simulate the 2-MBT concentration range generally found in rubber wastewater, 2-MBT was spiked at 100 mg L^{-1} into the sample prior to the experiment. For biodegradation assay, the loading amount an initial bacterial consortium at approximately 10^7 CFU mL^{-1} was inoculated into a 250-mL flask containing 100 mL of the 2-MBT-amended wastewater. The wastewater samples were consisted of into non-sterile and sterile wastewater conditions to investigate the effect of indigenous microorganisms on the added bacterial consortium. Additionally, non- sterile wastewater without bacterial inoculation was used to evaluate the efficiency of natural attenuation.

All experimental samples were incubated at 30 ± 2°C and 150 rpm and performed in triplicate. During the treatment period, the sample was analyzed the COD and 2-MBT concentrations. At the end of the experiment, benzothiazoles, TKN and sulfate were analyzed. Finally, the dominant population of the bacterial consortium was determined by quantitative PCR.

3.2.3 Characterization of bacterial community structures and functional genes in 2-MBT degrading bacterial consortia

Microbial community structure and functional genes in bacterial consortium influence the performance of wastewater reactor and have an impact on the treated water quality (Henze, van Loosdrecht, Ekama, & Brdjanovic, 2008). This phase was separated into 2 main parts to identify the dominant populations and functional genes within the bacterial consortia.

3.2.3.1 Identification of bacterial community structures in original sludge and enriched 2-MBT-degrading bacterial consortium by 16s rRNA gene sequencing technique

Bacterial community structure of the two original sludges and bacterial consortia from enrichment and acclimatization process were investigated and compared. The obtained data from this part was used to describe the biodiversity and the potential active bacteria in each bacterial consortium. Moreover, the results of metagenomics profiling were used as a supporting data for selecting the efficient bacterial consortium.

3.2.3.1.1 DNA extraction

Briefly, 0.25 g of rubber wastewater sludge and 1.5 mL of bacterial consortium were collected and extracted to obtain genomic DNA by using a PowerSoil® DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's protocol. Prior to the extraction, lysozyme was added to the samples at 35 mg mL^{-1} to remove the cell wall of gram-positive bacteria, and the samples were incubated for 1 h at 37°C (Sarma, Nava, Manriquez, Dominguez, & Lee, 2019). Then, the quality and quantity of the extracted DNA were measured using a NanoDrop™ND-1000 (Thermo Scientific, USA) and stored at -20°C before analysis.

3.2.3.1.2 PCR amplification, sequencing, and data analysis

In this work, the extracted genomic DNA was amplified by primers targeting the V4 region of 16s rRNA gene, because these primers are recommended to assess environmental sample (Kuczynski et al., 2011). They also provide the short-read length that has sufficient resolution for the accurate taxonomic analysis. The specific primers targeting the V4 region (515F-806R) with the barcode are shown in Table 3.2 as previously described (Caporaso et al., 2011). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Then, the mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen,

Germany). The libraries were generated with DNA Library Prep Kit, quantified, and analyzed by Illumina platform.

Table 3.2 PCR primer sequences used in this study

Primer name	Primer sequence	Reference
515F	GTGYCAGCMGCCGCGGTAA	Caporaso et al. (2011)
806R	GGACTACNVGGGTWTCTAAT	Caporaso et al. (2011)

3.2.3.1.3 Bioinformatics analysis

The resulting sequence read files were processed by Mothur software (Version 1.39.5). Sequences with 97% similarity were assigned to the same OTUs. Taxonomic classification of each OTU was assigned by the SILVA database (Version 132). Alpha diversity was measured to obtain the number of observed OTUs, representing the microbial richness. In addition, the bacterial community richness was obtained by Chao1, and the community diversity was obtained by the Shannon diversity index. The sequencing data were deposited into the European Nucleotide Archive (ENA) database with submission number ERP120594 and BioProject ID PRJEB37286.

3.2.3.2 Functional gene analysis in the 2-MBT degrading bacterial consortium by metatranscriptome analysis

The selected bacterial consortium (EN) was investigated for the full set of genes by the metatranscriptome analysis and compared with the original sludge from rubber industry E. This experiment was examined on RNA-seq platforms. The result of this part was used to measure gene expression of all populations in the bacterial consortium and to predict genes involved in 2-MBT biodegradation.

Functional genes in the entire microbial community of the efficient bacterial consortium were analyzed to understand the mechanisms involving 2- MBT biodegradation.

3.2.3.2.1 Sampling of cultures

Prior the RNA extraction, the EN consortium was cultivated in the N medium under aerobic condition at 30 ± 2°C. When the cells are growing, inducible promoters that related to the metabolism-linked genes are highly expressed during late log phase. Preliminary result showed that cell suspensions should be collected at 21-30 h (late log phase) for RNA extraction. The analysis of 2-MBT and cell growth was described in topic 3.4. The experiment was carried out in triplicates.

3.2.3.2.2 RNA extraction, library preparation and sequencing

For RNA extraction, 2 mL of culture suspensions and 0.25 g of original sludge were extracted by Total RNA Miniprep Kit (NEB) following manufacturers' protocol. The RNA samples were eluted using salt buffer and re-suspended in Rnase-Free water. Then, the RNA extracting samples were treated with Dnase to remove DNA contamination. Quantity of total RNA samples were assessed by three main methods including Nanodrop, Agarose Gel Electrophoresis for tests RNA degradation and potential contamination and measurement of RNA integrity and quantitation. After the quality of RNA assessment, the samples were further reversed to cDNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), following manufacturers protocol. The double-stranded cDNA library of samples was completed through size selection and PCR enrichment. Then, the qualified libraries were subsequence fed into Illumina sequencers.

3.2.3.2.3 Bioinformatic analysis

The fastQ files from paired end sequencing will be analyzed using MG-RAST server (http://metagenomics.nmpdr.org) (Meyer et al., 2008). The

protein sequence homology was investigated using M5NR database that integrates Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

3.2.4 Development of an immobilized 2- MBT degrading bacterial consortium for applying in rubber processing wastewater

The selected bacterial consortium was maintained in minimal medium supplemented with 50 ppm 2-MBT and preserved in 30% glycerol at -80℃ refrigerator prior to use. This study aimed to develop an immobilized 2- MBT degrading bacterial consortium for applying in rubber processing wastewater. The efficient bacterial consortium that obtained from 3. 2. 2 was immobilized in a porous carrier under optimum conditions. Moreover, this phase optimized the preparation process of immobilized cells and evaluated the treatment performance of 2- MBT in rubber wastewater. The work focused on immobilization- based bioaugmentation in the bioreactor with the indigenous microbes for treating 2- MBT and other organic compounds in real wastewater. Thus, this study proposes the use of porous carriers as a suitable matrix for bacterial consortium immobilization and its bioaugmentation application in treatment of high strength rubber processing wastewater.

3.2.4.1 2-MBT adsorption capacity of aquaporousgel

The adsorption process was carried out to investigate sorption capacity of a porous carrier. Two g of aquaporousgel was used in 100 mL of minimal medium containing various concentrations 100 mg L^{-1} of 2-MBT (pH 7.20) at 150 rpm and room temperature for at least 48 hours to ensure that adsorption equilibrium was achieved. The obtained result of abiotic sorption was used to estimate the carrier concentrations in bioreactor. The result obtained as an amount of solute adsorbed onto the carriers as followed the equation 2.

$$Qe = ((C0 - Ce)V0) / m \qquad \text{(eq. 2)}$$

When Q_e is the amount of solute adsorbed per weight of solid at equilibrium, C_0 is the initial concentration of the 2- MBT, C_e is the equilibrium concentration of solute remaining in solution, V_0 is initial volume of the solution and m is the weight of porous carriers added in the solution.

3.2.4.2 Optimization of cell immobilization conditions

3.2.4.2.1 Medium cultivation condition

Since rich medium may interfere with enzyme involved in the degradation of aromatic compounds (Díaz, Jiménez, & Nogales, 2013). This study investigated the influence of rich medium and minimal medium on growth and degradation activity of EN consortium. Benzoate was demonstrated to induce *catechol dioxygenase* (Cenci, Caldini, & Biotechnology, 1997) and it is used as a co-carbon source in benzothiazole degradation (Haroune et al., 2002). Thus, this work aimed to use benzoate to induce the enzymes in 2-MBT metabolic pathway. The effects of benzoate on 2-200 mg L^{-1} MBT biodegradation and cell growth were investigated. This 2-MBT concentrations were chosen based on the highest degrading activity of EN consortium. The experiment was conducted in 250 mL Erlenmeyer flask containing 100 mL of minimal medium supplemented with benzoate at 2, 5, and 7. 5 mM, and then incubated at 150 rpm shaking condition at 30 ºC. The cell suspension was collected every 24-h for analysis of 2-MBT and cell growth. The analysis was described in topic 4.3. The optimum condition was selected for preparing immobilized bacteria in section 3.3.3.2.3.

3.2.4.2.2 Cell concentration and incubation time

In this part, cell loading, and incubation time were varied to determine an optimum cell density inside the porous carrier. Different concentrations

of inoculants (2%, 5%, and 10% v/v) were added into the N medium under aerobic condition. The incubation time was tested at 16, 24, and 48 h. The suitable immobilized bacteria refer to those with high cell density in a carrier and high degradation activity in a shorter time. Briefly, the inoculum was added to 100 mL medium which containing 2 % (w/v) sterilized porous carrier in order to provide good mixing of submerged carriers in the medium and then incubated with shaking at 150 rpm at 30°C. After incubation, the immobilized cells were filtered through a sterilized stainless-steel sieve and washed twice with a normal saline (0.85% w/v NaCl). All steps were performed under sterilized condition. The determination of cell concentration in the porous carriers was described in analytical methods. In addition, the morphology and microstructure of the porous carrier-immobilized cells was observed by SEM analysis.

3.2.4.3 Biodegradation of 2-MBT by free and immobilized EN consortium under batch test

After the optimum immobilization condition was obtained. This experiment compared the biodegradation ability of suspended and immobilized cells on 2-MBT biodegradation. Moreover, the stability of immobilized cells was also investigated. This obtained results can be used to predict the immobilized cell activity in further work.

3.2.4.3.1 Suspended and immobilized EN cells preparation

For suspended cell preparation, 5%, v/v inoculum (2×10^6 CFU mL^{-1}) of the cell starter of EN were transferred into 100 mL of 5 mM benzoate containing MSM and incubated for 18 h at 30 ± 2°C and 150 rpm. Benzoate can be acts as a stimulatory substrate for inducing enzymes which involved in the 2-MBT metabolism (Haroune et al., 2004). The cells were harvested by centrifugation at 10,000 rpm, for 10 min, washed twice with sterile 0.85% NaCl and then resuspended in freshly MSM to use as an active cell in degradation batch test. For immobilized cell

preparation, EN consortium was added at 5% (v/v) into 100 mL of 5 mM benzoate-containing MSM which containing 2 % (w/v) sterilized porous carrier and incubated for a suitable incubation time at 150 rpm shaking condition at 30 ± 2°C.

3.2.4.3.2 Comparison of suspended and immobilized EN consortium on 2-MBT biodegradation

The immobilized cells at 2% (w/v) represented a 10^{12} CFU mL^{-1} of EN cells were transferred to 100 mL MSM containing 300 mg L^{-1} 2-MBT in 250 mL Erlenmeyer flask. Similarly, the suspended cells at the same cell concentration were added to another flask. Biodegradation tests operated under growth dependent condition because this work aimed to measure the cell viability during biodegradation assay. The concentration of 2-MBT was selected based on the previous result by the authors. All experiment tests were carried out at 30 ± 2°C and 150-rpm. The supernatant of the cultured broth was taken at the indicated times for 2-MBT analysis and determination of biomass concentration in the porous media. Control experiment was also carried out using heat-killed immobilized cells to investigate the abiotic in a sterile medium. All experiments were conducted in triplicates.

3.2.4.4 Reusability of immobilized EN consortium in a batch test

This experiment investigated the long term and operational stability of 2-MBT biodegradation by immobilized cells. After 80% of degradation in the first cycle of batch test, 300 mg L^{-1} 2-MBT was added into the spent medium, designed as second batch cycle. Since the effect of spent medium recycle might increase the 2-MBT accumulation that might adversely effect on bacterial activity. In the repeated batch test, the immobilized cells were removed from the spent medium after 10-day operation time, washed with 0.85% NaCl and added into fresh medium containing 300 mg L^{-1} of 2-MBT for the third recycle. The reusability of immobilized cells was continued to the fourth recycle under the same condition, until low degradation activity was investigated. All experiment batch tests were incubated at 30 ± 2°C and 150-rpm and conducted in triplicated. SEM analysis was used to monitor the cell

damage and cell abundance of reused immobilized cells and compared with the initial immobilized cells.

3.2.4.5 Performance of immobilized EN cells in a continuous test

Although the suspended EN cells were proved to be efficient in the effluent from rubber wastewater treatment plant, the continuous operation of biological processes may face the challenges of system instability and low performance biodegradation under high strength of rubber processing wastewater. Bioaugmentation-based immobilization has been used to resolve the potential risk of functional failure and bioaugmented cell loading retention (Q.-Q. Zhang et al., 2017). In addition, advantages of the immobilized cells are a long shelf life and the capability to recycle during treatment operation and to reduce the costly processes of cell recovery (Bayat et al., 2015).

The present work determined the possibility of augmenting immobilized EN bacterial consortium in activated sludge to enhance removal of 2-MBT and other organic compounds from high strength rubber processing wastewater. A porous carrier immobilized EN bacterial consortium was applied in bioreactor containing activated sludge. It was hypothesized that the application of porous carrier did not only support bacterial consortium colonization but also provide space for activated sludge attachment and retention. Consequently, the system simultaneously increased the 2-MBT, COD and other toxic compounds under continuous operation.

3.2.4.5.1 Efficiency of immobilized EN consortium in synthetic wastewater

To compare 2-MBT biodegradation performance of EN cells and indigenous microorganisms in activated sludge from rubber processing industry, three lab-scale stirred reactors (R1, R2 and R3) were used. Stirred tank bioreactors have been used in aerobic treatment for treatment of industrial effluents (Schirmer et al., 2018). An impeller is generally used to disperse the oxygen bubbles by agitation and thus to promote mass transfer of the gas bubbles through the gas-liquid (culture medium) (Narayanan & Narayan, 2019; Schirmer et al., 2018). Although sole agitation in the

bioreactor limits the oxygen transfer rate across the medium surface in the bioreactor (Huang, Chen, & Chen, 2006), this system would support the growth of facultative bacteria existing in the activated sludge as well as in the EN consortium. Consequently, this work used a stirred tank bioreactor that was made from borosilicate glass with four of side arms and a Teflon impeller was installed to provide vigorous mixing. The working volume was 1.8 L. The first reactor contained the immobilized AS only (R1), the second one contained both the suspended activated sludge and EN consortium (R2) and the third one contained both immobilized- activated sludge and EN consortium (R3) (Figure 3.6). The concentration of attached biomass within 20 g L^{-1} of porous carriers was approximately 2.5 g VSS L^{-1}. As the previous work suggested that the mixed ratio of exogenous bacterial consortium and indigenous bacteria should be 1:2 (w/w) (Tao et al., 2016), thus the activated sludge were added at approximately 5 g VSS L^{-1}. These inoculum amounts were used as a seed starter and added into the individual bioreactor containing N medium. The pH was adjusted to 7.0 with the addition of sodium bicarbonate. The 2-MBT was used as a sole carbon source and fed from 50-150 mg L^{-1}, which were represented the 2-MBT concentration in real rubber wastewater as previously reported.

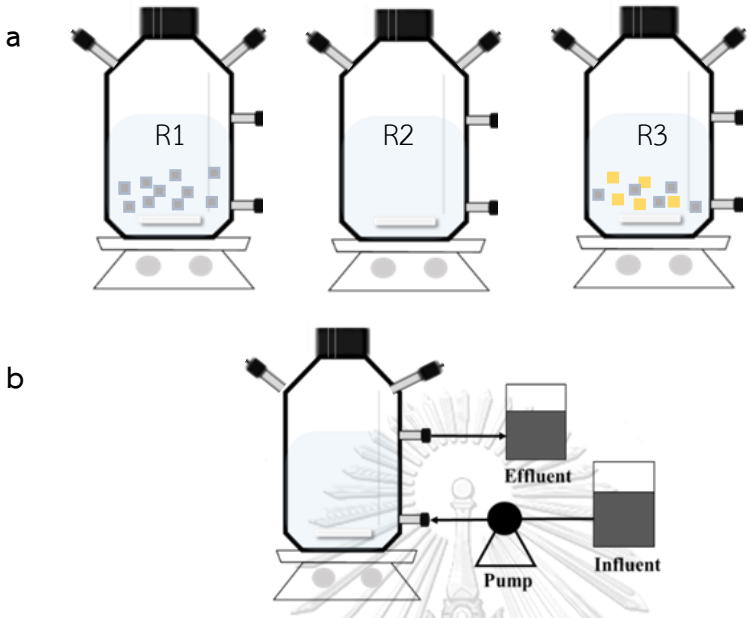


Figure 3.6 Scheme of bioreactor which had side arms for adding influent and taking effluent out while the upper arms (left and right) were used to provide airflow (a). The reactor was fed with wastewater and mixed by an impeller to reach the speed of 200 rpm (b).

All the bioreactors were carried out with the organic loading rate (OLR) of 0.25, 0.75 and 1.0 kg COD.m^{-3}. d^{-1} and at hydraulic retention time (HRT) of 6, 4 and 4. All bioreactors were incubated at room temperature under stirring condition and conducted in a closed chamber to avoid possible photolysis. Since the hydraulic retention time (HRT) was closely related to the amount of influent, the flow rate of influent at 300 ml d^{-1} was initially fed by peristaltic pump (Master flex L/S), while an equal volume of medium in reactor were removed as effluent to analyze parameter analysis.

3.2.4.5.2 Efficiency of immobilized EN consortium in rubber processing wastewater from factory E

The bioaugmentation of R3 reactor was continued to operate with real rubber wastewater at the OLR from 1.0 to 3.0 kg $COD.m^{-3}.\ d^{-1}$ with decrease in HRT from 4 to 3 days. Consequently, the flow rate of rubber wastewater was 0.6 L which was fed into the bioreactor. The concentration of 2-MBT was varied from 20 to 100 mg L^{-1} amended in the rubber wastewater to obtain the concentration of COD in range 3000-9000 mg L^{-1} (Figure 3.7).

Organic loading rate (OLR) and hydraulic retention time (HRT) are regarded as most of important operating parameters affecting the performance of wastewater treatment system (Z. Wang et al., 2014). The work further increased the OLR to 3 kg $COD.m^{-3}.\ d^{-1}$ and decreased the HRT to 3 days. As a matter of fact, higher OLR and shorter HRT values represented great performance of bioreactor treatment. Shorter HRT is desirable due to the reduction of cost of wastewater treatment (Shi et al., 2017). However, too short HRT might cause incomplete biodegradation (Magdalena, Greses, & González-Fernández, 2019). Consequently, the performance of the bioaugmentation of a bioreactor with immobilized EN consortium (R3) was conducted to evaluate its performance removal for real 2-MBT-contaminated rubber wastewater at different OLR (Figure 3.7). The bioreactor was conducted in a closed reactor with a commercial black bag to avoid possible photolysis (Figure 3.8).

Briefly, the treated rubber wastewater from activated sludge process in factory E (Table 4.1) was used to simulate the COD concentration of 3,000-4,000 mg L^{-1} in the laboratory bioreactors resulted in OLR 1 kg $COD.m^{-3}.\ d^{-1}$. When the performance became stable, an OLR was further increased to 2 and up to 3 kg $COD.m^{-3}.\ d^{-1}$. This step was used the COD concentration of 6,000-9,000 mg L^{-1} (Figure 3.7). This work used the real rubber processing wastewater from the production process of factory E (Table 4.1) as diluted wastewater for studying the real performance of the bioreactor to treat the rubber wastewater. The 2-MBT residual and the COD concentration were observed during operation

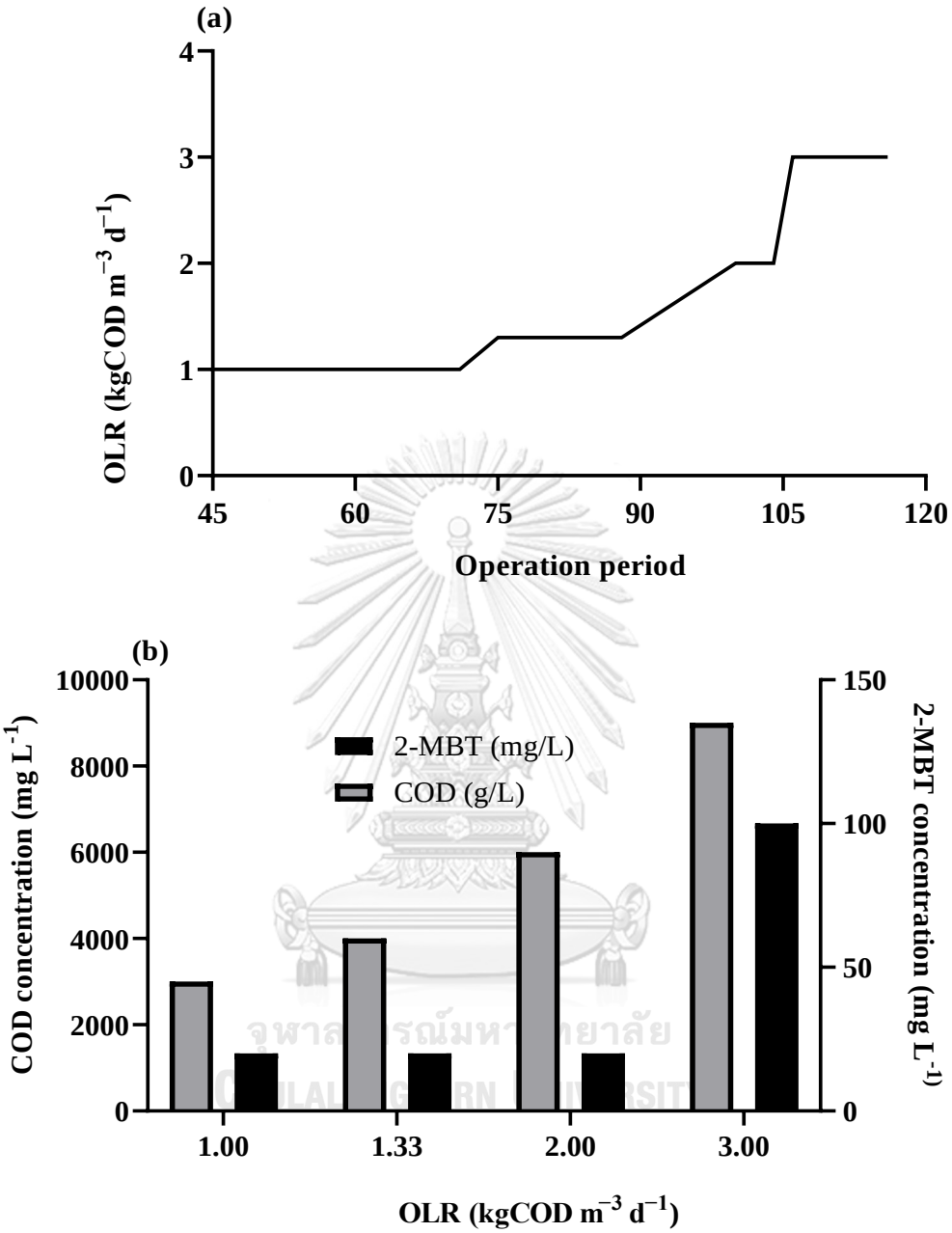


Figure 3.7 The operating conditions of organic loading rate (OLR) during periods (a) and the COD and 2-MBT concentrations were operated at different OLR (b).

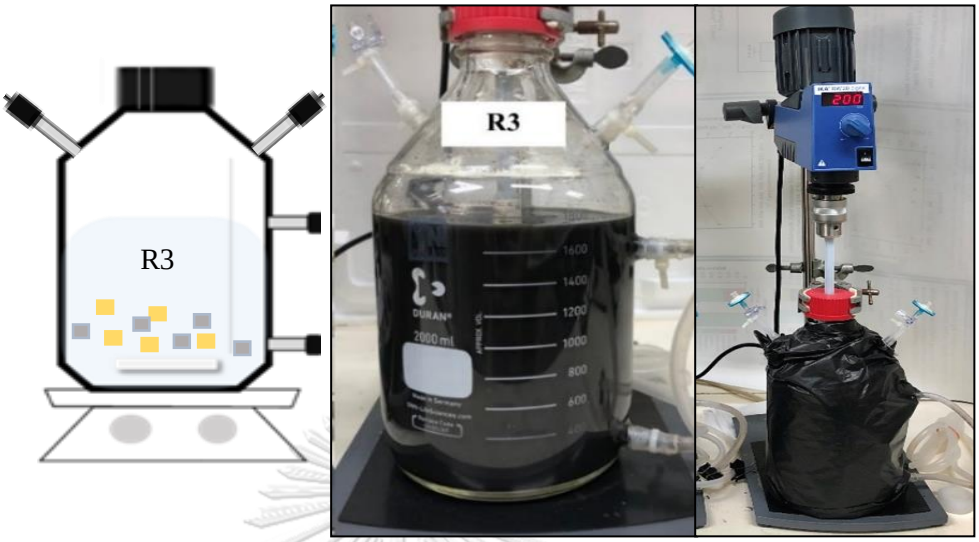


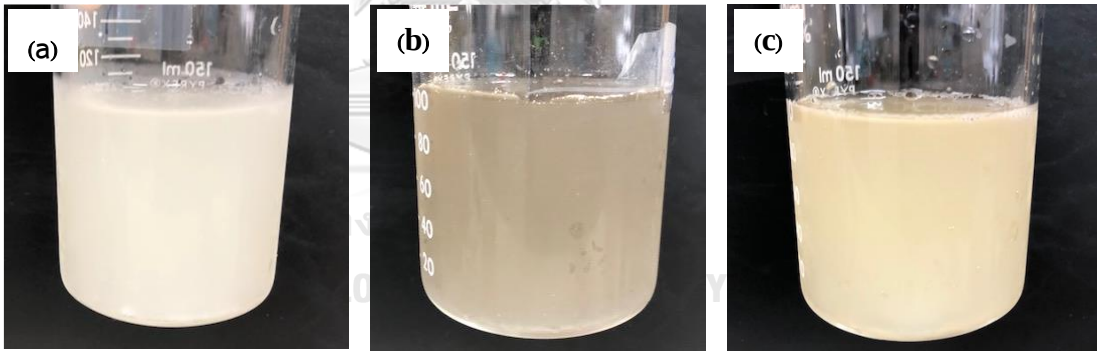
Figure 3.8 Photograph of the bioaugmentation reactor with immobilized EN consortium (R3) for treating rubber wastewater at different OLRs with HRT 4 days under agitation by a controllable turbine of 200 rpm.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Wastewater characterization

4.4.1 Wastewater characteristics

The raw wastewater samples from production process of two rubber manufacturing industries including the concentrated latex (factory S) and the block rubber production (factory E). The wastewater was generated during manufacturing processes at approximately 500 and 800 m^3 day^{-1} from S and E factories, respectively. The major sources of the wastewater of both factories were from the washing and cleaning processes, centrifugation of concentrated latex process, rubber content separation and crushing process which was released after the coagulation with the acid. Figure 4.1 shows the wastewater samples from rubber processing factories.



Influent wastewater from factory E | **Effluent wastewater from factory E** | **Influent wastewater from factory S**

Figure 4.1 Wastewater samples from rubber processing factories.

The influent wastewater samples (Figures 4.1a and c) were collected from wastewater receiving tank which derived from the rubber production in factories E and S, respectively. The effluent wastewater of factory E was collected at the secondary clarify which was treated by activated sludge system. The color of both influent wastewater samples was more turbidity than the effluent. The influent of factory E

was milky white color, whereas the color of influent from factory S was light brown. Moreover, the influent from factory E had stronger odor than the effluent from factory S. It was associated with noxious smell of rotten-egg gas, which could be the hydrogen sulfide gas. Similar to previous reports, the rubber sheet wastewater can contribute to odors which was influenced significantly by organic compounds loading (Kornochalert, Kantachote, Chaiprapat, & Techkarnjanaruk, 2014; Tekasakul & Tekasakul, 2006b).

As presented in Table 4.1, the wastewater characteristics from both industries were different, the COD and suspended solid concentrations in factory E was higher than the factory S. This was caused by the larger size of factory E, which generated larger amount of wastewater than concentrate latex in factory S. After treatment, the effluent from factory E had neutral pH; however, the COD and suspended solid concentrations did not meet the Thai standard (i.e. less than 120 mg L^{-1} of COD and 50 mg L^{-1} suspended solid). The results suggested that the effluent should be treated further, or the current wastewater treatment process should be improved.

Table 4.1 Characteristics of wastewater samples from rubber processing factories.

Parameters (unit)	**Production process wastewater from factory S**	**Production process wastewater from factory E**	**Effluent from factory E**
pH	4.84-4.94	4.4-5.15	7.6-7.8
Total COD (mg L^{-1})	9,900-11,700	32,500-35,000	2,500-3,100
Soluble COD (mg L^{-1})	8,700-9,100	25,500-29,200	1,900-2,850

Table 4.1 Characteristics of wastewater samples from rubber processing factories. (Cont.)

Parameters (unit)	Production process wastewater from factory S	Production process wastewater from factory E	Effluent from factory E
Sulfate (mg L^{-1})	364-396	323-370	46.17-62.80
TKN (mg L^{-1})	542	1,707-2,293	1,145-1,236
2-MBT (mg L^{-1})[a]	n.d.	118.7	14.1
Suspended solid	820	800	650

[a] Concentration was detected by GC-MS analysis which compared with the standard of 2-MBT.

4.4.2 Identification of organic compounds in the rubber processing wastewater by GC-MS

The characterization was performed in order to identify the organic contaminant compounds in the rubber wastewater. By using the information from the wastewater characteristic, the further experiments would be designed accordingly. The influents from both E and S factories and the effluent from factory E were collected and analyzed by gas chromatographic mass spectrometry (GC-MS) with scan mode. The results of the identified organic compounds in wastewater from two rubber factories were shown in Figure 4.2 and 4.3.

As presented in Figure 4.2 and 4.3, 28 and 34 identified of hazardous organic compounds contaminated in the influent wastewater from S and E factories, respectively. Benzothiazole family was found at 34% of the total area in influent from factory E, while the influent from factory S was shown approximately 2.2%. Figure 4.3

shows the peak of 3-(methylsulfanyl)-4,5,6,7-tetrahydro-2,1-benzisothiazole (1.65%) and 2-(methylmercapto) benzothiazole (0.67%), whereas the major compounds of benzothiazoles group include benzothiazole (28.6), 2-mercaptobenzothiazole (4.0%) and 2-(methylmercapto) benzothiazole (0.6%) were the dominant fractions in the influent from factory E. Benzothiazole and 2-MBT are used as vulcanization catalyst to add in the concentrated latex and produce the block rubber. In addition, the benzothiazole compounds can be used in rubber product industry (i.e. rubber glove industry). The main source of wastewater is from the production process that used the 2-MBT as a chemical additive to form the hand-shaped model in the production of glove.

From Figure 4.2 and 4.3, two dominated species in the influent from factory S were benzene methanol and cyclohexane carboxylic acid, which show high relative peak abundance of 31.1% and 11%, respectively (Figure 4.2). Conversely, the benzene methanol in the influent from factory E was found only 2.5%. The benzene methanol is an aromatic alcohol used to extend and soften rubber formulations in the rubber process. Moreover, indole and its derivatives include 3-methly indole and 1H-indole were found in both influent wastewater samples, they could be obtained from the tryptophan biodegradation during in the rubber serum. The compounds are known as carcinogen to human and cause bad smelling problem in rubber industry. Moreover, petroleum hydrocarbon compounds such as medium- and long-chain alkanes were also found in both influent wastewater samples. Both influent wastewater samples contained many fatty acids, which have been frequently found in the natural rubber latex processing wastewater.

Figure 4.2 Organic compounds in influent wastewater obtained from wastewater receiving tank in factory S as evidenced by GC-MS.

Figure 4.3 Organic compounds in influent wastewater obtained from wastewater receiving tank in factory E as evidenced by GC-MS.

Since the existing wastewater treatment system in the rubber industry could treat the COD at lower concentration (Table 4.1) and could not completely degrade emerging contaminants. The effluent was also full of recalcitrant compounds from GC-MS analysis (Figure 4.4). Phenol and its derivatives were the predominant toxic compounds in the effluent derived from factory E. In addition, the effluent wastewater contained both benzothiazole (2%) and benzothiazolone (4.8%). Indole and phthalic acid esters were also detected. Since phthalates could be leached from the adhesive and PVC products, suggesting that these compounds were leached from non-point sources (Wongniramaikul, 2006). Consequently, the post treatment of rubber processing wastewater was recommended for efficiently treating the target compound and excessive COD to meet the standard. The biological treatment process using efficient microorganisms is an alternative approach for solving these problems.

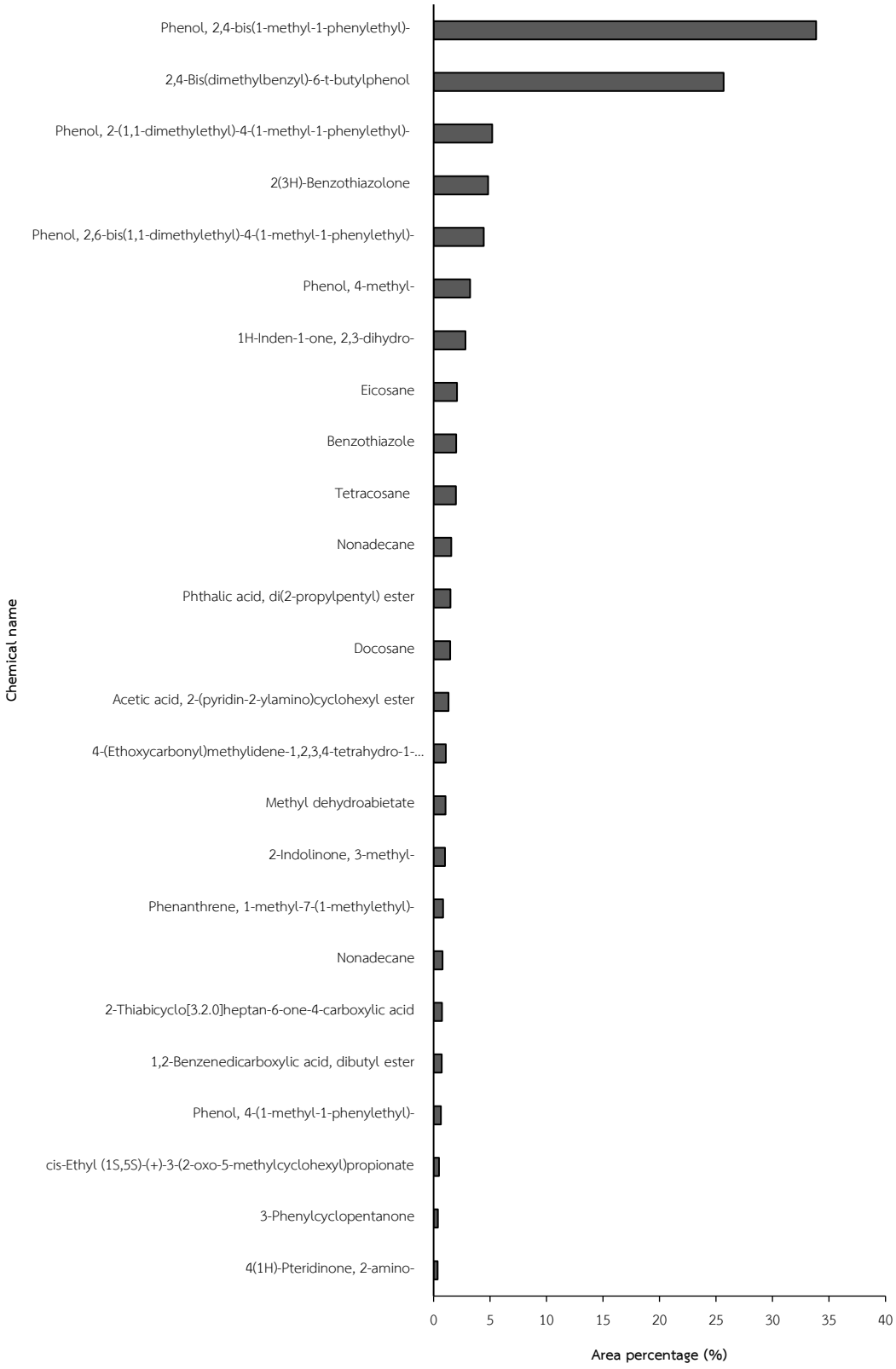


Figure 4.4 Organic compounds in treated wastewater obtained from activated sludge process (effluent) in factory E as evidenced by GC-MS.

4.2 Enrichment, acclimatization and characterization of 2- MBT degrading consortium

4.2.1 Enrichment of 2-MBT degrading bacteria

This part of research aimed to use an enrichment approach for obtaining efficient 2-mercaptobenzothiazole-degrading microorganisms. The gradual increasing of 2-MBT concentrations was used to allow the adaptation of microorganisms to 2- MBT in the original sludges. The research initially constructed the enrichment system from two sources of rubber wastewater sludges, which were collected from wastewater treatment plants with high and low benzothiazoles contamination. The system was initially conducted in N medium supplemented with 25 mg L^{-1} 2-MBT as a sole carbon source for 14 days, then the enriched sludge was transferred at 10% v/v to a new medium containing 50 mg L^{-1} 2-MBT and incubated for another 14 days.

The 2- MBT biodegradation efficiency of enriched sludge E was higher than that of enriched sludge S (Figure 4. 5) . The results corresponded with the higher concentrations of 2- MBT and various benzothiazoles in the production process wastewater from the factory E than that from the factory S (Figures 4.2 and 4.3). Several studies have shown the exposure to environmental pollution in pristine sites can be a major condition leading to bacterial adaptation to pollutant degradation (Itrich et al., 2015; Tezel & Pavlostathis, 2015; van der Meer, 2006).

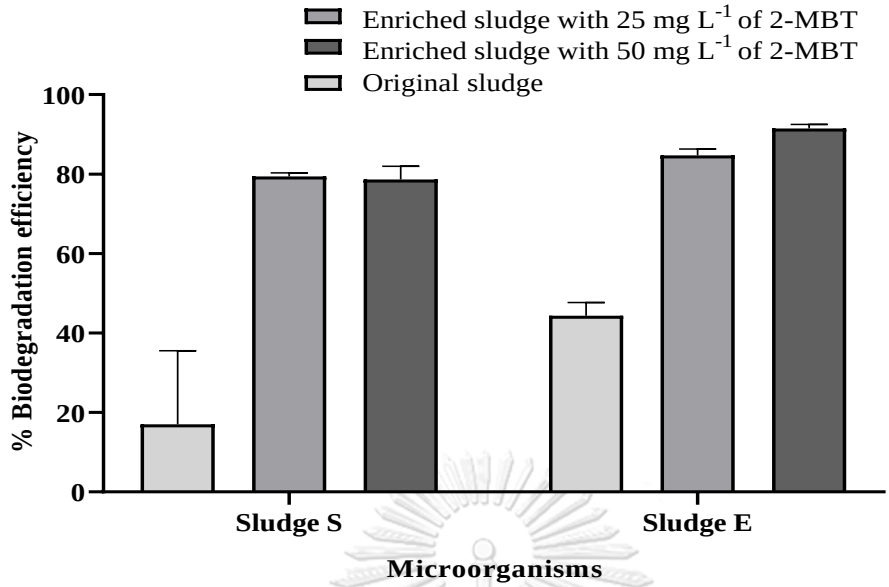


Figure 4.5 2-MBT biodegradation efficiency of microorganisms in original sludge and enriched sludge samples which were incubated in each 25 mg L^{-1} of 2-MBT and 50 mg L^{-1} containing N medium.

As shown in Figure 4.5, after the first 14-days of incubation, 25 mg L^{-1} 2-MBT decreased over 80 % of the total concentration in all batch reactors. After 28 days of enrichment incubation, enriched sludge E in the enrichment reactor degraded 91.5% of 50 mg L^{-1} 2-MBT, while the enriched sludge S showed ability to degrade 2-MBT at 78.6%. The highest biodegradation capability of the enriched sludge samples was observed in the enrichment sludge system with 50 mg L^{-1} of 2-MBT (Figure 4.5), indicating that the bacterial cells in the rubber wastewater sludge required the time of incubation for their bacterial adaptation to degrade and tolerate the higher 2-MBT concentrations. This suggestion was in agreement with other similar studies (Fosso-Kankeu, Marx, & Brink, 2017; van der Meer, 2006), they had indicated that substrate concentration and exposure time clearly stimulated biotransformation in the bacteria. In respect to biodegradation results, microorganisms in rubber wastewater sludge may display different levels of 2-MBT and its derivative degradation with respect to changing their physio-biochemistry under 2-MBT-containing system for 28 days. In addition, the biomass concentrations of enriched sludge in both reactors were increased to

approximately 3 g_{vss} L^{-1}. In the present work, the influences of the inoculum (sludge) source and pre-exposure of original sludges to 25 and 50 mg L^{-1} of 2-MBT were found to increase the 2-MBT biodegradation ability of enriched sludges.

4.2.2 Effect of nutrient supplement on 2-MBT biodegradation of enriched sludge under batch test

Although, the factors affecting the biodegradability of various recalcitrant compounds have been characterized, the key factors affecting 2-MBT biodegradation have not yet been elucidated. To increase the biodegradation efficiency of the enriched sludge (Section 4.2.1), this experiment investigated the factors affecting 2-MBT degradation including exogenous nitrogen and co-carbon substrate during the enrichment of 2-MBT degraders.

Table 4.2 Efficiency of original and enriched sludges on degrading 50 mg L^{-1} 2-MBT in different media after 96-h incubation.

Sludge source	Nitrogen availability	Carbon availability	Enrichment code	2-MBT Biodegradation efficiency (%) *	
				Original sludge	Enriched sludge
Factory S	N	W/o AC	SN	28 ± 3.5^{Aa}	82 ± 0.4^{Bc}
		W/ AC	SN-AC^{+}	59 ± 0.9^{Ac}	80 ± 0.2^{Bc}
	NF	W/o AC	SNF	41 ± 5.5^{Ab}	31 ± 0.5^{Aa}
		W/ AC	SNF-AC^{+}	60 ± 1.2^{Bc}	35 ± 1.8^{Aab}
Factory E	N	W/o AC	EN	42 ± 3.3^{Ab}	86 ± 1.7^{Bc}
		W/ AC	EN-AC^{+}	40 ± 8.2^{Ab}	81 ± 3.3^{Bc}
	NF	W/o AC	ENF	56 ± 2.5^{Bc}	46 ± 1.7^{Ab}
		W/ AC	ENF-AC^{+}	46 ± 1.6^{Ab}	41 ± 1.5^{Aab}

N; nitrogen supplement in MSM (N): NH_4Cl (1 g L^{-1}) and yeast extract (1 g L^{-1})
NF; without nitrogen supplement in MSM
AC; 10 mM sodium acetate was supplemented in MSM
Superscript capital letters (A and B) represent statistically significant differences ($P<0.05$) between the results obtained by original and enriched sludges. Both treatments were cultured in the same medium (row). The superscript lowercase letters (a, b, c) indicate statistically significant differences ($P<0.05$) of the results from the same sludge source among different media (column). Data are the mean and SD of the three replicates. Tukey's multiple comparisons following a two-way ANOVA was used.

Table 4.2 presents the biodegradation efficiencies of enriched sludges and original sludge from both sources of sludge under various media conditions which containing 2-MBT at 50 mg L^{-1}. Original sludge samples collected from the E and S rubber factories degraded 50 mg L^{-1} 2-MBT at efficiencies ranging from 28 to 60% when applied as inocula in various cultured media (Table 4.2). Both sludge samples gave a higher 2-MBT degradation efficiency after cultivation in NF medium than in N medium. The results suggested that the original sludges had sufficient nitrogen concentrations to support bacterial activities and that the excess nitrogen in N medium might interrupt their activity. However, the nitrogen remaining in the sludge samples was diluted after transfer to the new enrichment medium; thus, an additional nitrogen source was required.

The enriched sludges from both the E and S rubber factories had significantly higher 2-MBT biodegradation efficiencies in media supplemented with a nitrogen source regardless of acetate addition, among which SN, SN-AC^+, EN and EN-AC^+ degraded 80-86% 2-MBT. These results indicated that the nitrogen content in the inoculating sludge was not sufficient for the 2-MBT-enriched cultures. Moreover, in this study, acetate addition enhanced 2-MBT degradation only in the original sludge from the factory S. The organic substrate might be a co-substrate or might stimulate the co-metabolism activity of some microorganisms in the system. External organic substrates

(Feng et al., 2019) and nitrogen sources (Xiong, Kurade, Kim, Roh, & Jeon, 2017) allow microorganisms to transform persistent organic compounds for growth and induce non-specific enzymes for co- metabolism of recalcitrant substrates. Thus, ammonium chloride and yeast extract positively affected the growth of the 2- MBT- degrading bacterial community. Consequently, these nitrogen sources were used as auxiliary nutrients in the following experiments.

4.2.3 Effect of acclimatization stage on 2-MBT biodegradation

To further increase the efficiency of the enriched sludge samples, a series of acclimatization processes in N medium with/without sodium acetate was conducted by applying a stepwise increase in 2- MBT concentration, with values of 50, 100 and 200 mg L^{-1}. The time courses of 2-MBT degradation, COD removal and bacterial growth from the last round of acclimatization in stage II (100 mg L^{-1}) and III (200 mg L^{-1}) are shown in Figure 4.6.

A similar time course pattern was also found in other acclimatization rounds. In general, the abiotic control using heat-killed cells showed a minor reduction in 2-MBT and COD over time, while the SN, SN- AC^{+}, EN and EN- AC^{+} acclimatized cultures exhibited gradually decreasing concentrations of 2- MBT and COD. The biomass from all acclimatized cultures increased without a lag period for both 2-MBT concentrations, but the higher 2-MBT concentration required a longer time (Figure. 4.6.).

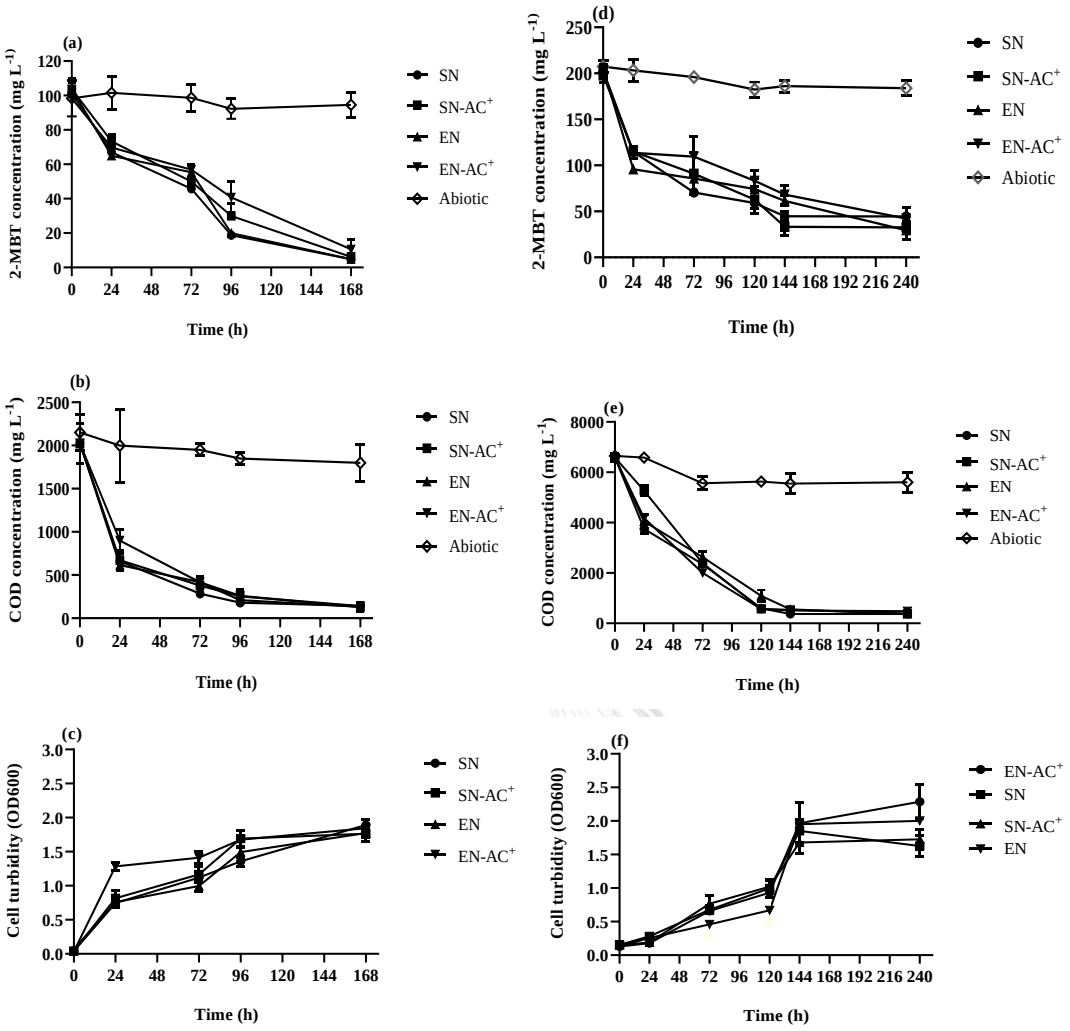
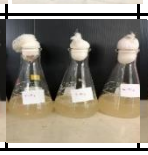


Figure 4.6 Time course of 2-MBT biodegradation (a, d), COD removal (b, e) and bacterial growth (c, f) when applied the bacterial consortia from different acclimated conditions in the MSM medium with 2-MBT at a concentration of 100 mg L^{-1} (left) and 200 mg L^{-1} (right). Experiments with heat-killed cultures were used as abiotic control.

Importantly, approximately 60 mg L^{-1} sulfate in all consortia was released into the medium during the last round of acclimatized stage III. The results confirmed that the disappearance of 2-MBT occurred due to the biological activity of the acclimatized sludge. Similarly, a previous report showed that the degradation of 2-MBT was observed with the liberation of sulfate in the system (Umamaheswari & Rajaram, 2017). Although the activities of acclimatized samples from different media were quite similar at the end of the experiment (Figure. 4.6), the color of the samples (red and green)

changed over time and depended on the culture conditions and sludge sources (Table 4.3).

Table 4.3 Enrichment sludge and acclimated bacterial consortia in different culture conditions

Stages	**2- MBT Concentration in N medium (mg L^{-1})**	**Period (day)**	**Conditions**			
			Sludge S		**Sludge E**	
Enrichment	25-50	28				
Acclimation	2- MBT Concentration in N medium (mg L^{-1})	Periods (day)	SN	SN-AC^{+}	EN	EN-AC^{+}
I	50	28				
II	100	21				
III	200	25				

The red color of bacterial culture during 2-MBT biodegradation (Table 4.3) is suggested to be due to complexes between in the medium or the formation of ferrous isothiocyanate after the release of thiazole rings (Umamaheswari & Rajaram, 2017). When comparing values of biodegradation efficiency between acclimatization stages, the 2-MBT biodegradation efficiencies of all samples were in the range of 78-90%

(Figure 4.7). The biodegradation efficiencies and specific degradation rates were not significantly different among the cultures with or without acetate carbon in this experiment. The specific degradation rates significantly increased (P<0.05) from acclimatization stage I to III (Figure 4.7).

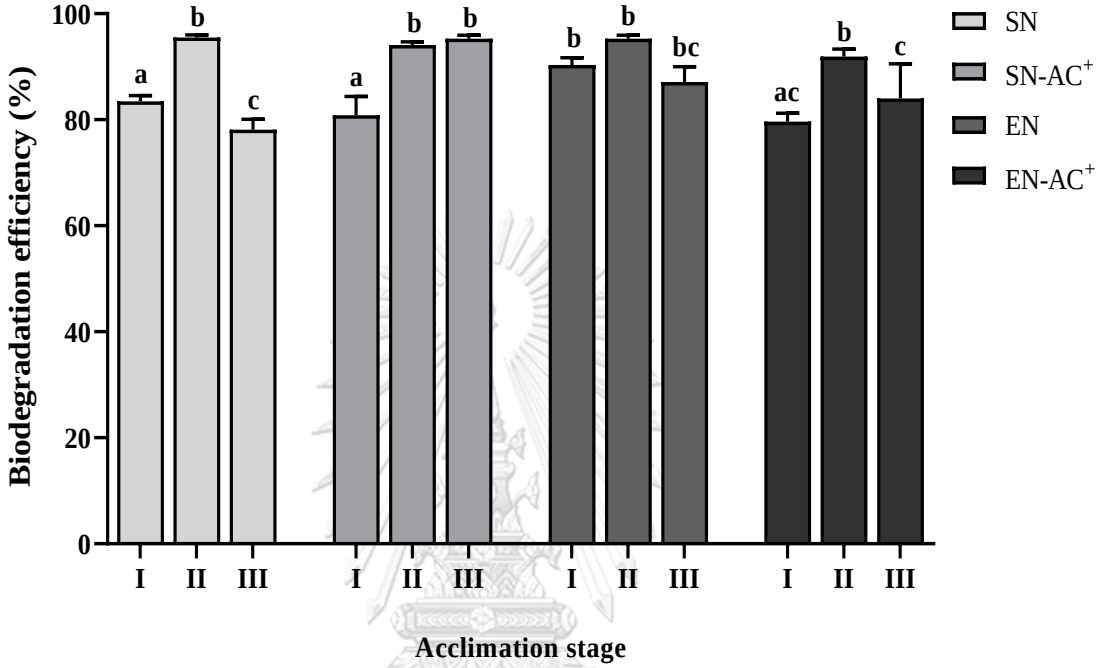


Figure 4.7 Percentage of biodegradation efficiency of acclimated bacterial consortia in stage I-III under different acclimated conditions. Different letters represent significant differences (P<0.05).

The bacterial consortia from acclimatization stage III were exposed to 2-MBT for 76 days. They had the highest degradation rates of 1.8-2.3 mg L^{-1} d^{-1} mg cell $prot^{-1}$ (Figure 4.8), which were approximately 61% higher than those of the acclimatization stage I consortia. This result indicated that the 2-MBT biodegradation rate significantly improved by bacterial acclimation. The acclimatized consortia from stage III were therefore selected for further experiments.

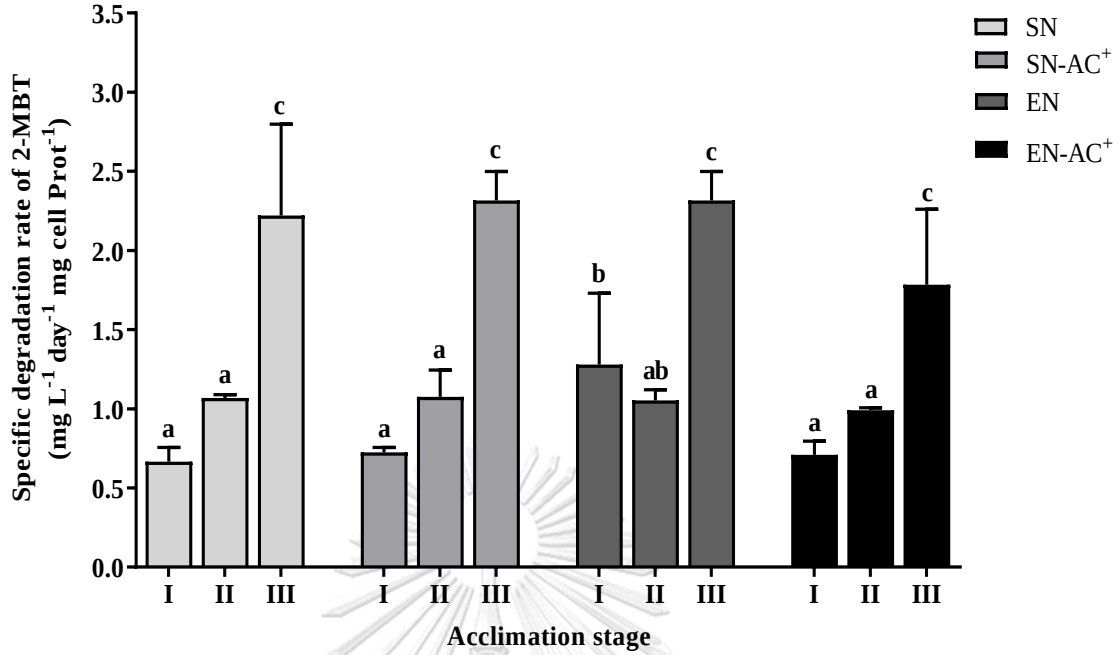


Figure 4.8 The specific biodegradation rates of acclimatized consortia were calculated as the average of slopes within the first 72 hour of acclimation periods and were normalized with total protein concentration. Different letters represent significant differences ($P<0.05$).

Similarly, several studies have suggested that long periods of acclimatization can select for specific populations and increase the magnitude of microbial adaptation in the cultivated community (Elcey & Kunhi, 2010; Herzog, Yuan, Lemmer, Horn, & Müller, 2014; Poursat, van Spanning, de Voogt, & Parsons, 2019). In addition, induction of bacterial adaptation by subsequent transfer during long-term exposure can be selected specific populations in order to be applied in environmentally realistic conditions (Poursat et al., 2019). In this work, the stepwise acclimation strategy was the major factor leading to an adaptation at the community level which undergone a series of enzyme induction process and resulting the biodegradation of 2-MBT changes. Our results confirmed that the sequential enrichment and acclimatization process allowed for the development of efficient 2-MBT-degrading bacterial consortia.

4.2.4 2-MBT biodegradation rates of bacterial consortia in resting cell assay

To further compare the activity of the SN, SN-AC$^+$, EN and EN-AC$^+$ acclimatized bacterial consortia, the effect of the initial 2-MBT concentration on the specific biodegradation rate was investigated using a resting cell assay in N medium (Figure 4.9).

Figure 4.9 shows the EN bacterial consortium had a higher 2-MBT degradation efficiency than the other consortia, with the highest specific biodegradation rate of 5.2 ± 0.5 mg L^{-1} day^{-1} mg cell protein^{-1} and an optimal 2-MBT concentration of 300 mg L^{-1}.

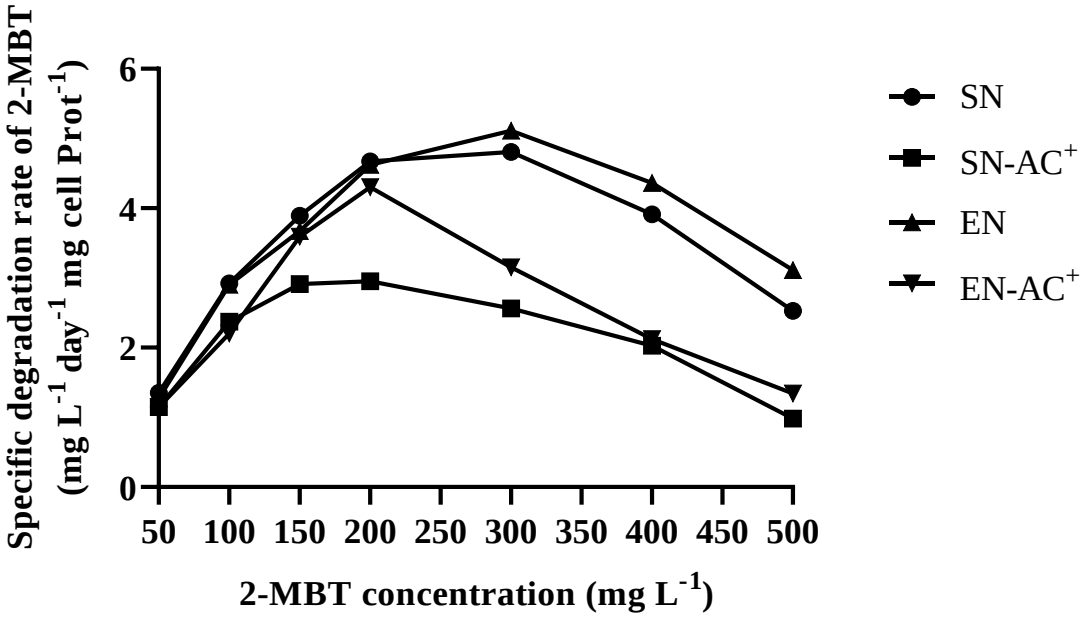


Figure 4.9 Effect of initial 2-MBT concentrations in the MSM medium on specific degradation rate of bacterial consortia from different acclimated conditions when used as resting cells. Specific degradation rates were calculated as the average of slopes within the first 72 hour of degradation periods and were normalized with total protein concentration.

The SN bacterial consortium had a slightly lower efficiency than the EN bacterial consortium, with a highest specific biodegradation rate of 4.8 ± 0.31 mg L^{-1} day^{-1} mg cell protein^{-1}. Interestingly, the bacterial consortia enriched and acclimatized in medium containing acetate, EN-AC$^+$ and SN-AC$^+$, had considerably low 2-MBT specific biodegradation rates and were effective at low 2-MBT concentrations (< 200 mg L^{-1}).

The results indicated that different bacterial communities were obtained whose 2-MBT degrading activity was probably dependent on the presence of acetate. For the non-inoculated experiment (abiotic control, approximately 5.1 2-MBT removal was observed at all 2-MBT concentrations.

Compared with the results of other studies, the EN consortium had higher 2-MBT degradation efficiency. This consortium was able to degrade 93% of 100 mg L^{-1} 2-MBT after 72 h (Figure 4.6a), while *Alcaligenes* sp. CSMB1 degraded only 34% of 100 mg L^{-1} 2-MBT (measured as TOC) (Umamaheswari & Rajaram, 2017). In addition, most isolated bacteria degrade 2-MBT at concentrations lower than 100 mg L^{-1} (El-Bassi et al., 2010; Kowalska & Felis, 2015). Such a result, most isolated bacteria and sludge samples degrade 2-MBT efficiently at concentrations lower than 200 mg L^{-1} (Table 4.4). The high efficiency of the EN bacterial consortium could be due to the activity of various bacterial strains that were acclimatized to high 2-MBT concentrations. The EN bacterial consortium was chosen for further investigation.

Table 4.4 Comparison of benzothiazole removal efficiency by the obtained bacterial consortia with those by bacterial isolates and sludge samples.

Microbial inoculum	Test system/ duration	Type of benzothi -azoles	Concentration (mg L^{-1})	Efficiency (%)	References
Rhodococcus rhodochrous OBT18	Aerobic batch with resting cells in water/ 128 h	2-MBT	251 (1.5 mM)	30	Haroune et al. (2004)
P*seudomonas putida* HKT554	Aerobic batch with resting cells in mineral medium/ 30 h	A mixture of benzothi azoles	2-MBT, 20 (120 µM) Benzothiazole, 19 (140 µM) 2-methylthiothia zole, 18 (100 µM)	ca. 80	El-Bassi et al. (2010)
Alcaligenes sp. MH146 strain CSMB1	Microaero bic batch with growing cells in mineral medium/ 72 h	2-MBT	50	86 (as TOC)	Umamah eswari & Rajaram (2017)

Table 4.4 Comparison of benzothiazole removal efficiency by the obtained bacterial consortia with those by bacterial isolates and sludge samples. (Cont.)

Microbial inoculum	Test system/ duration	Type of benzothi -azoles	Concentration (mg L^{-1})	Efficiency (%)	References
Activated sludge	Aerobic batch with activated sludge in mineral medium/ 25 days	2-MBT	167 (1 mM)	85	M. A. Gaja & Knapp (1998)
Anaerobic granular sludge	Anaerobic batch with mixed liquor suspended solids in synthetic wastewater/ 84 h	Benzothiazole	50	98	Y. Li et al. (2017)

Table 4.4 Comparison of benzothiazole removal efficiency by the obtained bacterial consortia with those by bacterial isolates and sludge samples. (Cont.)

Microbial inoculum	Test system/ duration	Type of benzothi -azoles	Concentration (mg L^{-1})	Efficiency (%)	References
Diluted river mud	Microbial electrolysis cells (MECs) conducting in fed-batch mode with mineral medium/ 30 h	2-MBT	8.35	70 (as TOC)	San-Martin et al. (2020)
Consortium SN-AC^{+}	Aerobic batch with resting cells in mineral medium/ 72 h	2-MBT	300	56	This study
Consortium SN		2-MBT	300	82	
Consortium EN-AC^{+}		2-MBT	300	68	
Consortium EN		2-MBT	300	85	

4.2.5 Application of a bacterial consortium for the removal of 2-MBT and benzothiazoles in rubber processing wastewater

The EN bacterial consortium was initially applied to rubber processing wastewater (influent) from factory E, but the microorganisms did not survive. This result could be due to the high strength of the influent wastewater (Table 4.1). Consequently, wastewater sample was collected from the effluent of the activated sludge plant at factory E. The wastewater was used to determine the activity of EN bacterial consortium in the treatment of 2-MBT and benzothiazoles as a post-treatment process. The effects of indigenous microbes on the performance of EN bacterial consortium were also evaluated in a comparative study using non-sterile and sterile wastewater. Moreover, the control was non-sterilized wastewater (3.7×10^4 CFU/mL) without EN bacterial inoculum, representing the effect of natural attenuation after treated effluent is discharged to the environment.

After 7 days of operation, the 2-MBT and COD removal efficiencies of EN consortium were higher than those of natural attenuation, which displayed only 7% removal of both 2-MBT and COD compared with the initial concentrations in wastewater (control in Figures. 4.10a and 4.10b). The biodegradation profiles of EN-inoculated sterile and non-sterile wastewater revealed the retention of 22-37 and 253-407 mg L^{-1} 2-MBT and COD, respectively, indicating that biodegradation proceeded by metabolic reaction of the bacterial communities in the EN bacterial consortium. This conclusion is consistent with previous work suggesting that the removal of 2-MBT can proceed through biological reactions in conventional wastewater treatment but 2-MBT cannot be efficiently removed due to its tolerance to biological degradation (H. De Wever & Verachtert, 1997). EN bacterial consortium showed a faster 2-MBT degradation rate in sterile wastewater than in non-sterile wastewater (Figure 4.10). At the end of the study, 80% and 65% of 112 mg L^{-1} 2-MBT was removed from sterile and non-sterile wastewater, respectively (Figure 4.10a). These results indicated competition between the EN bacterial consortium and the indigenous microorganisms in the wastewater. On the other hand, there was no significant difference in COD removal efficiency between sterile and non-sterile wastewater samples, where the EN bacterial consortium

removed 90-93% of ~4,000 mg L^{-1} COD (Figure 4.10b). Thus, most organic contaminants in the activated sludge-treated wastewater were readily biodegradable, while 2-MBT was refractory and could be used only by the inoculated bacterial consortium.

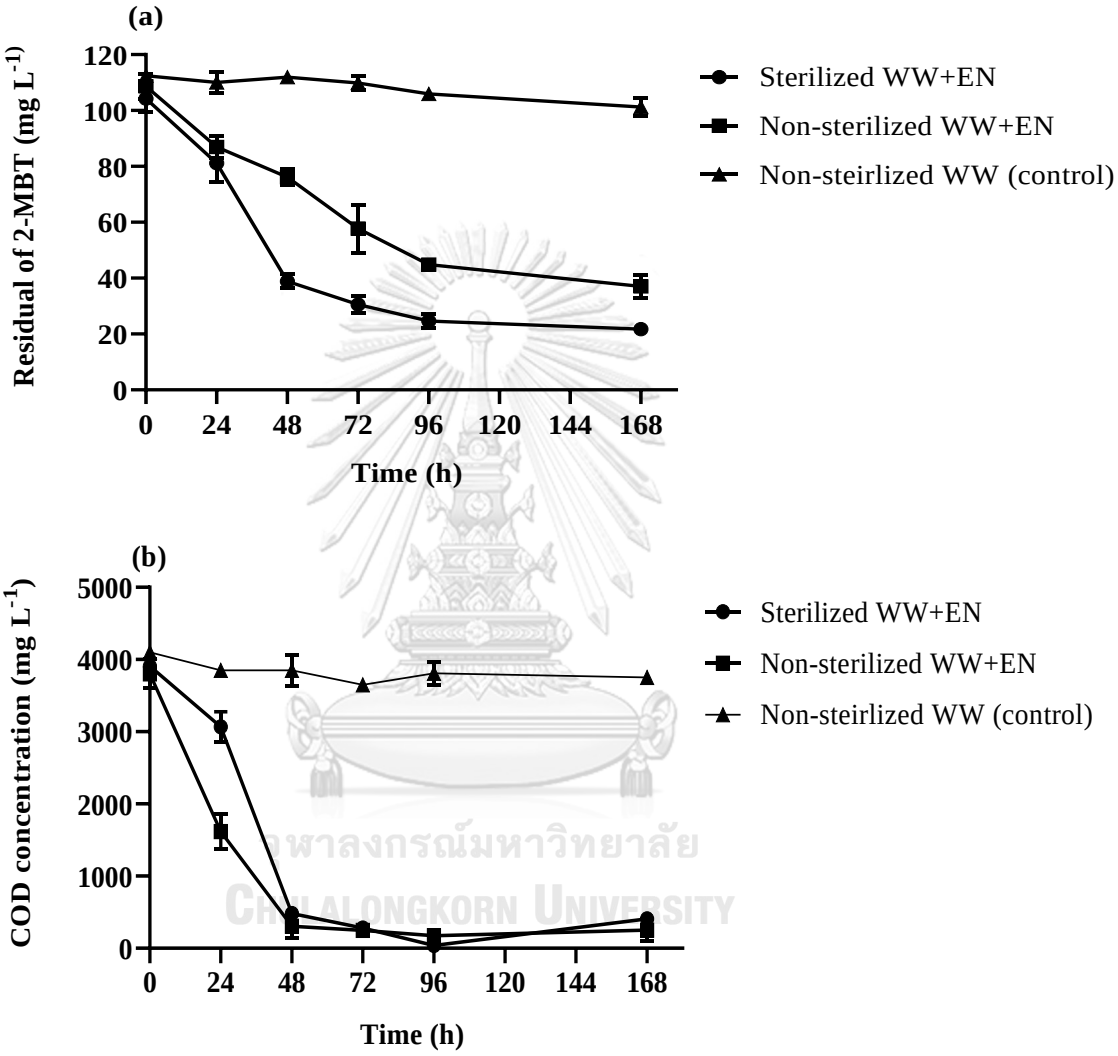


Figure 4.10 Time course of 2-MBT biodegradation (a) and COD removal (b) when applied the EN bacterial consortium in the sterilized and non-sterilized treated rubber wastewater from activated sludge process. Control was the non-sterilized wastewater without EN culture inoculation. Different letters denote significant differences among treatments ($P < 0.05$).

The high 2- MBT removal efficiency of EN bacterial consortium in sterile wastewater corresponded with the presence of the dominant bacterial genus *Pseudomonas* until the end of the experiment. On day 7, the number of *Pseudomonas* spp- specific 16S rRNA genes in sterile wastewater was maintained at 2.2×10^{10} copies number $mL^{-1,}$ while the number in non- sterile wastewater effluent significantly decreased to 3×10^{8} copies number mL^{-1} ($P < 0.05$) (Figure 4.11). Many studies have shown that complex interactions, including interference from toxic substances and indigenous microbes in real wastewater effluent, might be responsible for reducing the performance of augmented cultures (Abtahi et al., 2020; Viero et al., 2008). Notably, the number of *Pseudomonas* in non-sterile wastewater determined by qPCR could be higher than that indicated by the plate counting technique due to the unculturable fraction and multiple numbers of 16S gene copies in this genus (Stoddard, Smith, Hein, Roller, & Schmidt, 2015).

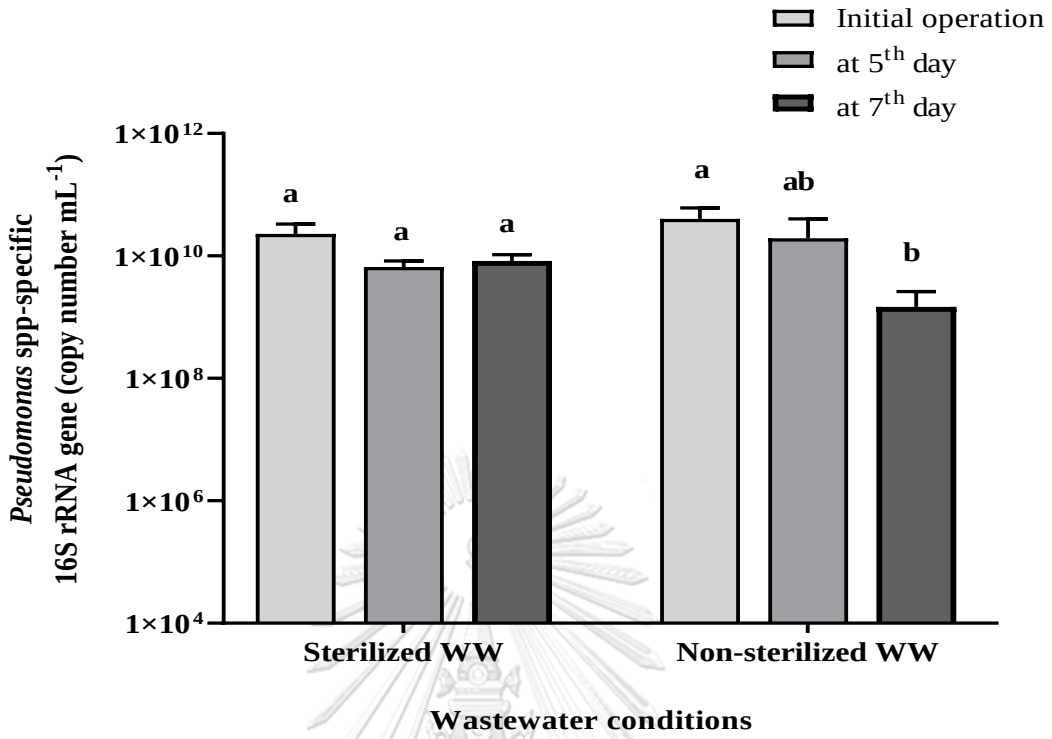


Figure 4.11 Quantity of *Pseudomonas* spp. population when applied the EN bacterial consortium in the sterilized and non-sterilized treated rubber wastewater from activated sludge process.

GC-MS chromatograms were used to confirm the degradation of 2-MBT and other benzothiazoles resulting from the addition of EN bacterial consortium. The relative abundances of 2-MBT (no. 18) and benzothiazole (no. 6) were significantly decreased in both treated wastewater samples (Figures 4.12b and 4.12c) compared with those in untreated wastewater (Figure 4.12a). In addition, intermediate compounds such as 2-aminobenzothiazole (no. 23), 2-hydroxybenzothiazole (no. 24) and methylbenzothiazole-2-thione (no. 25) were observed in low abundances in both wastewater conditions (Figures 4.12b and 4.12c). 2-MBT bacterial metabolism can lead to the formation of several metabolite compounds (Umamaheswari & Rajaram, 2017). Interestingly, a decrease in the relative abundance of 2-(methylthio) benzothiazole (no. 13) was observed when sterile wastewater was treated in comparison with that in non-sterile wastewater. The cooperative behaviors among bacterial populations in the

consortium under sterile conditions were probably more efficient than those under non-sterile conditions; thus, 2-MBT was transformed into 2-(methylthio)benzothiazole through a thiol S-methyltransferase reaction as previously described (Drotar et al., 1987a).

According to the GC-MS analysis, catechol was not detected after 7 days of operation. This could be due to the degradation of catechol by the activities of catechol 1,2-dioxygenase (Setlhare, Kumar, Mokoena, & Olaniran, 2019). In addition, the result of metatranscriptomic showed high level of catechol 1,2 dioxygenase at approximately 363±30 reads (Table 4.13).

Under natural conditions, such as non-sterile wastewater, the diverse bacterial communities could facilitate the development of competitive strategies to face a constant battle for resources and compete with their neighbors for space (Freilich et al., 2011; Hibbing, Fuqua, Parsek, & Peterson, 2010). Another beneficial effect of EN bacterial consortium was the reduction of sulfate and TKN in the wastewater from 46.15-62.80 and 1,145-1,235 mg L^{-1} to 9.5- 20.5 and 76-80 mg L^{-1}, respectively (Table 4.5). The sulfate removal efficiency of the bacterial consortium in sterile wastewater (79%) was higher than that in non-sterile wastewater (56%), while the TKN removal efficiencies in both wastewater samples were similar at > 90%.

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Table 4.5 Rubber processing wastewater treatment efficiency for the performance parameter in the effluent and after applying EN bacterial consortium.

Parameters (unit)	Effluent from activated sludge treatment plant at factory E	
	Before treatment	After applying EN consortium[b]
pH	7.6-7.8	7.4- 7.6
Total COD (mg L^{-1})	2,500-3,100	n/a
Soluble COD (mg L^{-1})	1,900-2,850	253-407
Sulfate (mg L^{-1})	46.17-62.80	9.5- 20.5
TKN (mg L^{-1})	1,145-1,236	76-80
2-MBT (mg L^{-1})[a]	14.1	21.7-37.1

n.d., compound analyzed but not detected; n/a, total COD not analyzed.

[a] Concentration was detected by GC-MS analysis which compared with the standard of 2-MBT.

[b] Ranges of average data obtained from sterile and non-sterile wastewater which were spiked with 100 mg L^{-1} of 2-MBT after 7-day treatment.

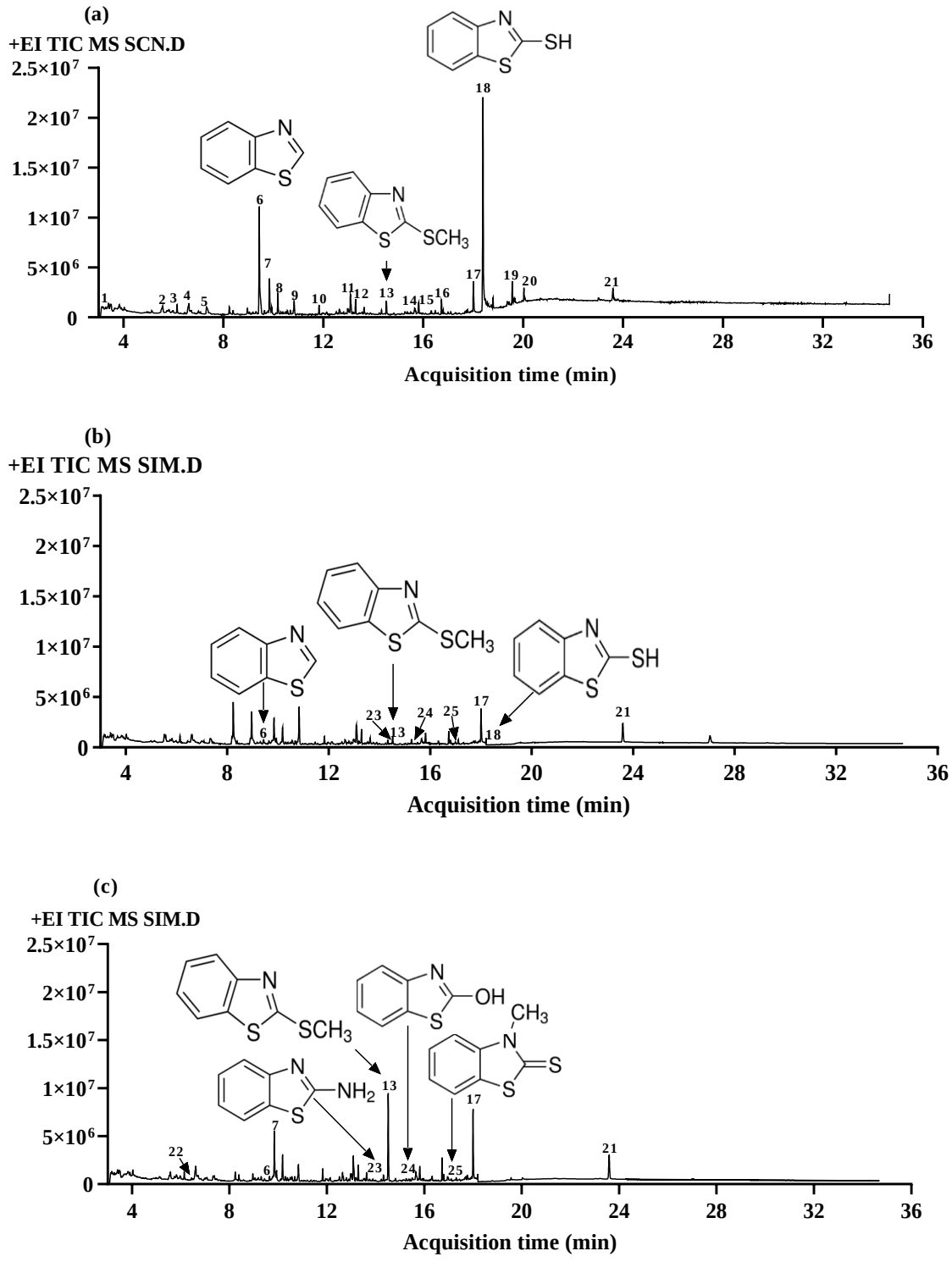


Figure 4.12 Profile of GC-MS chromatogram presented 21 out of 43 organic compounds (Table D-1 in appendix D) account for the majority of the total organic contaminants using 1% of area across the original rubber processing wastewater sample (a) and

treated wastewater after 7 days of operation; sterilize (b) and non-sterile (c) wastewater samples. The compound identification was performed with scan mode in the same samples— 1:Ethylbenzene; 2:Decane; 3:1-Hexanol, 2-ethyl-; 4:Undecane, 2-methyl-; 5:Benzoic acid, methyl ester; 6:Benzothiazole; 7:Benzene, 1,3-bis(1,1-dimethylethyl)-; 8:Pentadecane; 9:unidentified; 10:Tetradecane; 11:unidentified; 12:Phenol, 2,4-bis(1,1-dimethylethyl)-; 13: 2-(methylthio)-Benzothiazole,; 14: Heptadecane; 15: unidentified; 16: 1-Octadecanol; 17: Hexadecanoic acid, methyl ester; 18: 2-Mercaptobenzothiazole; 19: Methyl stearate; 20: unidentified; 21: Di- n- octyl phthalate; 22: Benzaldehyde, 2-hydroxy- ; 23: 2- Aminobenzothiazole; 24: 2- hydroxy- 1,3- benzothiazole; 25: 2(3H) -Benzothiazolethione, 3-methyl-.

4.3 Characterization of the bacterial community structures and functional genes of 2-MBT degrading bacterial consortia by molecular analyses

4.3.1 Identification of bacterial community structures in original sludge and enriched 2-MBT-degrading bacterial consortium by 16S rRNA gene sequencing technique

The shifts in bacterial populations in response to 2- MBT and different acclimatization conditions were explored by 16S rRNA gene amplicon sequencing of the sludge samples (S and E) and acclimatized bacterial consortia, i.e., SN, SN-AC$^+$, EN and EN- AC$^+$. A total of 403,286 quantified sequences were generated by high-throughput sequencing, which were assigned to 5,265 OTUs in all samples. According to the obtained OTUs, sludge S had the richest diversity, followed by sludge E and the acclimatized bacterial consortia (Table 4.6). The low diversity in the acclimatized bacterial consortia was due to the selection of 2-MBT-degrading bacterial communities during the long-term acclimatization process.

Table 4.6 Alpha-diversity indices of the bacterial diversity of the original sludge and acclimated bacterial consortia samples.

Samples	High-quality sequence numbers	Observed OTUs	Richness	Diversity Index
Original sludge S	92,934	2406	2792.252	5.254318
SN-AC^{+}	39,842	63	99.14286	0.66382
SN	32,923	73	115.2727	0.895895
Original sludge E	76,996	2530	2772.612	5.576249
EN-AC^{+}	58,954	83	114.1667	0.232455
EN	101,637	110	126.1111	1.000994

For the high-quality sequence (HQ) numbers which were presented in Table 4.6, they were obtained from pre-processing in the samples. The present work showed the HQ-sequences of the samples more than 80% to total of each sample while most of low-quality sequence (LQ) comes low quality score of each base in its sequence, indicating the sequencing error occur on PCR primer region (Figure 4.13).

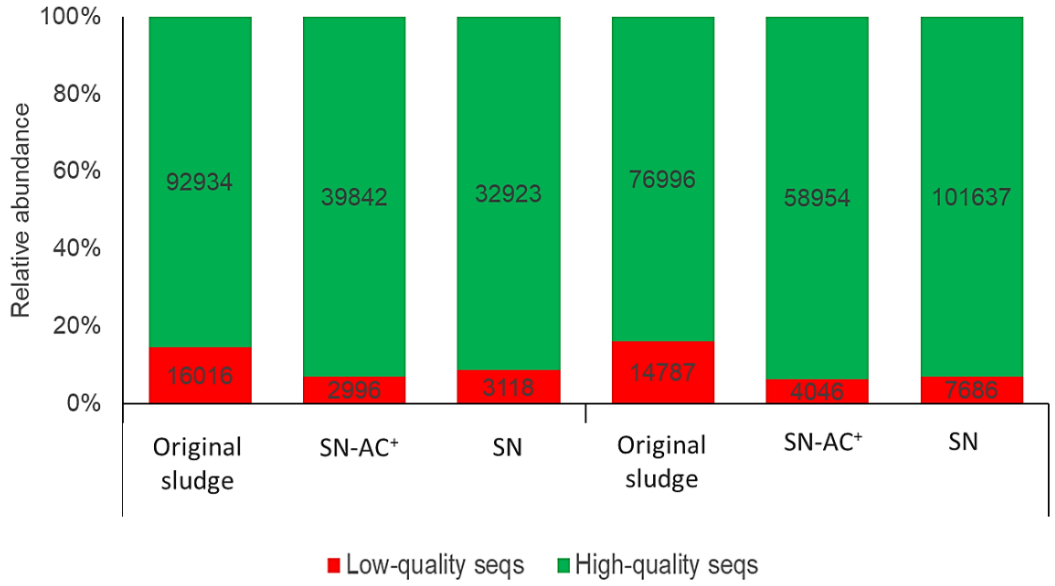


Figure 4.13 Mothur pre-processing steps represent the relative abundance of the sequence after the sequencing barcode and PCR primers were identified and removed.

The changes in the bacterial community at the phylum and genus levels are represented in Figures 4.14a and b. A total 13 out of 52 phyla are revealed, using 1% relative abundance across sample as criteria to determine dominant phylum (Fig. 4.14a). *Firmicutes* (E: 22.3% and S: 12.6%), *Proteobacteria* (E; 17.3%, S; 14.5%), *Bacteroidetes* (E: 14.3% and S: 13.8%), *Chloroflexi* (E: 8.2% and S: 17.4%) and *Euryarchaeota* (E: 9.7% and S: 11.2%) were found as the top five most abundant phyla in the original sludge from both factories (Figure 4.14a). Among phyla in original sludge samples, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the higher relative abundances in the sludge E than those in sludge S. In previous studies, these phyla are commonly found as the predominant members in sewage sludge and anaerobic digesters treating natural rubber wastewater (N. Li, Liu, Zhou, Dai, & Kong, 2019; Watari et al., 2016). On the other hand, *Chloroflexi* was the most predominant phylum in sludge S, accounting for 17.4%. This phylum was also reported to be the dominant populations in anaerobic digesters (Buettner & Noll, 2018).

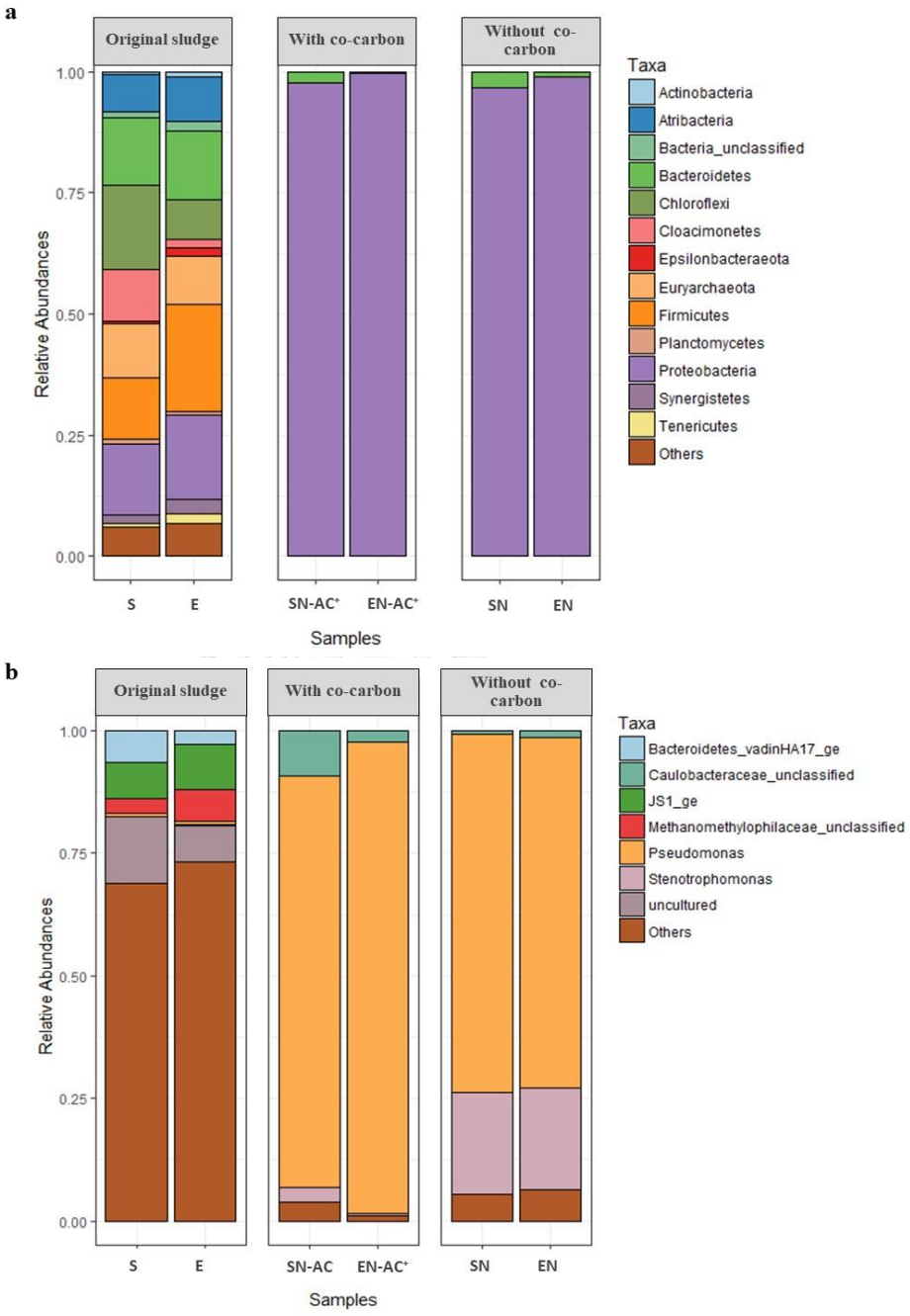


Figure 4.14 Comparison of bacterial compositions in original sludges from S and E rubber processing industries and bacterial consortia established after three stepwise acclimatization stages, presented on the phylum levels (a) and genus levels (b). The results determined based on the targeting the V4 variable regions of the 16S rRNA gene marker.

In addition, a high relative abundance of uncultured accounted for 20.9 and 15.9% of S and E, respectively, suggesting that the sludges from both factories are good reservoir of new microorganisms. After long-term acclimation period of this work, two phyla, *Proteobacteria* and *Bacteroidetes* were found to be dominant in consortia. Particularly, *Proteobacteria* phylum showed the highest relative abundant in the bacterial consortia, accounting for about 70-99% of the total bacteria, distinguishing from original sludge samples (Fig. 4.14a). Previous studied has reported that members of *Proteobacteria* were potential as persistence organic compound degraders (B. Li, Qiu, Zhang, Liang, & Huang, 2019; F. Wang, Li, Wang, Chen, & Huang, 2016).

A total of 663 genera were found in the original sludge samples, whereas a total of 29 genera were prevalent in the consortia. Seven dominant genera in each sample were revealed as dominant genera among samples using 5% relative abundance across samples for comparison, as shown in Figure 4.14b). The bacterial consortia were dominated by members of *Pseudomonas*, *Stenotrophomonas* and unclassified *Caulobacteraceae*. Most of the detected bacteria in the consortia of this study have been reported in heterocyclic aromatic-degrading bacterial consortia (Ahmad et al., 2019).

Among the acclimated consortia, distinct patterns of bacterial community profiles between different consortia were revealed at the OTU resolution, as illustrated in Figure 4.15. The relative abundance of *Pseudomonas* (OTU0001) was significantly increased in all of the acclimatized consortia, accounting for 71.36-96.07%, while the original sludge samples contained *Pseudomonas* at abundances in the range of 0.5-0.72%, as presented in Figure 4.15. Species belonging to the *Pseudomonas* genus have been proposed to degrade benzothiazoles through dioxygenase and methylation reactions (El-Bassi et al., 2010; Y. Li et al., 2017). *Stenotrophomonas* (OTU0002) was the second largest genus, contributing to high proportions of 20.89% and 20.59% in bacterial consortia SN and EN, respectively, which were much higher than those in the original sludges (0.05-0.14%) and bacterial consortia from the sodium acetate-containing acclimatization system (EN-AC^+: 0.51 and SN-AC^+: 3.07). All acclimatized bacterial consortia contained unclassified *Caulobacteraceae* (OTU0003) as the third

most abundant population. There was a higher relative abundance of unclassified *Caulobacteraceae* in SN-AC$^+$ (9.31%) and EN-AC$^+$ (2.35%) than in the other acclimatized consortia (0.60-1.47%) and the original sludges (0.01-0.02%). The additional supplementation of a co-organic carbon source in the acclimatization systems of sludge S and E might have led to an increase in unclassified *Caulobacteraceae* abundance. Members of unclassified *Caulobacteraceae* have also never been reported as 2-MBT biodegrading bacteria, but they are newly identified lignocellulose degraders (Puentes-Téllez & Salles, 2020; Wilhelm, Singh, Eltis, & Mohn, 2019).

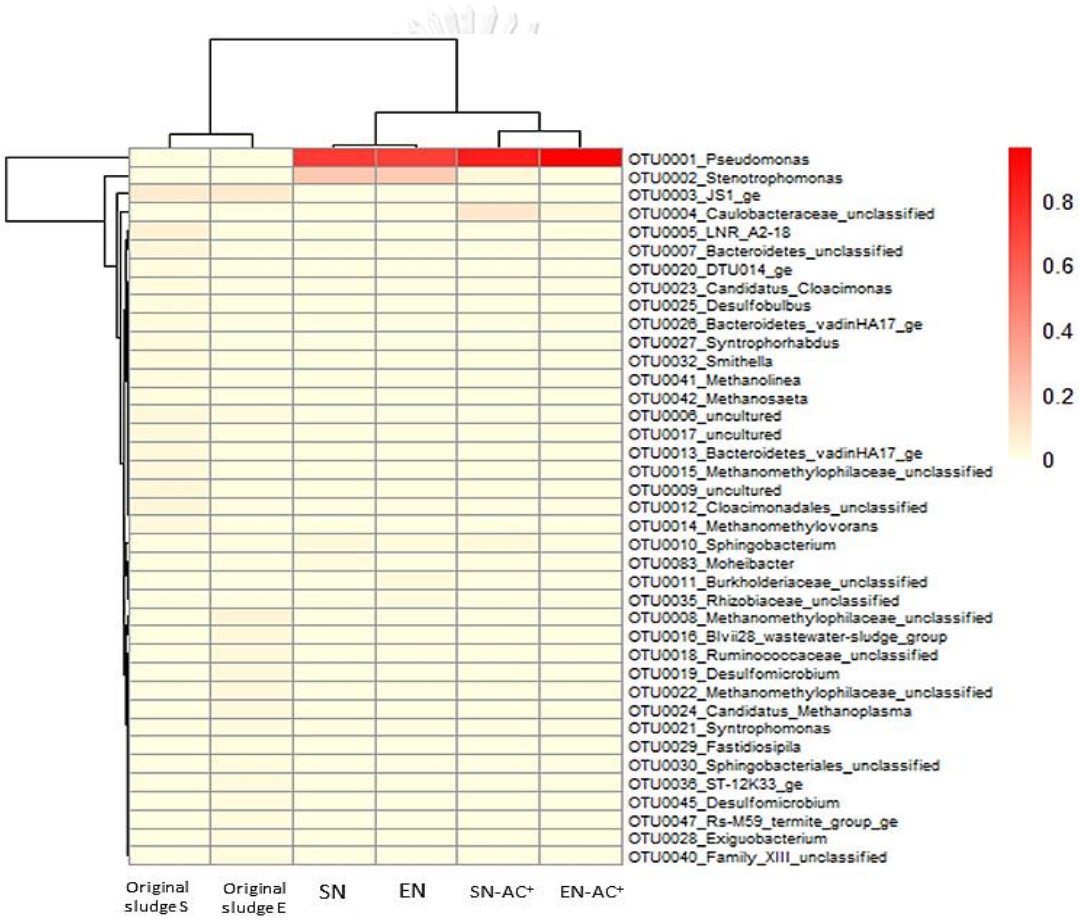


Figure 4.15 Heat map plot relied on relative abundance of OTU across samples. The horizontal and vertical dendrogram were calculated based on Bray-Curtis dissimilarity. Color represent the range of relative from 0 (light yellow) to 1 (red). The label represents the OTU with taxonomic annotation at genus level.

Interestingly, the *Stenotrophomonas* genus has never been reported to exhibit 2-MBT biodegradation, but they are known degraders of heterocyclic aromatic rings (Galíndez-Nájera et al., 2011; Rajini, Sasikala, & Ramana, 2010). Across all sites, the *Pseudomonas*, *Stenotrophomonas* and unclassified *Caulobacteraceae* genera were found in higher abundance in the consortia which were expected to be the major 2-MBT-degrading bacteria in the consortia. These results indicate that the stepwise acclimatization process played an important role in selecting for these bacteria and maintaining the community composition over long-term cultivation.

Currently, there is limited study on the microbial community composition of bacterial consortia capable of degrading 2-MBT. This study was the first to show the distinct shifts in bacterial community composition from sludge S and E to bacterial consortia SN and EN with high 2-MBT degradation efficiency under aerobic condition (Table 4.7).

Table 4.7 Bacterial community of published reports and the obtained bacterial consortia.

Microbial inoculum	Test system/ duration	Type of benzothiazoles	References
Anaerobic granular sludge with *Trichococcus* and *Clostridium sensu stricto* as the dominant populations	Anaerobic batch with mixed liquor suspended solids in synthetic wastewater/ 84 h	Benzothiazole	Y. Li et al. (2017)

Table 4.7 Bacterial community of published reports and the obtained bacterial consortia. (Cont.)

Microbial inoculum	Test system/ duration	Type of benzothiazoles	References
Diluted river mud with *Geobacter*, *Bacterioides* and *Rhodococcus rhodochrous* as the dominant populations	Microbial electrolysis cells (MECs) conducting in fed-batch mode with mineral medium/ 30 h	2-MBT	San-Martin et al. (2020)
Consortium SN-AC^{+} with *Pseudomonas*, *Stenotrophomonas* and *Caulobacteraceae* as the dominant populations	Aerobic batch with resting cells in mineral medium/ 72 h	2-MBT	This study
Consortium SN with *Pseudomonas* and *Stenotrophomonas* as the dominant populations			

Table 4. 7 Bacterial community of published reports and the obtained bacterial consortia. (Cont.)

Microbial inoculum	Test system/ duration	Type of benzothiazoles	References
Consortium EN-AC^{+} with *Pseudomonas*, *Stenotrophomonas* and *Caulobacteraceae* as the dominant populations	Aerobic batch with resting cells in mineral medium/ 72 h	2-MBT	This study
Consortium EN with *Pseudomonas* and *Stenotrophomonas* as the dominant populations			

On the other hand, various bacterial populations are reported during the biodegradation of benzothiazole and 2-MBT under anaerobic condition by mixed bacterial culture. For example, *Trichococcus* and *Clostridium sensu stricto* are the dominant populations in anaerobic granular sludge (Y. Li et al., 2017), while *Geobacter*, *Bacterioides* and *Rhodococcus rhodochrous* are the dominant populations in microbial electrolysis cells (MECs) (San-Martin et al., 2020) (Table 4.6). This study suggested that the mixed bacterial inoculum for the treatment of benzothiazole-contaminated rubber wastewater under aerobic condition should contain bacteria in the genera of *Pseudomonas* and *Stenotrophomonas*.

The cooperation of *Pseudomonas* and *Stenotrophomonas* for 2-MBT-biodegradation was first identified here. However, these two genera have been reported as dominant populations during the degradation of polycyclic aromatic hydrocarbons (PAHs) by a bacterial consortium isolated from petroleum polluted soil

(González et al., 2011). This study provided a better understanding of the microbial community structure relevant to 2-MBT degradation. This knowledge would facilitate the development of a bacterial inoculum for the treatment of thiazole-contaminated rubber wastewater. From these results, EN bacterial consortium was selected for treating 2-MBT in rubber processing wastewater due to its higher diversity and 2-MBT biodegradability than other consortia.

4.3.2 Functional gene profiling through metatranscriptome approach in the 2-MBT degrading bacterial consortium community

Gene expression associated with 2-MBT degradation in EN bacterial consortium was examined. In this study, the metatranscriptomics was used to profile and characterize the difference in gene expression in response to both original sludge and EN bacterial consortium.

4.3.2.1 Effect of cell growth state on 2-MBT degradation activity

In order to collect the EN bacterial consortium for obtaining high concentration of RNA and further identify the transcript genes among diverse microorganisms in the EN bacterial consortium, the state of the cells was investigated in response to 2-MBT degrading activity. Growth rate of bacterial cells is phenotypic characteristics that has potentially impact on specific gene expression and gene regulation system (Wytock & Motter, 2019).

Figure 4.16 depicts bacterial growth and degradation curves of the bacterial consortium when incubating in the N medium containing 2-MBT at 100 mg L^{-1} as the sole carbon source. During the incubation period, the growth result was obtained by measuring the growth of cell population increase over time. The result showed no noticeable lag phase during growth period due to the initial inoculum size of this work was 5% (v/v) that contained more than 10^8 CFU mL^{-1} cells in the culture medium. Moreover, the acclimated consortium can be physiologically adapted themselves to synthesize the appropriate enzymes for 2-MBT metabolism. The length of the exponential growth appeared between 3 h to 30 h of incubation periods. This phase can be divided into three sub phase by microbial biomass at the point of time including

early log (0 h-6 h), mid-log (6 h-24 h) and late log (24 h-30 h). Nadaf & Ghosh (2011) suggested that the genes were potential expressed given by late log phase growth. Consequently, the cell suspension was collected at time point of 21-24 h of incubation for RNA extraction which was used to compare with the transcript genes of original sludge.

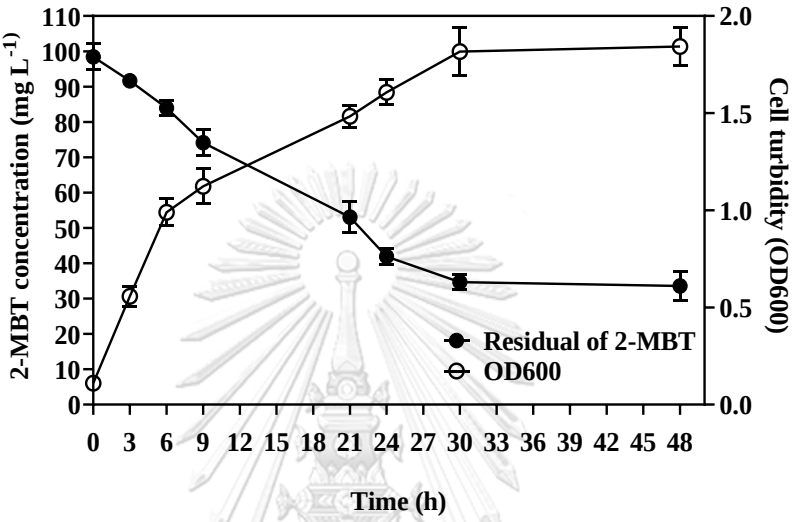
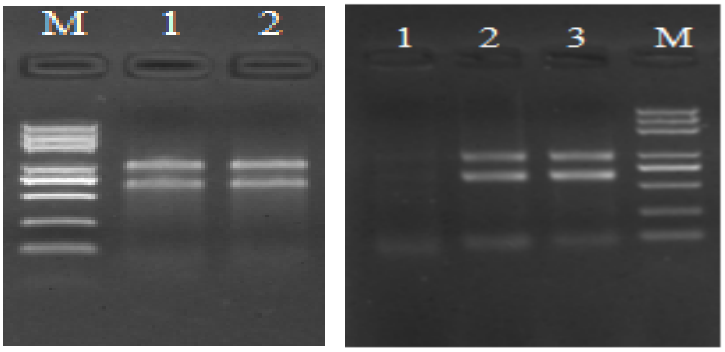


Figure 4.16 Representative growth curve of 2-MBT-grown cells (○) is measured as the OD_{600} and the residual of 2-MBT presented in the N medium (■). Error bars represent errors (*n* = 3).

The RNA of the consortium was extracted and further analyzed the quality and quantity of the extracted RNA by agarose gel electrophoresis, as shown in Figure 4. 17. This method can be used to test the RNA degradation and the RNA contamination. In addition, overall results of the RNA quality of the original sludge and the EN bacterial consortium were presented in Table 4.8.



Original sludge E EN bacterial consortium

Figure 4.17 Agarose Gel Electrophoresis for RNA quality of the original sludge from E industry (left) and the bacterial consortium EN (right). M: Trans 2K plus DNA ladder, 1 loaded is 2 µL.

Table 4.8 Overall results of RNA concentration, rRNA ratio and RNA integrity number for the original sludge and acclimated consortium

Sample name	RNA concentration (ng/µl)	Amt (ng)	OD260/280	OD260/230	RIN
EN cells, R1	378	15.88	2.03	2.15	9.70
EN cells, R2	298	5.66	2.04	1.62	9.80
Original sludge E R1	306	5.8	1.94	1.70	8.40
Original sludge E R2	306	5.8	2.04	1.65	8.40

4.3.2.2 Functional and phylogenetic characteristics of the 2-MBT degrading bacterial community

The goal of this experiment was to compare the transcript levels between original sludge (non-acclimatized cultures) and EN bacterial consortium (acclimatized cultures). In this study, RNA-seq metatranscriptomic approach was used to quantify the level of a specific degradation genes in a complex community and compare gene expression profiles of both original sludge and EN bacterial consortium. Functional

genes associated to metabolism of 2-MBT in the EN consortium were probably determinant factors controlling benzothiazoles biodegradation. This work employed metatranscriptomic analysis as a promising approach to shed new light on the metabolic processes involved in the response of microbial communities to benzothiazole adaptation.

After normalizing the sequence counts for each taxonomic by the total reads. The paired reads of *Pseudomonas* genera were merged into 2,650,288 and 2,203,473 of EN consortium R1 and R2, respectively. The sequences were compared against the original sludge, as presented in Table 4. 9. The sequences from metatranscriptomic data matched well with data of 16S rRNA sequencing which revealed that *Pseudomonas* was the most abundant population at community level.

Table 4.9 Relative abundance of genera involving 2-MBT degradation in EN consortium and original sludge based on metatranscriptomic analyses

Sample	Total read	Reads		
		Pseudomonas	*Stenotrophomonas*	Shared protein between *Pseudomonas* and *Stenotrophomonas*
EN R1	2,650,288	2,150,164	8,306	5,392
EN R2	2,203,473	2,000,677	10,016	6,607
Original sludge E	1241079	2618	34	0

The genus-level analysis showed that duplicate result of 81% and 91% of *Pseudomona*s was among most abundant genera that exist in the EN consortium. *Stenotrophomonas* was 0.3% and 0.45% in R1 and R2, respectively.

Interestingly, this work also indicated that the cooperation between *Pseudomonas* and *Stenotrophomona*s was observed the shared protein together in

the EN consortium, accounting to 0.20% (5,392 reads) and 0.29% (6,607), in R1 and R2, respectively (Table 4.10). Thus, the functional capabilities of bacterial communities, especially *Pseudomonas* and *Stenotrophomonas* had potential impact on 2-MBT metabolism.

Table 4.10 Ratio of reads associated to genera in the EN consortium and the original sludge based on metatranscriptomic analyses

Samples	% ratio of reads			
	Pseudomonas	*Stenotrophomonas*	Shared protein between *Pseudomonas* and *Stenotrophomonas*	Other genera
EN R1	81.1294	0.3134	0.2034	18.3538
EN R2	90.7965	0.4546	0.2998	8.4491
Original sludge E	0.2109	0.0027	0	99.7864

The annotation of contigs of the EN bacterial consortium more highly transcribed during 2-MBT metabolism. This result revealed the adaptation of bacterial community in the EN bacterial consortium after long-term acclimatization process. Most of the detected protein contigs related to a more resistance proteins. As illustrated in Table 4.11, the observed protein contigs in the EN bacterial consortium were also found in specific xenobiotic degraders. For example, hypothetical protein (AGA75498.1) of an antibiotic resistance gene (Molina et al., 2014) was the highest abundance in the EN bacterial consortium (3,779,405) compared with the original sludge (1,252). In addition, the EN bacterial consortium were dominated by conserved exported protein (SPO57603.1) and hypothetical protein (SDM81684.1) that containing 288,831 and 212,290.5 abundances, respectively. These predicted proteins have been expressed in *Pseudomonas* sp. strain Chol1, which is a model microorganism for the

naphthalene degrading bacteria and bile salt-degrading bacteria (Holert et al., 2013). Moreover, the other hypothetical proteins were expressed in the bacterial consortium that were related to the xenobiotic degraders (S. Li, Zhao, Li, Niu, & Cai, 2012).

Table 4.11 Difference in top ten contigs of original sludge and EN bacterial consortium

Accession number	Reads			Annotation
	EN R1	EN R2	Original sludge E	
SDM81684.1	263389	161192	293	hypothetical protein SAMN05660875_109145 [*Pseudomonas balearica* DSM 6083]
AGA75498.1	259189	496692	1252	hypothetical protein B479_23045 [*Pseudomonas putida* HB3267]
SPO57603.1	217512	360150	191	conserved exported protein of unknown function [*Pseudomonas* sp. JV551A1]
SPO57553.1	105858	102643	42	conserved protein of unknown function [*Pseudomonas* sp. JV551A1]

Table 4.11 Difference in top ten contigs of original sludge and EN bacterial consortium (Cont.)

Accession number	Reads			Annotation
	EN R1	EN R2	Original sludge E	
EKM96396.1	98302	91792	312	hypothetical protein C211_08439 [*Pseudomonas* sp. Chol1]
WP_133975529.1	88066	115322	41	hypothetical protein [*Pseudomonas inefficax*]
WP_003248687.1	80171	67150	31	MULTISPECIES: hypothetical protein [Gammaproteobacteria]
SPO61030.1	74975	85286	33	conserved protein of unknown function [Pseudomonas inefficax]
WP_003253897.1	66919	40905	26	MULTISPECIES: hypothetical protein [*Pseudomonas*]
WP_134939841.1	50256	59778	23	hypothetical protein [*Pseudomonas veronii*]

In order to assign annotation to potential genes of interest, all filter contigs were blasted and determined the putative function. The differential gene expression analysis between original sludge and EN bacterial consortium was analyzed and compared. The majority of these contigs were annotated as gene involved in 2-MBT metabolism. In general, the oxygenase enzymes including monooxygenase and dioxygenase were up-regulated which have been associated with the breakdown of 2-

MBT (El-Bassi et al., 2010; Haroune et al., 2004; Haroune et al., 2002; Umamaheswari & Rajaram, 2017).

In this study, metatranscriptome results revealed transcripts mapped to oxidation reaction including monooxygenase and dioxygenase, which were expressed at higher level than in the original sludge as presented in Table 4.12. The EN bacterial consortium could convert the 2-MBT into two metabolites included a cis-dihydrodiol derivative and a hydroxylated compound and further degraded by catechol 1, 2 dioxygenases, as given in Table 4.13. Other metabolites might be produced later by various microorganisms. In this work, the possible route of 2-MBT biodegradation by the EN consortium was based on the result of transcript genes that existed in the consortium (Figure 4.18). However, the biodegradation pathway should be further investigated in minimal salt medium and metabolites analysis.

Interestingly, the genes involved in methylation reaction were observed at great abundance in the EN bacterial consortium (Table 4.14). Methyltransferase has been proposed in 2-MBT biodegradation pathway (Reemtsma et al., 1995; Umamaheswari & Rajaram, 2017). Umamaheswari & Rajaram (2017) suggested that inorganic compounds including ammonia and sulfate were released during biodegradation process. In this study, metatranscriptomic results also revealed the genes associated with sulfite, sulfate, and ammonia degradation. Transcripts genes mapped to ammonium transportation and sulfate/sulfite metabolism are provided in Tables 4.15 and 4.16.

Table 4.12 Difference in reads of EN bacterial consortium (duplicates) and original sludge, using 5 reads as cut-off point.

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Oxidation (Monoxygenase)	AEJ15752.1	44	48	0	putative flavin-binding monooxygenase [*Pseudomonas putida* S16]
	WP_015271638.1	150	191	0	antibiotic biosynthesis monooxygenase [*Pseudomonas*]
	AEJ15752.1	44	48	0	putative flavin-binding monooxygenase involved in arsenic resistance [*Pseudomonas putida* S16]
	WP_137164335.1	37	55	0	nitronate monooxygenase [Pseudomonas asiatica]

Table 4.12 Difference in reads of EN bacterial consortium (duplicates) and original sludge, using 5 reads as cut-off point. (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Oxidation (Dioxygenase)	WP_112254175.1	35	42	0	TauD/TfdA family dioxygenase [Pseudomonas putida]RAM66298.1 SyrP [*Pseudomonas putida*]
	WP_038409121.1	19	27	0	dioxygenase [*Pseudomonas putida*]
	WP_033699720.1	14	19	0	MULTISPECIES: TauD/TfdA family dioxygenase [*Pseudomonas*]
	OII58920.1	14	17	0	dioxygenase [*Pseudomonas putida*]
	WP_085706364.1	4	7	0	dioxygenase [*Pseudomonas* sp. B8(2017)]
	AZR96215.1	5	4	0	dioxygenase [*Bordetella trematum*]

Table 4.13 Difference in reads of EN bacterial consortium (duplicates) and original sludge, using the abundance of 5 reads as cut-off point.

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Ring- hydroxylating dioxygenase	WP_085615638.1	8	5	0	MULTISPECIES: ring-hydroxylating dioxygenase ferredoxin reductase family protein [*Pseudomonas incertae sedis]*
	WP_061305294.1	5	4	0	ring-hydroxylating dioxygenase ferredoxin reductase family protein [*Pseudomonas monteilii*]

Table 4.13 Difference in reads of EN bacterial consortium (duplicates) and original sludge, using the abundance of 5 reads as cut-off point. (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Ring- hydroxylating dioxygenase	AAF19975.1	5	9	0	putative class I ring-hydroxylating dioxygenase, partial [*Pseudomonas putida*]
	WP_004576275.1	6	5	0	MULTISPECIES: ring-hydroxylating dioxygenase ferredoxin reductase family protein [*Pseudomonas*]
Intradiol dioxygenase using an Fe^{3+} cofactor.	WP_102083660.1	120	95	0	catechol 1,2-dioxygenase [*Pseudomonas plecoglossicida*]
	WP_013973065.1	99	90	0	MULTISPECIES: catechol 1,2-dioxygenase [*Pseudomonas*]
	WP_108480622.1	52	54	0	catechol 1,2-dioxygenase [*Pseudomonas plecoglossicida*]

Table 4.13 Difference in reads of EN bacterial consortium (duplicates) and original sludge, using the abundance of 5 reads as cut-off point. (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Intradiol dioxygenase using an Fe^{3+} cofactor.	WP_047603049.1	32	33	0	catechol 1,2-dioxygenase [*Pseudomonas putida*]
	WP_133326620.1	36	35	0	catechol 1,2-dioxygenase [*Pseudomonas putida*]
	WP_023118542.1	21	22	0	MULTISPECIES: catechol 1,2-dioxygenase [*Pseudomonas*]
	WP_003120081.1	16	20	0	catechol 1,2-dioxygenase [*Pseudomonas aeruginosa*]
	WP_075045181.1	9	9	0	catechol 1,2-dioxygenase [*Pseudomonas putida*]
	HBK49450.1	7	7	0	catechol 1,2-dioxygenase [*Pseudomonas* sp.]

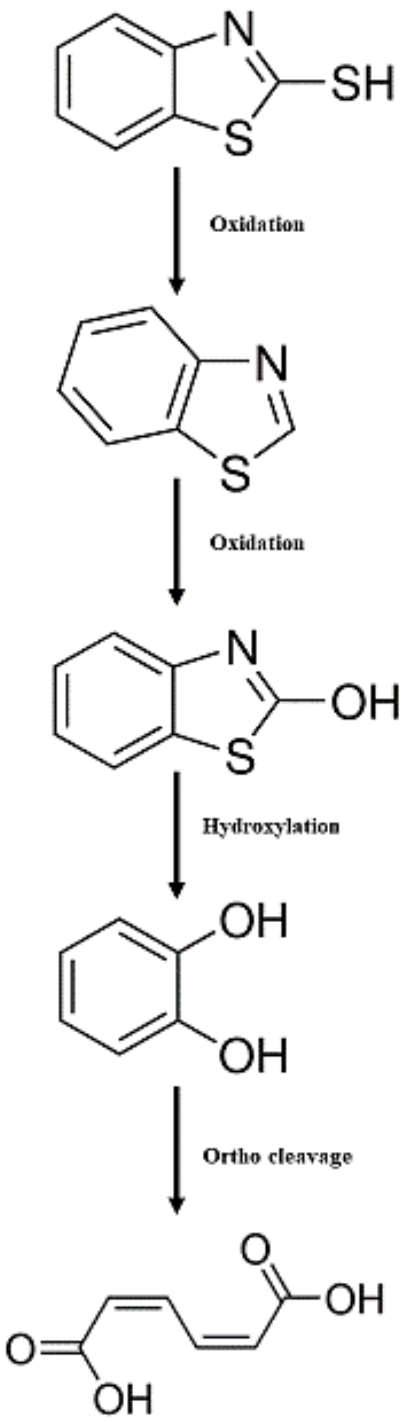


Figure 4.18 The possible route of 2-MBT biodegradation pathway based on prediction of transcribed genes expressing in the EN bacterial consortium.

Table 4.14 Predicted putative genes for methyltransferase route in 2-MBT metabolism, using the abundance of 5 reads as cut-off point.

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
Conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates	WP_123085110.1	17	25	0	glutathione S-transferase [*Pseudomonas putida*]
A class of enzymes that catalyze the transfer of a methyl group from methyl donor S-adenylyl-l-methionine (SAM) to their substrates	WP_135002037.1	10	18	0	methyltransferase [*Pseudomonas putida*]
	EJT85006.1	20	29	0	type 11 methyltransferase [*Pseudomonas putida* S11]
	WP_054573585.1	8	12	0	Bifunctional cobalt-precorrin-7 (C(5))-methyltransferase/cobalt-precorrin-6B (C(15))-methyltransferase [*Pseudomonas putida*]

Table 4.14 Predicted putative genes for methyltransferase route in 2-MBT metabolism, using the abundance of 5 reads as cut-off point. (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
A class of enzymes that catalyze the transfer of a methyl group from methyl donor S-adenylyl-l-methionine (SAM) to their substrates	WP_102083892.1	19	23	0	MULTISPECIES: class I SAM-dependent methyltransferase [*Pseudomonas*]
	WP_151345791.1	26	48	0	methyltransferase domain-containing protein, partial [*Pseudomonas putida*]
	WP_015270906.1	15	20	0	MULTISPECIES: class I SAM-dependent methyltransferase [*Pseudomonas*]

Table 4.15 Predicted putative genes for sulfate/ sulfite metabolism and ammonium transportation, using the abundance of 5 reads as cut-off point.

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
An enzyme transfers a sulfate group from phenolic sulfate esters	AEJ14547.1	8	11	0	conserved hypothetical protein [*Pseudomonas putida* S16]
	WP_012273773.1	7	9	0	aryl-sulfate sulfotransferase [*Pseudomonas putida*]
	WP_125033136.1	8	7	0	aryl-sulfate sulfotransferase [*Pseudomonas aeruginosa*]
	WP_054901098.1	196	274	0	IscS subfamily cysteine desulfurase [*Pseudomonas incertae sedis*]
	WP_090261455.1	15	25	0	IscS subfamily cysteine desulfurase [*Pseudomonas*]
	WP_126867387.1	22	30	0	IscS subfamily cysteine desulfurase [*Pseudomonas aeruginosa*]

Table 4. 15 Predicted putative genes for sulfate/sulfite metabolism and ammonium transportation, using the abundance of 5 reads as cut-off point. (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
An enzyme transfers a sulfate group from phenolic sulfate esters	WP_060479412.1	28	38	0	IscS subfamily cysteine desulfurase [*Pseudomonas monteilii*]
An enzyme of the esterase class that catalyze sulfate esters.	BBH46543.1	29	42	0	alkyl sulfatase [*Pseudomonas* sp.]
	OII56077.1	28	39	0	sulfatase, partial [*Pseudomonas putida*]
An enzyme catalyzes the formation of disulphide bonds.	WP_012271357.1	24	36	0	Si-specific NAD(P)(+) transhydrogenase [*Pseudomonas*]
Ammonium transport (Amt) proteins for uptake and assimilation of nitrogen	TXI07667.1	11	12	0	ammonium transporter, partial [*Pseudomonas*]
	WP_086974186.1	5	6	0	ammonium transporter [*Pseudomonas putida*]

Table 4.16 Predicted putative genes for sulfate/ sulfite metabolism and ammonium transportation, using the abundance of 5 reads as cut-off point

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
An enzyme that catalyzes the chemical reaction ATP + sulfate, pyrophosphate + adenylyl sulfate	WP_039613009.1	12	21	0	sulfate adenylyltransferase subunit CysN [*Pseudomonas* sp. C5pp]
	WP_070331229.1	25	35	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas aeruginosa*]
	WP_099775736.1	21	33	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas incertae sedis*]
	WP_056834179.1	15	23	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas*]
	WP_134691113.1	15	28	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas* sp. RIT623]

Table 4.16 Predicted putative genes for sulfate/ sulfite metabolism and ammonium transportation, using the abundance of 5 reads as cut-off point (Cont.)

Reaction	Accession number	Reads			Annotation
		EN R1	EN R2	Original sludge E	
An enzyme that catalyzes the chemical reaction ATP + sulfate, pyrophosphate + adenylyl sulfate	WP_023378799.1	9	19	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas*]
	WP_027918026.1	9	12	0	sulfate adenylyltransferase subunit CysD [*Pseudomonas incertae sedis*]
	WP_112252290.1	44	68	0	sulfite exporter TauE/SafE family protein [*Pseudomonas putida*]
	WP_054572886.1	30	49	0	PQQ-dependent catabolism-associated CXXCW motif protein [*Pseudomonas*]
	WP_013974448.1	19	26	0	sulfurtransferase [*Pseudomonas*]
	WP_061303896.1	31	26	0	thiosulfate sulfurtransferase GlpE [*Pseudomonas*]
	WP_132846689.1	12	22	0	molybdopterin-synthase adenylyltransferase MoeB [*Pseudomonas putida*]

2-MBT is reported for antimicrobial activities and has a potential to inhibit several membrane proteins like acyl coenzyme A cholesterol acyltransferase, monoamine oxidase, heat shock protein, cathepsin D, and c-Jun N-terminal kinases (Azam & Suresh, 2012). Moreover, 2-MBT may interact with membrane bound protein system and reduce the level of flavoproteins or Quinone and Fe-S clusters (H. De Wever et al., 1994). For this work, metatranscriptomic profiling revealed dominant proteins that related to membrane bound system in the EN bacterial consortium at higher relative abundance than that in the original sludge (Table 4.17). Previous reports showed that several microorganisms use multidrug resistance proteins to persist intrinsic agents with adapted efflux pump resistance mechanisms (Daniels & Ramos, 2009). The membrane protein (WP_025341148.1) was found only in the acclimatized EN consortium (Table 4.17) but not in the original sludge.

This might contribute to the ability to resistant the 2-MBT in the EN bacterial consortium. In addition, the AdeC/ AdeK/ OprM family multidrug efflux complex outer membrane factor, multidrug transporter was found in the EN bacterial consortium at higher abundance than in the original sludge (Table 4.12). This type of protein is one of the mechanism that bacteria use to evade the toxic effects of the bioactive agents (Putman, van Veen, & Konings, 2000). According to Table 4.17, this work indicated that the bacterial cells in the EN bacterial consortium had developed various ways to resist the toxic effects of 2-MBT and their derivatives during acclimatization process.

Table 4.17 Predicted membrane bound proteins in the EN bacterial consortium compared with the original sludge.

Accession number	Reads			Annotation
	EN R1	EN R2	Original sludge E	
WP_054573880.1	431	949	0	MULTISPECIES: DUF485 domain-containing protein [*Pseudomonas*]
WP_025341148.1	120	150	0	MULTISPECIES: TIM44-like domain-containing protein [*Pseudomonas*]
WP_046614836.1	63	86	0	MULTISPECIES: AdeC/AdeK/OprM family multidrug efflux complex outer membrane factor
WP_056801870.1	54	75	0	chaperonin GroEL [*Pseudomonas* sp. Leaf58]
WP_033701830.1	120	157	0	MULTISPECIES: membrane integrity-associated transporter subunit PqiC [*Pseudomonas*]
WP_025340591.1	52	69	0	efflux transporter outer membrane subunit [*Pseudomonas* sp. FGI182]

Table 4.17 Predicted membrane bound proteins in the EN bacterial consortium compared with the original sludge. (Cont.)

Accession number	Reads			Annotation
	EN R1	EN R2	Original sludge E	
WP_112254220.1	67	91	0	outer membrane protein assembly factor BamC [*Pseudomonas* putida]
WP_015272354.1	292	380	0	MULTISPECIES: GlsB/YeaQ/YmgE family stress response membrane protein [*Pseudomonas*]
WP_012051471.1	55	97	0	MULTISPECIES: OprD family porin [Pseudomonas], outer membrane porin [*Pseudomonas putida* F1]
WP_027615814.1	41	52	0	MULTISPECIES: outer membrane protein assembly factor BamA
WP_003253367.1	147	131	0	MULTISPECIES: TonB system transport protein ExbD [*Pseudomonas*]

Table 4.17 Predicted membrane bound proteins in the EN bacterial consortium compared with the original sludge. (Cont.)

Accession number	Reads			Annotation
	EN R1	EN R2	Original sludge E	
WP_019097259.1	52	89	0	MULTISPECIES: toluene efflux RND transporter periplasmic adaptor subunit TtgA [*Pseudomonas]*
WP_003259010.1	63	100	0	MULTISPECIES: hypothetical protein [*Gammaproteobacteria*]
WP_046613482.1	90	122	0	MULTISPECIES: DUF924 domain-containing protein [Gammaproteobacteria]
AAF64240.1	95	148	0	outer membrane porin, partial [*Pseudomonas putida*]

4.4 Development of an immobilized 2-MBT degrading bacterial consortium for applying in rubber processing wastewater

In this phase, the EN bacterial consortium was selected to apply as a bioaugmented culture for treating rubber wastewater under continuous operation. Since the colonization and maintenance of the active bacteria in complex rubber contaminated wastewater are important for efficient bioaugmentation, the embedded immobilization strategy was chosen for this study. In the present work, bacterial consortium was immobilized into a porous carrier as described in Chapter 3.

The objectives of this phase were: 1) to investigate the biodegradation of 2-MBT by suspended and immobilized EN bacterial consortium; 2) to determine the reusability of the immobilized EN bacterial consortium; 3) to start up lab-scale bioreactors to evaluate the performance of the immobilized bacterial consortium on real rubber processing wastewater treatment at different organic loading rates (OLR).

4.4.1 2-MBT adsorption capacity of the carrier

2-MBT adsorption capacity of the carrier was investigated by using 2 g of porous carrier (55-57 pieces) in 100 mL of minimal medium (pH 7.20). Figure 4.19 shows the concentration of 2-MBT in the solution as an equilibrium 2-MBT concentration. This result indicated that the porous carrier could adsorb the 2-MBT in low concentration when the 2-MBT concentration of 50-300 mg L^{-1} were tested. The result was corresponded with the high-water solubility of the 2-MBT, which were 118 mg L^{-1} and 230 mg L^{-1} in water and buffer medium at pH 7 and 7.5, respectively.

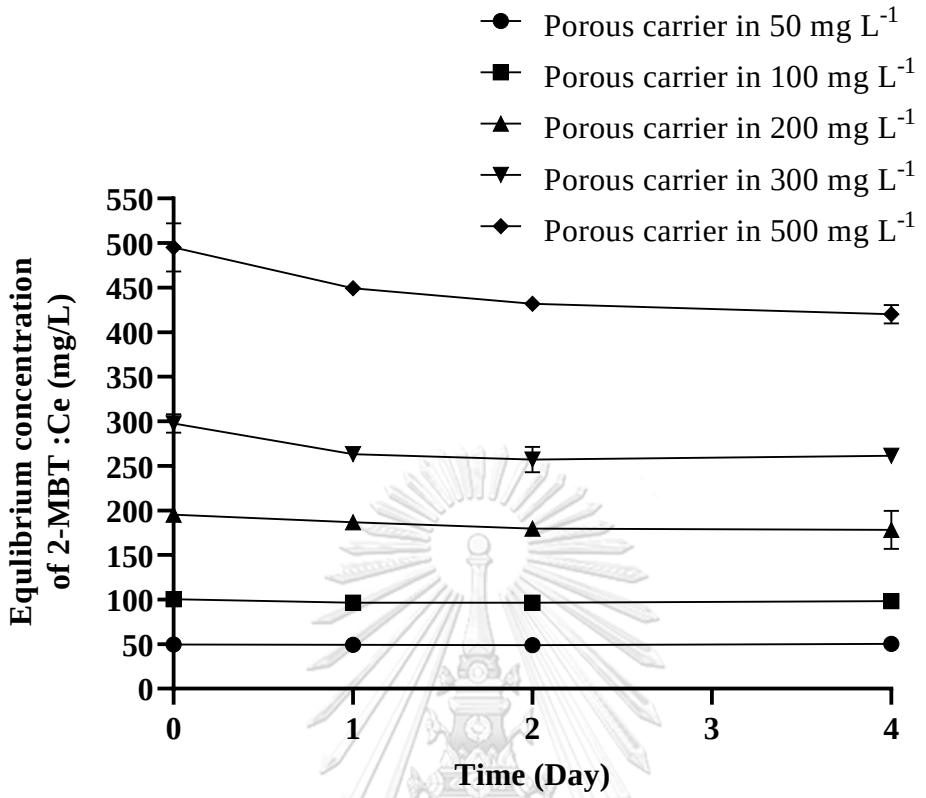


Figure 4.19 The equilibrium concentration of 2-MBT after applying the 2% (w/v) of porous carrier in the MSM containing 50 to 500 mg L^{-1} of 2-MBT at room temperature, 150 rpm for 4 days of operation.

Adsorption capacity (q_e) of the carrier as a function of time was later investigated in a batch mode. In this study, 2-MBT at 100 mg L^{-1} was chosen to mimic the real level of 2-MBT in the rubber processing wastewater. Figure 4.20 presents the adsorption capacity of the porous carrier at different time intervals. After the first 15-min of operation, the adsorption capacity (q_e) was fluctuated. This might be due to the adsorption of 2-MBT on rough surface of porous, thereafter the 2-MBT residual was released and detected. After 24-h operation, the adsorption capacity (q_e) was found to be 0.60-0.66 mg g^{-1} (mg of substrate adsorbed into 1 g of carrier). The low adsorption capacity was due to the high-water solubility of 2-MBT, even though the carrier has porous structure and high surface area. In addition, this type of material did not have swelling property because of high porosity and well-defined surface properties.

Previous work also suggested that the impact of narrow micro-pore and pore size distribution related to the adsorption capacity of organic compound (Lorenc-Grabowska, 2016). The porous carriers can easily adsorb diesel fuel or liquid oil component in the pores of the carrier (Atta, El-Hamouly, Al Sabagh, & Gabr, 2007; H.-D. Liu, Wang, Yang, & He*, 2014). The sorption capacity of the porous carriers could be varied depending on the type of the organic compounds, the volume of porous carriers and affinity to the pollutant. Given the low 2-MBT adsorption capacities, the toxicity of 2-MBT to immobilized bacteria on porous carrier would be low. These results suggested that the porous carrier was suitable for applying in the bioreactor.

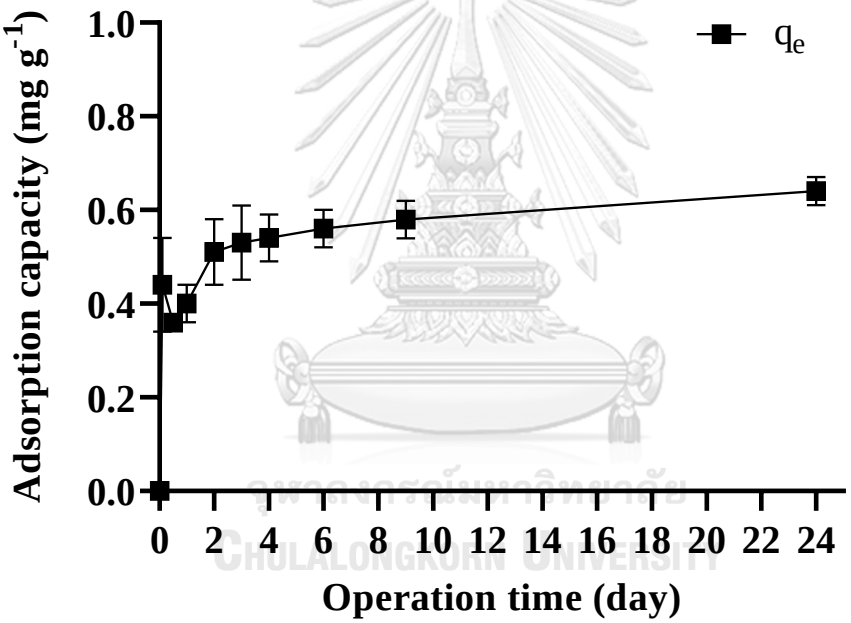


Figure 4.20 Adsorption capacity (*qe*) of 2 % (w/v) porous carriers in medium solution containing 100 mg L^{-1} 2-MBT under batch mode.

4.4.2 Optimization of cell immobilization conditions

Cell immobilization has been described to be the most efficient and cost effective strategy to overcome the problems faced by suspended cells in bioreactor for wastewater treatment (Farag, Soliman, & Abdel-Fattah, 2018). In order to obtain the high density of bacterial cells in the supporting material, this work used benzoate as

co-carbon source to produce high cell concentration and enhance 2-MBT degrading activity. The results suggested that 5 mM benzoate promoted the highest growth and did not inhibit 2-MBT degradation activity (Figure 4.21). It was possible that co-metabolic degradation of 2-MBT was occurred by the EN bacterial consortium in the presence of 5 mM of benzoate as an additional carbon source. This co-substrate has been proposed to support cell growth and increase biomass concentration, and also induce some speficic enzymes (Nowak & Mrozik, 2016). These bacteria growth profiles suggested that 48-h incubation was optimum for bacterial culturing and immobilization processes.

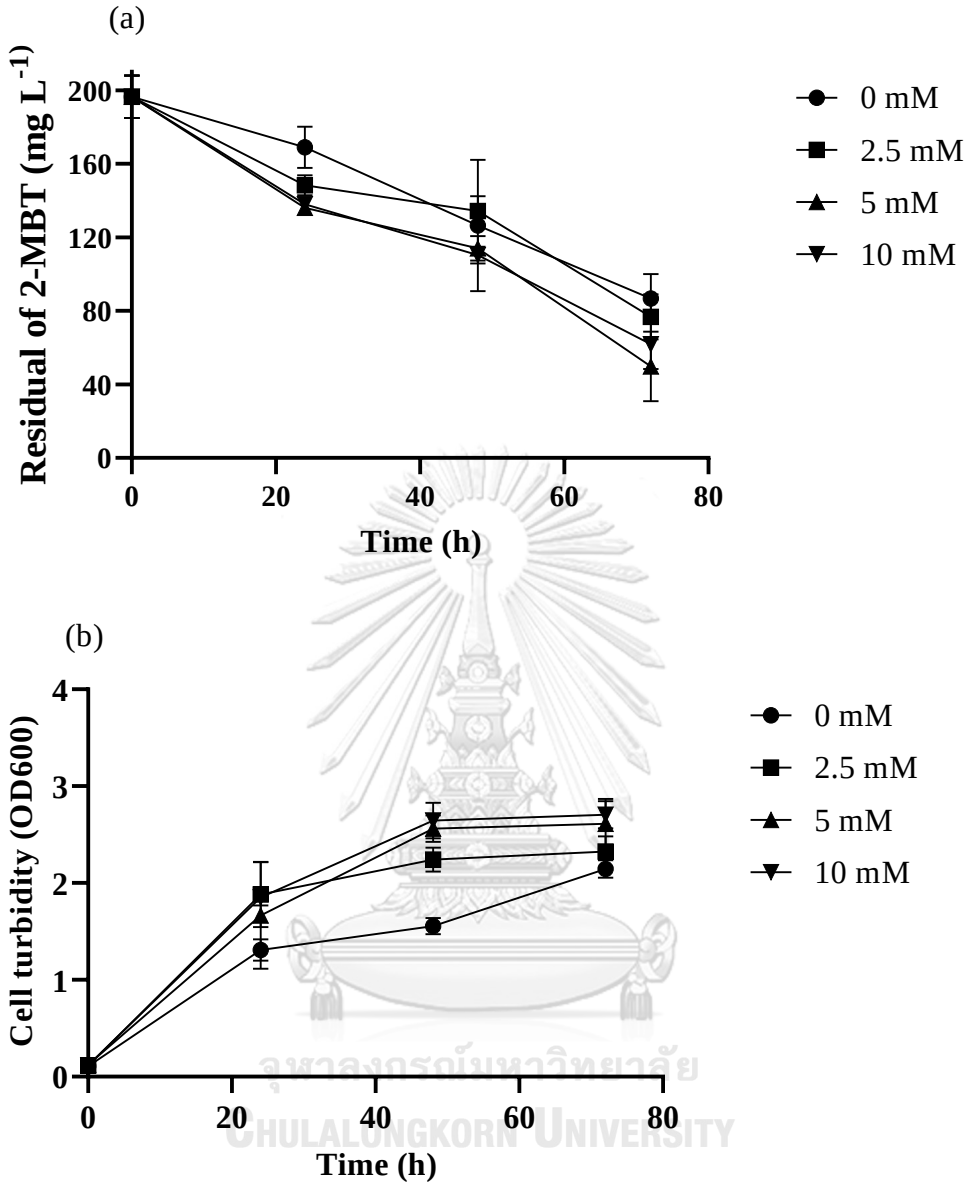


Figure 4.21 Time courses of 200 mg L^{-1} 2-MBT degradation (a) and cell growth (OD600) (b) for EN bacterial consortium in MSM medium supplemented with different sodium benzoate concentrations.

Among three initial cell concentrations, the number of cells significantly increased in the medium inoculated with cell starter of 5% and 10% (v/v) while the attached growth in porous carriers from 2% (v/v) exhibited at lower concentration (p<0.05) (Figure 4.22). The 5% and 10% of inoculum gave similar cell concentration of 2 x10^{13} CFU g carrier ^{1}. Similarly, other work indicated that polyurethane,

polyacrylamide and agar in MSM containing optimum carbon source can immobilize bacterial cells at 1.7×10^{12}, 1.8×10^{12} and 1.6×10^{12} CFU g carrier^{-1}, respectively (Tallur, Mulla, Megadi, Talwar, & Ninnekar, 2015). Consequently, 5% (v/v) of cell starter was chosen for cell immobilization prepreration.

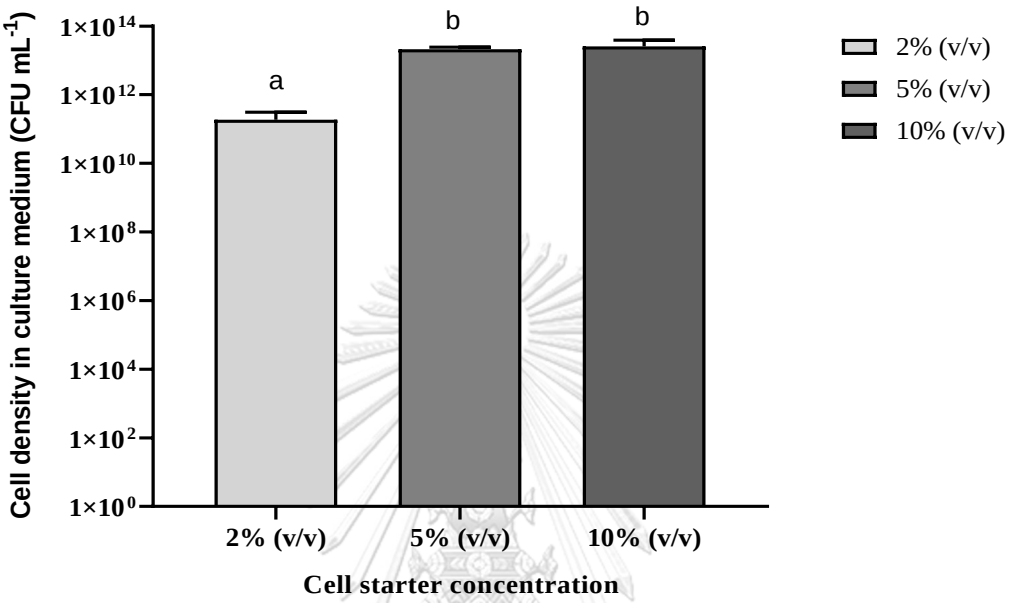


Figure 4.22 Numbers of bacteria in N medium containing 5 mM benzoate after 48-h immobilization of EN bacterial cells (in terms of Log CFU mL^{-1}) in porous carriers with different cell loadings.

4.4.3 Biodegradation of 2-MBT by suspended and immobilized bacterial consortium in batch test

This experiment was conducted to compare the 2-MBT biodegradation ability of suspended and immobilized EN consortium. The initial concentrations of suspended and immobilized EN bacterial consortium were approximately 10^{12}-10^{13} CFU mL ^{1}. The cell numbers were determined from both attached cells in porous carrier and suspended cells. The 2-MBT was added at 300 mg L^{-1} into the 100 mL of medium suspension. Figure 4.23a shows the course of 2-MBT biodegradation. The immobilized EN bacterial consortium showed higher degradation efficiency than that of suspended cells. It has ability to degrade 77.9% of 300 mg L^{-1} 2-MBT, while the efficiency of suspended cells was only 35.9% (Figure 4.23).

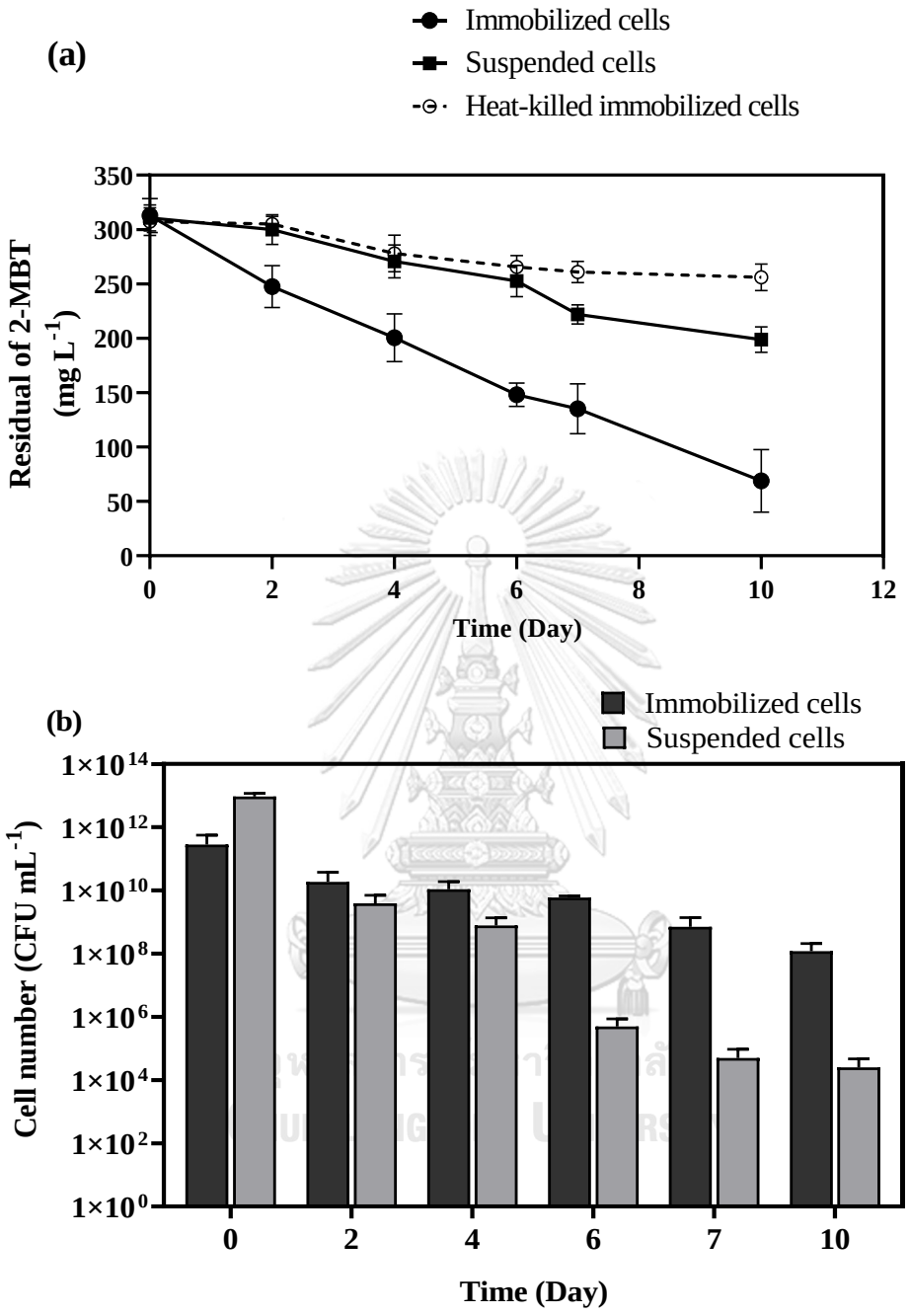


Figure 4.23 Time course profiles for removal of 300 mg L^{-1} 2-MBT (a) and number (b) of both immobilized and suspended cells of EN bacterial consortium during batch degradation test. The cell numbers were determined from both attached cells in porous carrier and suspended cells.

Previous work by Dzionek, Wojcieszyńska, Hupert-Kocurek, Adamczyk-Habrajska, & Guzik (2018) suggested that naproxen (6-methoxy-2-naphthyl) propionic acid) was completely biodegraded using cell immobilization onto sponge material. The good performance of immobilized cells is caused by the synthesis of large amounts of extracellular polymeric substance (EPS) that increased the tolerance of the bacterial cells to the substrate (Dzionek et al., 2018). In addition, the viable cell number was determined by plating on LB agar for overnight, at room temperature, immobilized EN bacterial consortium exhibited the higher cell number concentrations than the suspended cells (Figure 4.23b). These results suggested that the immobilized cells could better tolerate and more degrade than freely suspend cells. Our results were consistent with previous works suggested that the use of porous carriers including polyurethane foam (Kotresha & Vidyasagar, 2017) and loofa sponge (Y.-K. Liu, Seki, Tanaka, & Furusaki, 1998) showed faster degradation than free cells and the porous carriers immobilized cells capable of degrading high concentration of xenobiotic compounds. Similarly, previous work also indicated that the bacterial cells are retained better in the immobilization form (S. Martins, Martins, Oliveira, Fiúza, & Santaella, 2013).

4.4.4 Reusability of immobilized EN bacterial consortium in batch test

After completing the test in section 4.4.3, 2-MBT at 300 mg L^{-1} was further added into the fresh medium solution in order to determine the reusability of the immobilized EN bacterial consortium. This observation could be used to estimate the stability of the immobilized EN bacterial consortium in the bioreactor. The immobilized cells showed up to 84-77% biodegradation of 2-MBT in all 4th repeated uses (Figure 4.24). Further increase in the cycle of test, decreased the rate of degradation, which was 14.24, 16.99, 14.80 and 11.34 mg L^{-1} d^{-1} g carrier^{1}, respectively. Thus, the porous carrier immobilized cells could be reused for up to 4 cycles at the initial 2-MBT concentration of 300 mg L^{-1}. It shows that the immobilized EN bacterial consortium could be recycled with more than 70% of 2-MBT removal. Additionally, the cell number of immobilized bacteria in the system was decreased from 10^{12} to 10^{8} CFU mL^{-1} after reaching the 4th cycle (Figure 4.24). Although this attached cell concentration

in a porous carrier was decreased, this concentration has been reported to be able to degrade xenobiotic compounds (Quek et al., 2006). Thus, this experiment might extend the incubation time of the 4th cycles to completely biodegrade the 2-MBT for obtaining greater biodegradation in later cycles. During 44 days of operation, the color of immobilized EN bacterial consortium was changed (Figure 4.24). To confirm that the microorganisms in the EN bacterial consortium can support colonization, scanning electron microscopy (SEM) was used in this study.

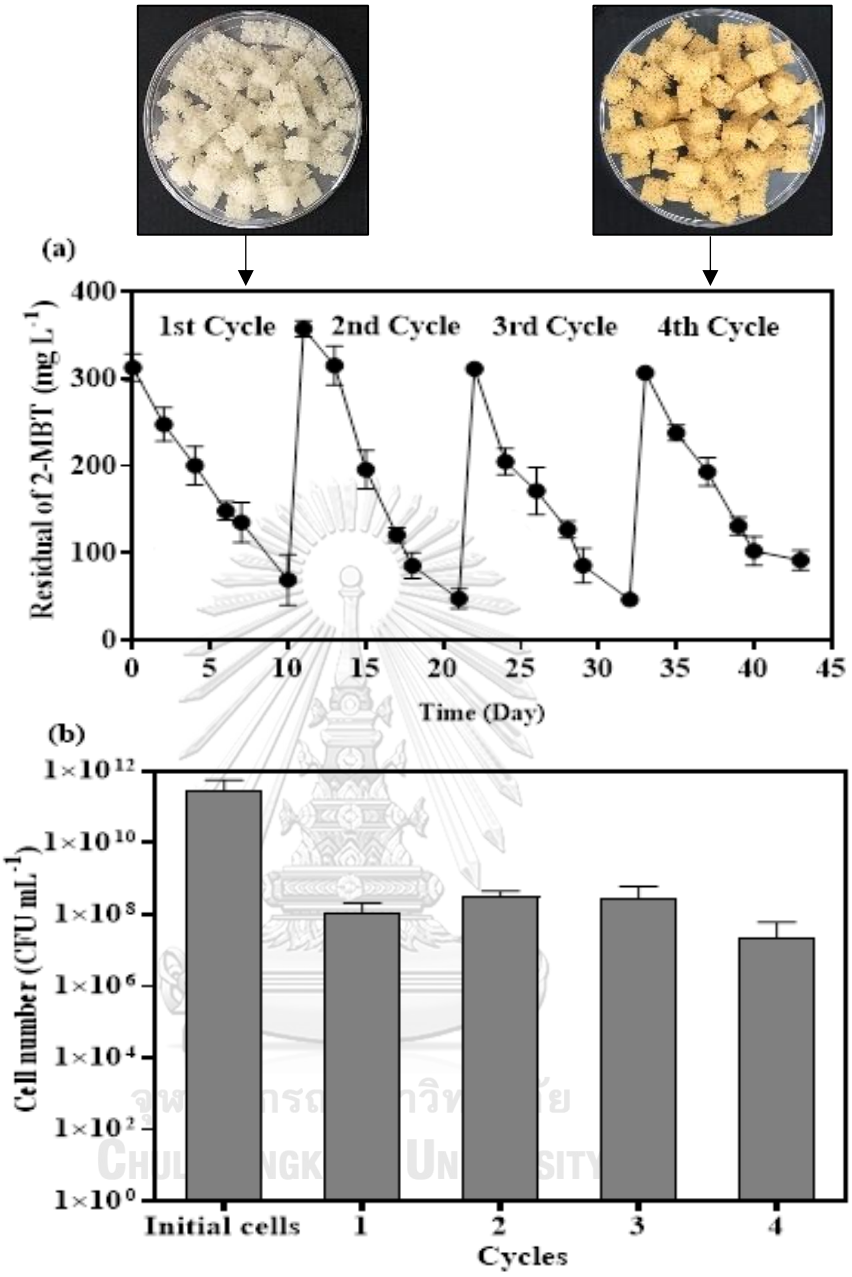


Figure 4.24 Reusability of immobilized EN bacterial consortium in a series of repeated batch-experiments with 2-MBT concentrations of 300 mg L^{-1} (a) and the number of bacterial cells in the immobilized system (b). The cell numbers were determined from both attached cells in porous carrier and suspended cells.

SEM images represented that bacterial cells completely adhered and attached in a porous carrier at the beginning of experiment (Figure 4.25a-d), whereas the bacterial cells in the 44-day old porous carrier were reduced and damaged (Figure

4.25e-h). This could be due to the extent of nutrient acquisition from support materials and loss of catalytic activity under high 2- MBT doses. This test used higher concentrations of 2-MBT than the real environmental contaminant. A longer period of operation and lower concentration of 2-MBT might prolong the efficiency of the 2-MBT biodegradation by immobilized EN bacterial consortium.

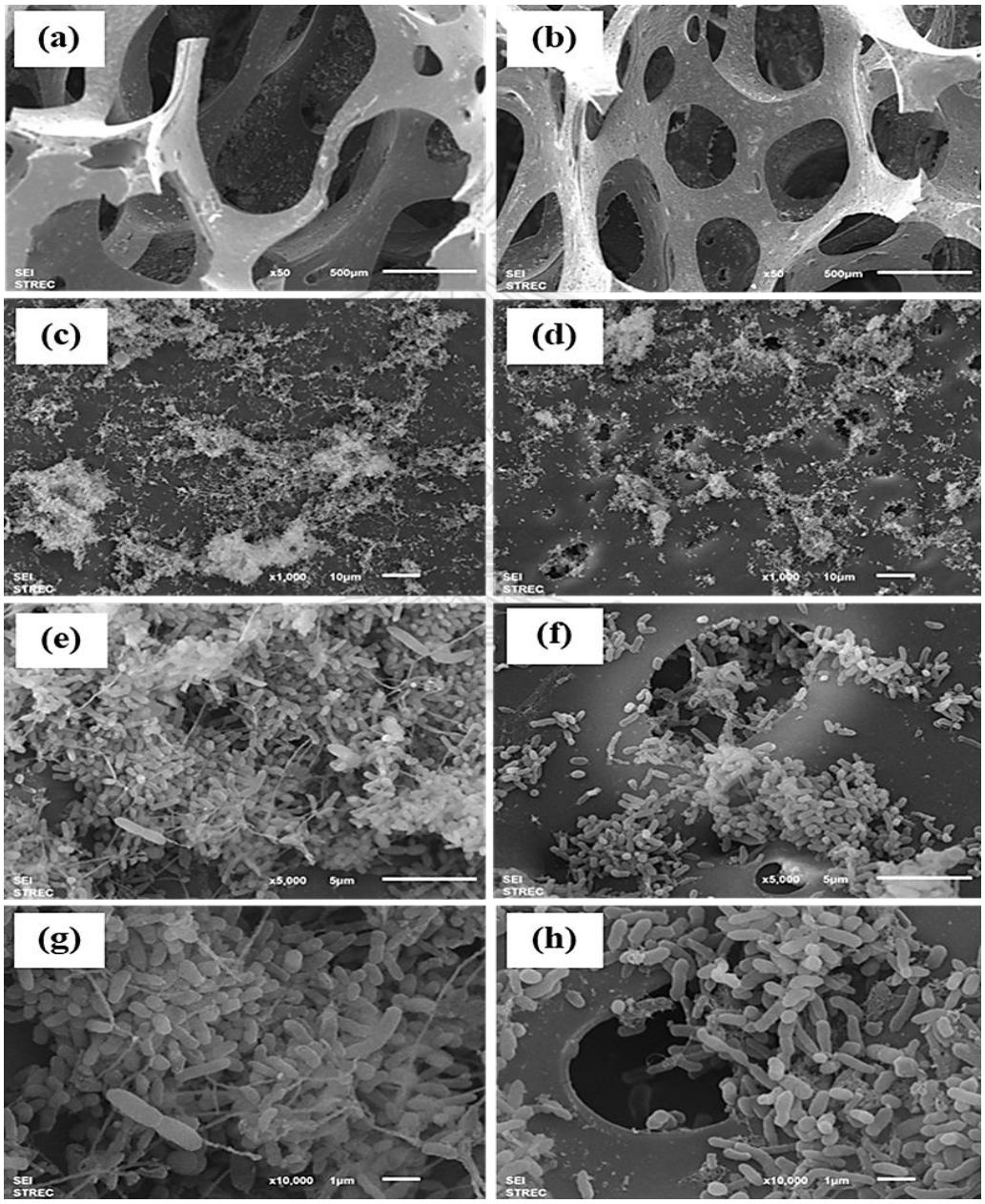


Figure 4.25 Photograph of SEM image of EN cells grown in porous carrier at 0- (left) and 44-day old porous carrier after repeated batch-experiments with 2-MBT (right); 50x (a, b), 1,000x (c, d), 5,000x (e, f) and 10,000X (g, h).

This study revealed that the EN bacterial consortium immobilized in the porous carriers was more effective for the degradation of 2-MBT than suspended cells. Although, the cell in the porous carriers could be damaged during reusability test. The immobilized bacterial cells had the advantage of an increase in degradation rate, resistant to a higher substrate concentration and its reusability.

4.4.5 Performance of immobilized EN consortium in continuous test

This study investigated the performance of EN consortium in bioreactor containing activated sludge to simulate the wastewater treatment plant with bioaugmented cultures. Laboratory bioreactors were designed to illustrate the bioremediation efficiency of the suspended cells and immobilized bacterial consortium. The experiment was divided into two main studies following the methodology in Section 3.2.4.5.

4.4.5.1 Efficiency of immobilized EN consortium in synthetic wastewater containing the 2-MBT

To determine the potential of immobilized EN bacterial consortium for treating the 2-MBT in wastewater, three laboratory scale bioreactors were performed: (1) immobilized AS only (R1), (2) suspended EN bacterial consortium and activated sludge (R2) and immobilized EN bacterial consortium and activated sludge (R3). The bioreactors with controllable stirring speed were run continuously and fed with artificial wastewater which containing organic load rate (OLR) at 0.25, 0.75 and 1 kg $COD.m^{-3}.d^{-1}$, and at HRT of 6, 4 and 4, respectively.

Figures 4.26-4.28 presents the 2-MBT and COD concentrations of the three bioreactors and their removal percentages at different OLR. When the OLR was 0.25 kg $COD.m^{-3}.d^{-1}$ and hydraulic retention time was 6 d, the 2-MBT concentration in R3 (Figure 4.28) was decreased in the average of 61.3% and corresponded to 80.2 % of the COD removal. While the other bioreactors, R1 and R2 had low 2-MBT degradation efficiencies of 41.4% and 34.5%, respectively (Figures 26 and 27). This result indicated that reactor with immobilized EN bacterial consortium and activated sludge exhibited a higher performance that that with suspended cells. This might be due to high porosity

of carrier, thus there was no mass transfer problem and it could support 2-MBT degrading to mitigate from indigenous microbes in the activated sludge. Thus, this work indicated that the immobilized EN bacterial consortium could contribute to rapid startup periods. These findings also correspond to various previous reports using porous carrier (Basak et al., 2019; Kurade, Waghmode, Xiong, Govindwar, & Jeon, 2019).

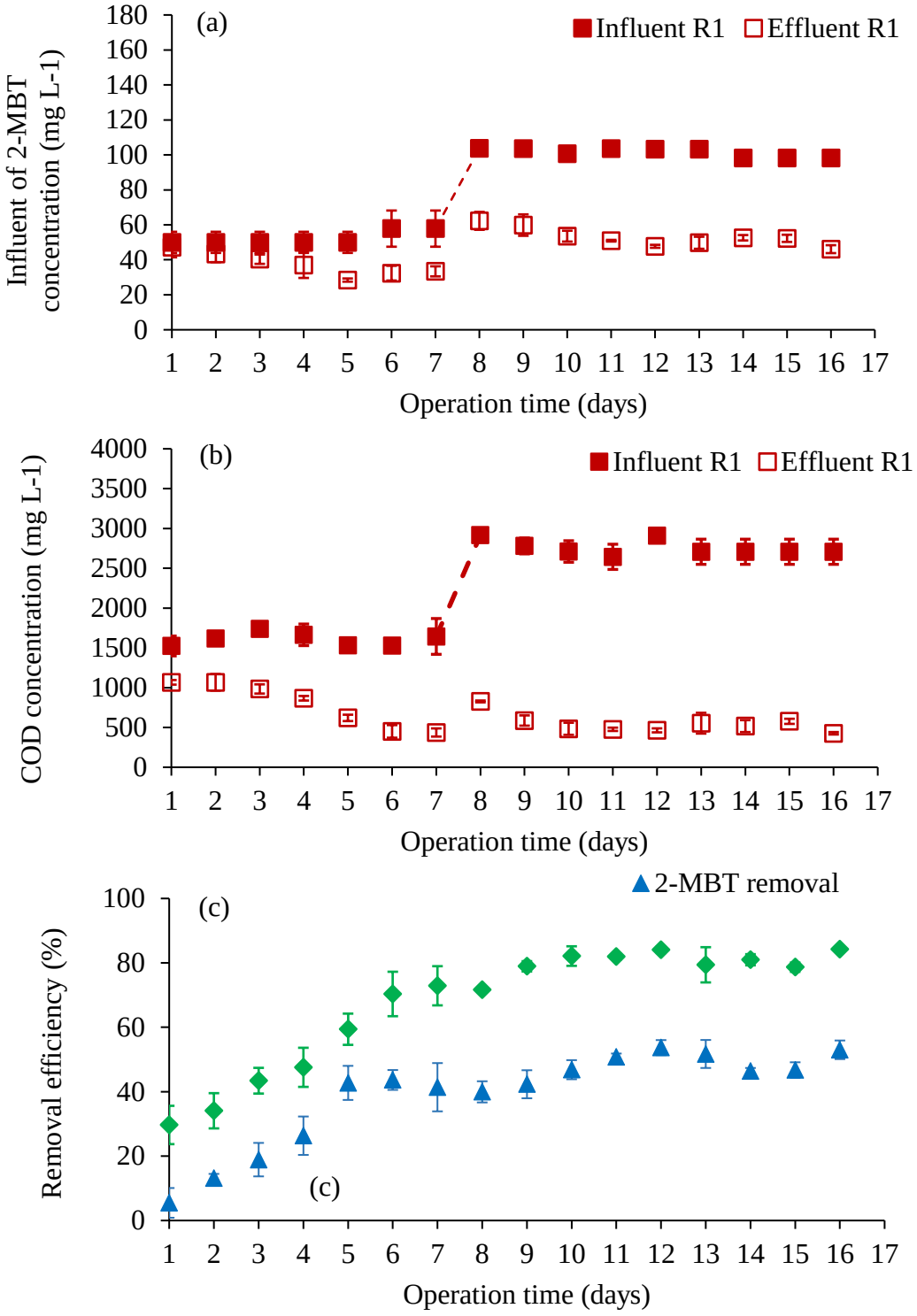


Figure 4.26 Profiles of 2-MBT (a) and COD (b) in the influent (■) and effluent (□), and their removal efficiencies of R1 bioreactor containing only immobilized activated sludge (c) during an experimental period of 16 d.

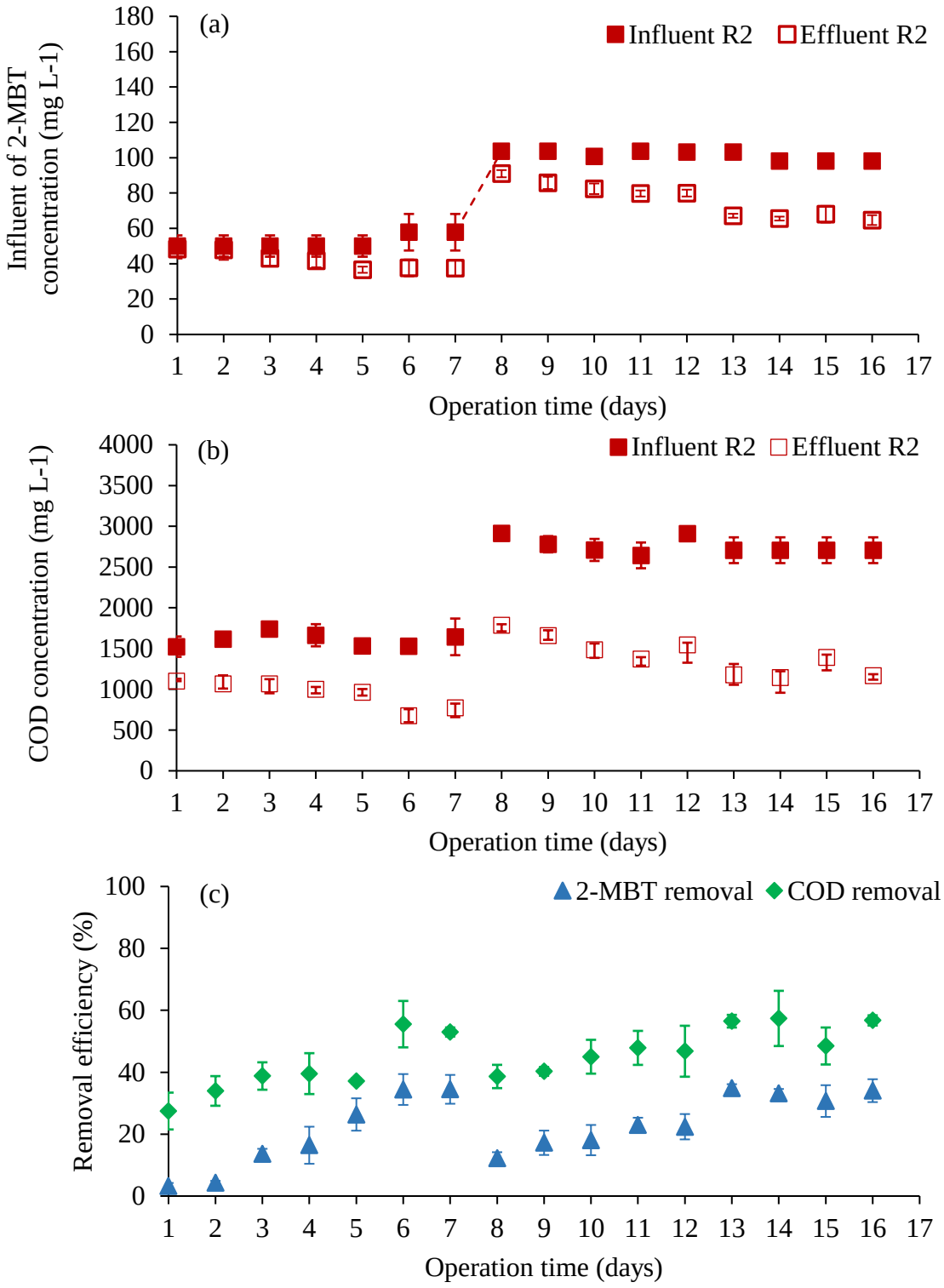


Figure 4.27 Profiles of 2-MBT (a) and COD (b) in the influent (■) and effluent (□), and their removal efficiencies of R2 bioreactor containing suspended EN bacterial consortium and activated sludge (c) during an experimental period of 16 d.

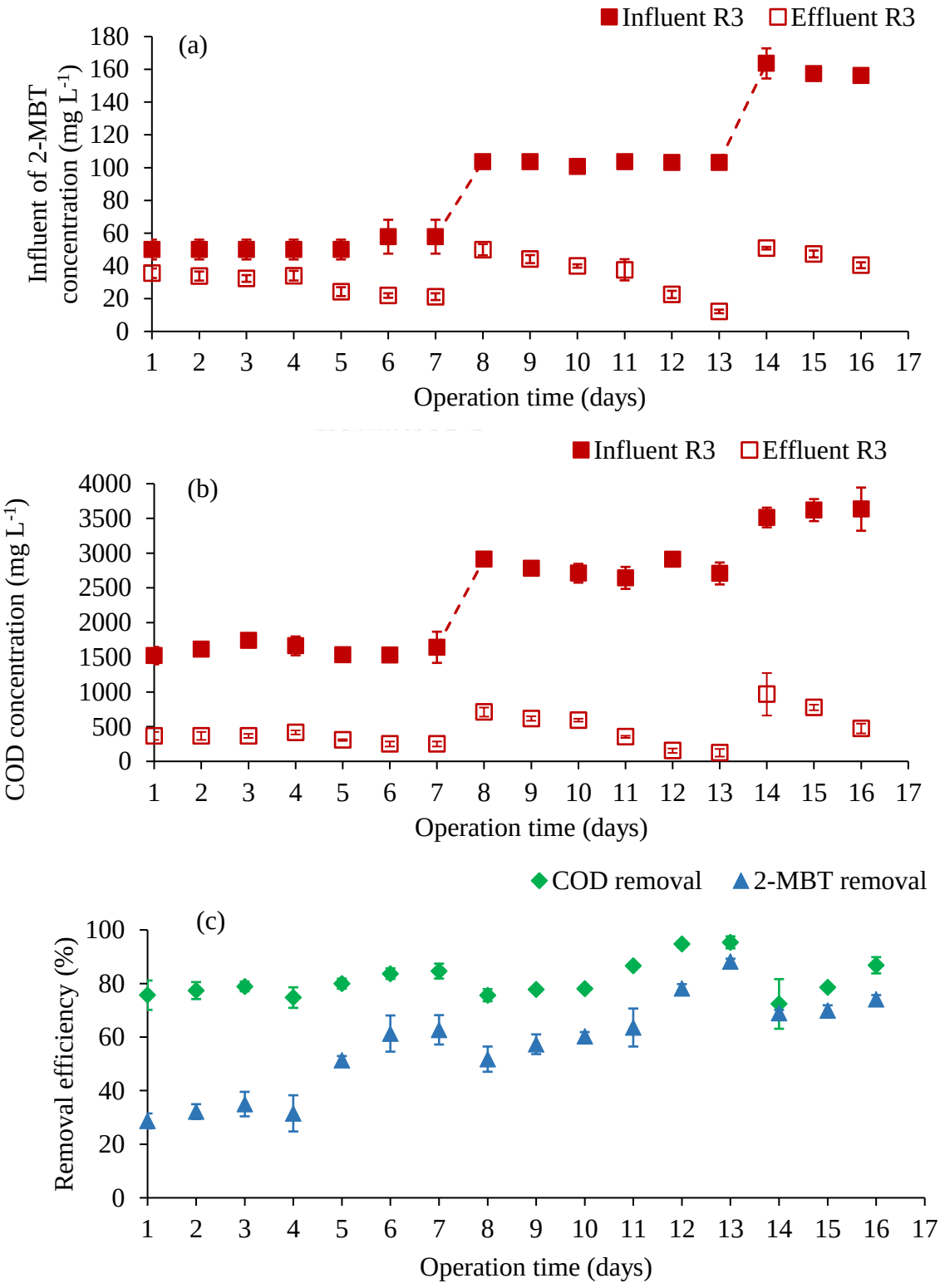


Figure 4.28 Profiles of 2-MBT (a) and COD (b) in the influent (■) and effluent (□), and their removal efficiencies of R3 bioreactor containing immobilized EN consortium and activated sludge (c) during an experimental period of 16 d.

When the OLR was increased to 0.75 kg COD.m^{-3}. d^{-1} (100 mg L^{-1} of 2-MBT), the average percentages of 2-MBT removal of R1 and R2 were 51.7 and 34.9% at a 7 day-operation respectively, while the R3 had the highest 2-MBT removal of 88.2 % with an average COD removal of 95.4 % (Figures 4.26 to 4.28). This result indicated that the increase in the OLR and decrease in the HRT led to the decrease in the degradation ability of suspended cells bioreactor (R2), while the degradation efficiencies of immobilized bacterial consortium (R3) were maintained. Consequently, R3 was chosen to explore at higher OLR of 1 kg COD.m^{-3}. d^{-1} at HRT of 4 days.

Figure 4.29 depicts the R3 bioreactor and immobilized EN augmented with activated sludge after operating with synthetic wastewater at OLR 1 kg COD. m^{-3}. d^{-1}. The porous carriers displayed a dark color, which caused from the activated sludge in the bioreactor. The degradation efficiency of immobilized bacteria and activated sludge in R3 also began to slightly reduce to reach 74.13% of 2-MBT removal. The effectiveness of immobilized bacterial consortium in the presence of activated sludge was comparable with those of the system containing only immobilized cells (Section 4.4.3).

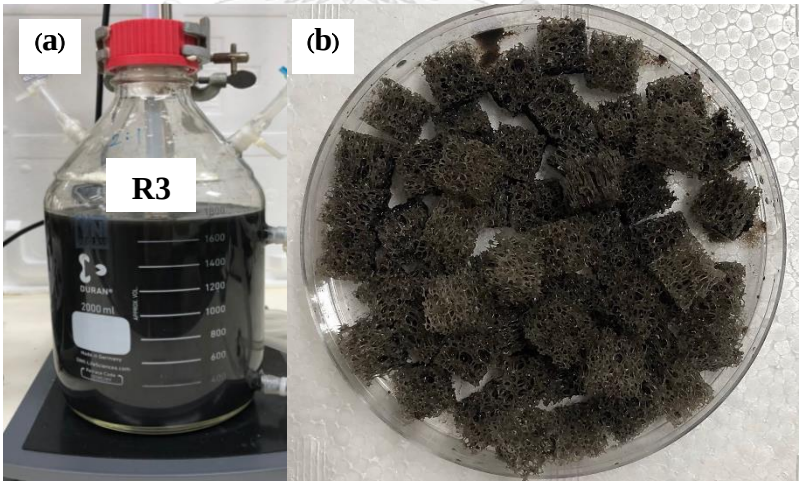


Figure 4.29 Photographs of the bioaugmentation reactor (R3) (a) which fed with synthetic wastewater at OLR 1 kg COD m^{-3} d^{-1} and contained activated sludge and immobilized EN consortium (b) after 16-day operation period.

Accordingly, the immobilized EN bacterial consortium could be applied to improve the efficiency of activated sludge system and accelerate the removal rate of 2-MBT in wastewater. In further investigation, the R3 was chosen to evaluate its performance in real rubber wastewater with higher OLR.

4.4.5.2 Efficiency of immobilized EN consortium in rubber processing wastewater from factory E

To successfully treat real rubber wastewater with high OLR and short HRT, this work evaluated the performance of bioaugmentation reactor (R3) fed with rubber wastewater at different organic loading rates (OLRs between 1 and 3 kg COD L^{-1} d^{-1}). The characteristic of the rubber processing wastewater from industry E was presented in Table 4.1. The effluent pH in the bioreactor ranged from 7.6-8.3 throughout the 71-day operation. These pH values are closed to the ranges that considered ideal for the wastewater treatment.

When OLR was 1 kg COD m^{-3} d^{-1}, this reactor exhibited high removal efficiencies of approximately 90% of COD and 80% 2-MBT at HRT of 3 days (Figure 4.30). Further increase in OLR from 1.33 to 3.0 kg COD m^{-3} d^{-1} (at COD concentration 4000-9000 mg L^{-1}), the 2-MBT and COD removal efficiencies were ranged 70-79% and 83-96%, respectively (Figure 4.30).

Throughout the 116-day operational period, the bioaugmentation reactor (R3) showed high performance with respect to the removal of 2-MBT and COD in the rubber processing wastewater. This result was consistent with the previous work studied by Loh, Chung, & Ang (2000), which proposed that the effectiveness of cell immobilization had found for delaying the effects of substrate inhibition. The obtained results indicated that the immobilized cells were superior to suspended cells for rubber wastewater treatment.

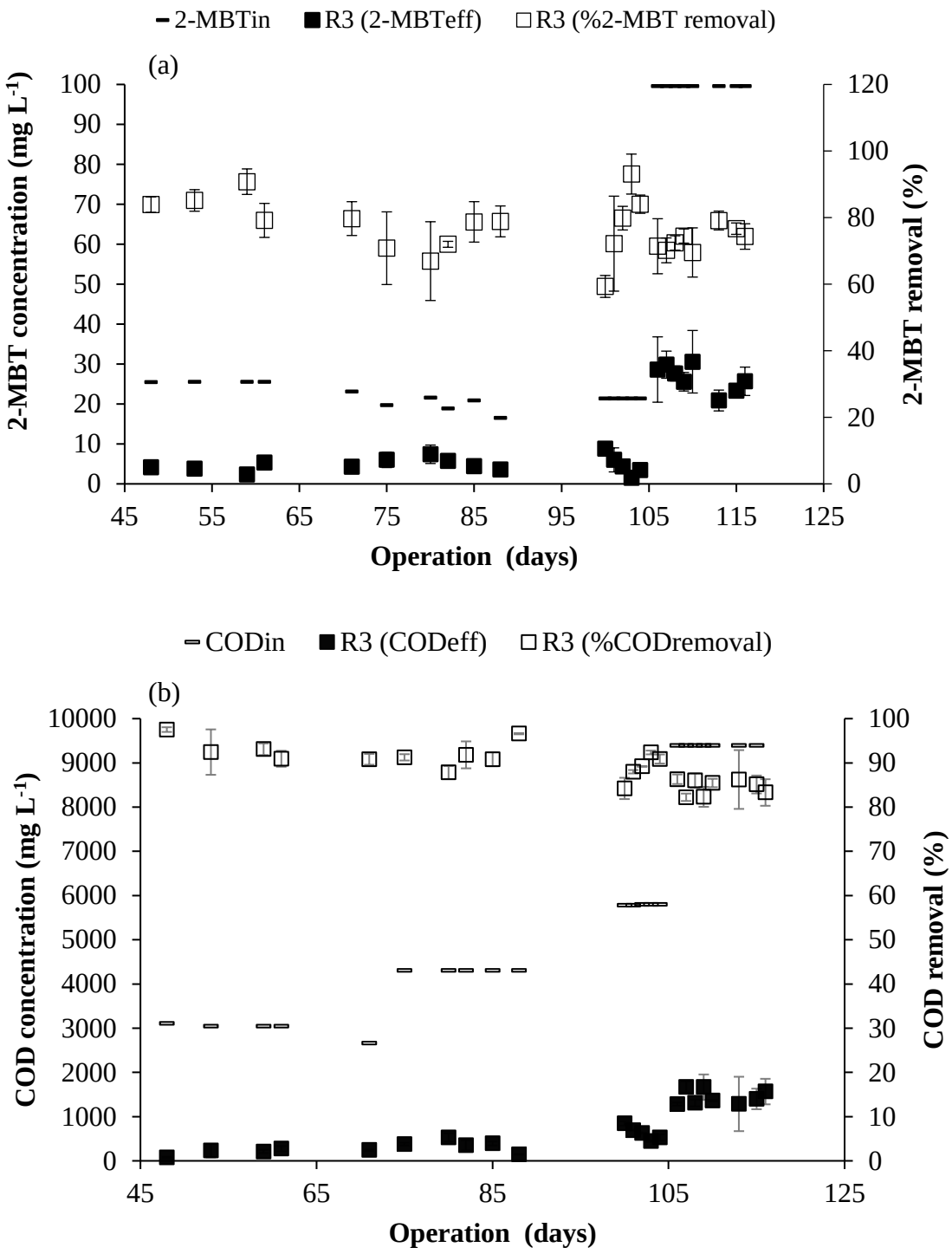


Figure 4.30 Removal of the chemical oxygen demand (COD) (a) and 2-MBT (b) from rubber wastewater at different organic loading rate (OLR) in the R3 bioaugmentation reactor.

The biomass concentration inside the bioreactor was determined from both attached cells in porous carrier and suspended cells. The 2% (w/v) filling density of porous carriers in the R3 reactor achieved the maximum biomass concentration at 6.68 g_{vss} L^{-1} (0.33 g_{vss} $g^{-1}_{carrier}$), while initial cells in porous carriers was 2.5 g_{vss} L^{-1} (0.12 $g_{vss}g^{-1}_{carrier}$) (Figure 4.31). The high bacterial density in carriers might be due to their high hydrophilicity and macrostructure, which can provide a large surface for bacterial cells of activated sludge to immobilize and colonize in the carriers. Previous work has shown that the amount of biomass as mass of biofilm on the surface of polyurethane in anaerobic continuous-flow packed-bed bioreactor was approximately 0.02 g $g^{-1}_{carrier}$ (Kerčmar & Pintar, 2017). They suggested that the surface architecture and pore size of support materials influenced the biofilm formation and metabolic activity. Similarly, a recent research has indicated that biofilm formation was important factor to enhance the bacterial cells attached in a support material like polyurethane in order to increase their microbial metabolic activity (Kerčmar & Pintar, 2017). This result indicated that the support material had an influence on the bacterial behavior to relocate themselves for cell proliferation.

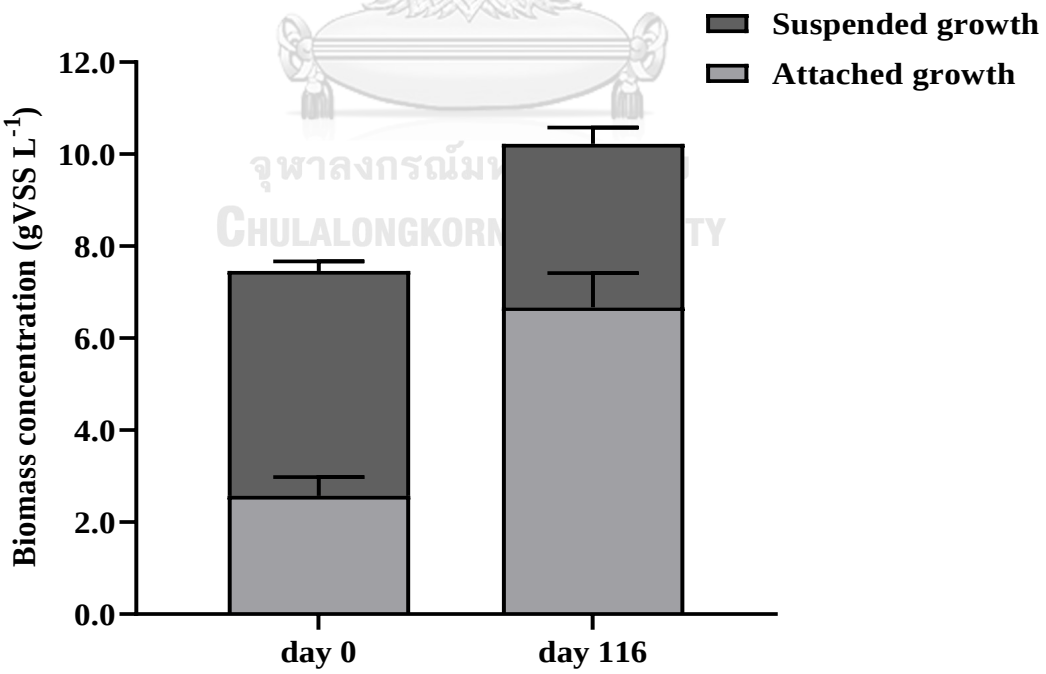


Figure 4.31 Total biomass concentration of the bioaugmentation reactor (R3), which included both attached and suspended cells after 116-day of operation.

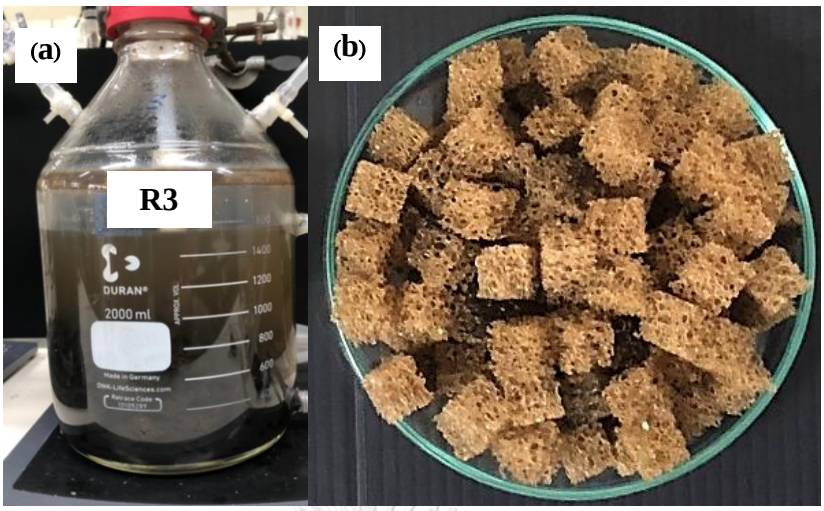


Figure 4.32 Photographs of the bioaugmentation reactor (R3) (a) and immobilized porous carriers after 116-day operation (b).

Figure 4.32 depicts the bioaugmentation reactor (R3) and immobilized cells after 116-day operation. The color of the immobilized cells after applying for real rubber wastewater treatment was different from the color of operation process with synthetic wastewater (Figure 4.29). The color change might be due to the physical and chemical properties of rubber processing wastewater and long operational period. In addition, the physical observation of the immobilized bacterial cells suggested that the porous carriers were not clogged and damaged.

Therefore, the ratio of 1:2 between immobilized bacterial consortium and activated sludge in porous carriers was sufficient for treatment of 2-MBT and COD in real rubber wastewater. Thus, the bioaugmentation reactor with immobilized EN bacterial consortium was advantageous for rubber processing factory.

CHAPTER 5
Conclusions and recommendations

5.1 Conclusions

This research successfully obtained a stable 2-MBT-degrading bacterial consortium by a sequential enrichment and acclimatization process when wastewater sludge containing benzothiazoles was used as the inoculum. The immobilization of bacterial consortium in porous carriers increased the consortium's efficiency on degrading 2-MBT and COD in rubber wastewater.

In conclusion:

1. The medium for enrichment, acclimatization and cultivation of the efficient bacterial consortium was composed of minimal salts, co-nitrogen sources and long-term of incubation amended with a gradual of 2-MBT concentrations as the sole source of carbon and energy.

2. Bacterial consortia possessed high biodegradation activities over a wide range of 2-MBT concentrations (50-200 mg L^{-1}), especially, EN consortium degraded up to 300 mg L^{-1} of 2-MBT, which had never been reported.

3. When applied to rubber wastewater, EN consortium removed 79 % 2-MBT and 93 % COD. Thus, the EN consortium could be an ideal inoculum for the post-treatment of benzothiazoles in rubber industrial wastewater.

4. The enrichment associated with long-term acclimatization had significantly increased bacterial adaptation to the use of higher concentration of the 2-MBT resulting in changes in abundances of the dominant bacterial populations and majority of genes.

5. The dominant populations in the consortia were *Pseudomonas*, *Stenotrophomonas* and *Caulobacteraceae.*

6. Abundance of the transcripts related to the degradation of aromatic compounds, benzothiazoles, benzoates, phenols, sulfate, sulfite, ammonia and other xenobiotic compounds indicated diverse capabilities of the EN bacterial consortium.

7. The expression of various outer membrane proteins in the acclimatized consortium was proposed as a part of the detoxification and tolerance mechanisms of bacterial cells.
8. The taxonomic classification of metatranscriptomic sequence data revealed *Pseudomonas putida* as a generalist participating in the initial degradation of 2-MBT.

9. EN consortium as immobilized cells in a porous carrier exhibited high cell density and could be reused for at least 4 cycles.

10. The bioaugmentation of the activated sludge system with immobilized EN consortium was able to treat rubber processing wastewater with high organic loading rate.

11. Porous carrier-immobilized cells at 2% (w/v) was an optimum filling density in a bioreactor to achieve high microorganism retention and treatment performance.

12. The biomass ratio of immobilized consortium and activated sludge at 1:2 (w/w) could accelerate and increase the efficiency of rubber wastewater treatment.
13. This study is the first to reveal the potential application of an efficient bacterial consortium for degrading 2-MBT and other benzothiazoles in rubber industrial wastewater.
14. These findings emphasized the advantages of using bacterial consortia for degrading recalcitrant compounds in industrial wastewater.

5.2 Recommendations for future work

1. There are other co-nitrogen sources such as KNO_2, KNO_3, NaH_2PO4, $(NH_4)2SO_4$, NH_4NO_3 (Feng et al., 2019; Heylen et al., 2006; Kwapisz, Wszelaka, Marchut-Mikolajczyk, & Bielecki, 2008) that could be supplied to replace the NH_4Cl and the yeast extract for enrichment of recalcitrant compound-degrading bacteria .
2. Other selection approaches such as dilution (Kang et al., 2020), toxicity acclimation (Ho, Chen, & Lee, 2010) and heat acclimation (Chaitanya et al., 2016; Fang et al., 2017) could be used to increase the desired microbial populations and to reduce adaptation period.
3. The medium containing sodium benzoate could be used to select the efficient 2-MBT degraders and reduce the period of acclimation process.
4. To confirm the important genes in the EN consortium, qPCR could be employed to quantify the levels of specific genes expressing during 2-MBT degradation.
5. Since the microbial consortium is a complicated system, the active degraders and its activity should be confirmed by DNA-stable isotope probing (DNA-SIP) and RNA-stable isotope probing (RNA-SIP) with labeled substrate (Aoyagi et al., 2018; J. Li et al., 2019; Sul et al., 2009).
6. In the bioreactors, the performance of EN consortium could be improved by increasing the biomass concentration and extending the hydraulic retention time for the effective implementation of 2-MBT bioremediation.
7. To apply bacterial community of the EN consortium as a bioagent product in the real fild, a defined bacterial consortium consisting of non-pathogenic bacterial species should be constructed. Briefly, the dominant populations should be isolated from the 2-MBT-degrading bacterial consortium and screened for non-pathogenic species before mixing them together. This approach would allow for the application of bacterial consortium in the wastewater treatment plant without potential environmental and health risks.

8. The defined immoblizing EN consortium could be applied as a post-treatment for treating residual of 2-MBT and other xenobiotic compounds in the treated rubber wastewater (Figure 5.1). In addition, the process could be extended to other benzothiazoles containing industrial wastewater.

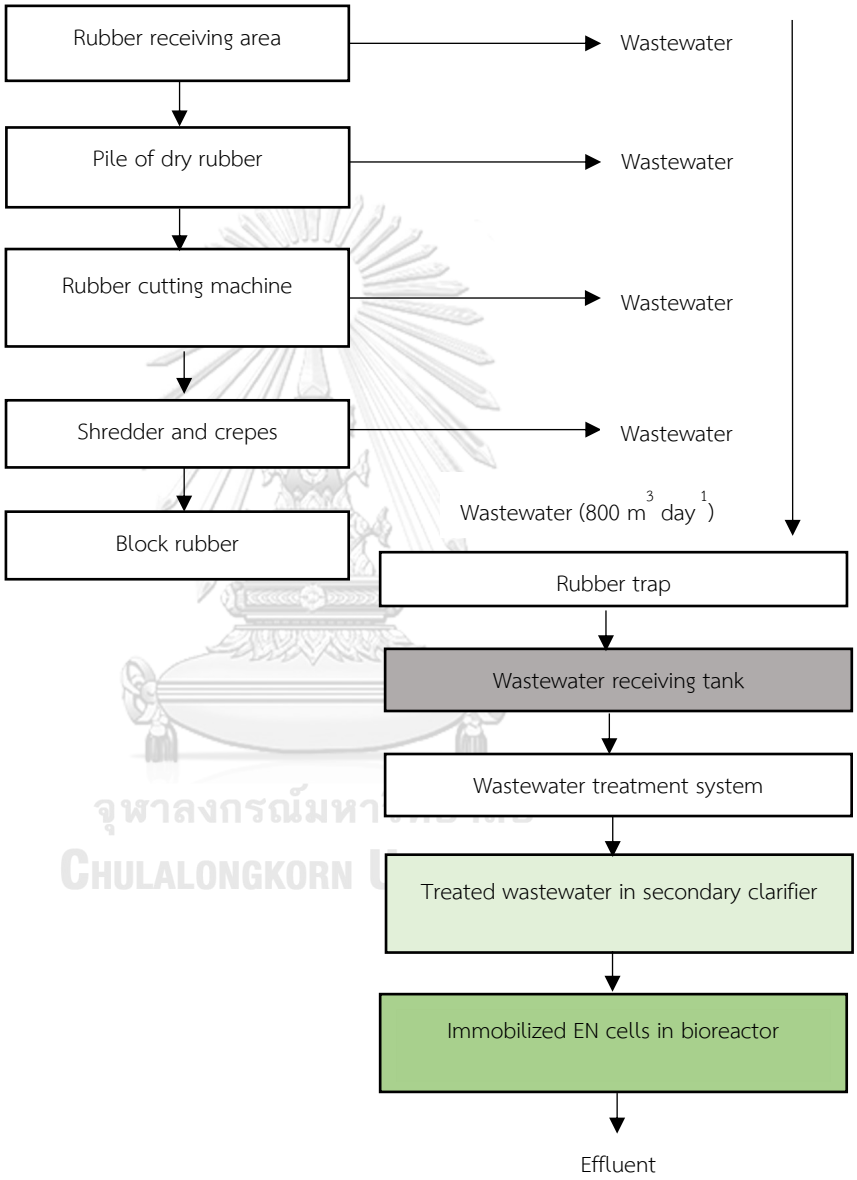


Figure 5.1 Application of immobilized EN cells system (Dark green) in the bioreactor for co-operating in wastewater treatment plant of rubber industrial wastewater treatment.

9. These immobilized cells with high mass loading rate could be applied in membrane bioreactor (Chaipon Juntawang, Chaiwat Rongsayamanont, & Eakalak Khan, 2017) in order to control biofouling of diverse microbiota in activated sludge (Xu, Zhao, Lee, Wang, & Xu, 2019).

10. For long-term performance, the dynamic of bacterial diversity in the consortium during treatment period should be identified by 16S rRNA sequencing analysis.

11. To reactivate the immobilized bacteria after long term treatment period, the washed immobilizing carriers could be incubated in benzoate containing medium for 48 h to promote bacterial growth and activity.

12. To apply the defined immoblizing EN consortium in an on-site pilot scale study, the effects of environmental stresses such as pH, salinity and temperature on 2-MBT biodegradation should be investigated.

13. The application of immoblizing EN consortium in various rubber industries could provide an easy access to module compartments of the reactors and options for membrane cleaning and replacement. The cost-benefit study of this process should be carried out.

14. Several researchers have conducted post-treatment studies on aerobic down-flow hanging sponge (DHS) reactor (Machdar, Onodera, Syutsubo, & Ohashi, 2018; Daisuke Tanikawa et al., 2020; D. Tanikawa et al., 2016). According to the manufacturer, this porous material could be used in the similar reactor for enhancing water distribution and mass oxygen dissolved in the wastewater. It is thus possible to apply the immobilized EN consortium in various types of bioreactor.

5.3 Benefits of the research

The outcome from this study is useful for the development of industrial wastewater treatment plant containing eco- friendly immobilized bioagent. The immobilized cells had been tested, verified, implemented as clean alternative, and cost effective bioagent for removal of benzothiazoles from contaminated wastewater without generating hazardous substances or toxic by-products. Therefore, the findings will be valuable for environmental decision-making to offer the opportunity to employ the treated rubber industrial wastewater for irrigation and other uses. This approach would reduce the conflicts between factories and surrounding communities. Additionally, the acquired knowledges will be published in international journals, while the technical knowledges will be used for intellectual property development and further commercialization.

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Further biodegradation studies at phenol concentrations of 2,000 and 3,500 mg/L were also performed to evaluate the effectiveness of cell immobilization for delaying the effects of substrate inhibition. Phenol could be completely degraded at both high concentrations. doi:doi:10.1061/(ASCE)0733-9372(2000)126:1(75)

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APPENDIX

APPENDIX A

MEDIA

Table A.1 LB broth (Luria-Bertani broth)

Component	**Amount (g)**
Tryptone	10.0
Yeast extract	5.0
Sodium Chloride	10.0

All components were dissolved in 1 L of distilled water

APPENDIX B

STANDARD CURVES

1. Standard curve of 2-MBT

Standard curve was used to analyst absorbance of spectrophotometry results in order to calculate the concentration of 2-MBT compound. The pure 2-MBT standard was prepared in methanol as a stock solution. Thus, 2-MBT at various concentrations was obtained by doing a serial dilution from the stock solution. The standard curve was plotted between absorbance at OD320 and concentration of 2-MBT. The graph was plotted as linear regression and displayed the R^2 value ≈ 1.

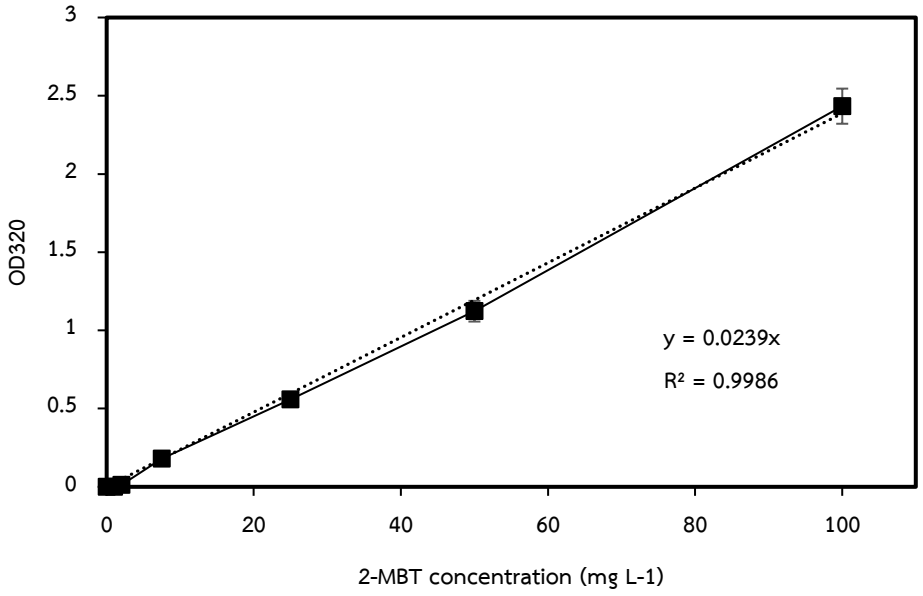

Figure B-1 2-MBT standard curve for calculation of 2-MBT concentration

The calculation to determine amount of 2-MBT in sample is follow

$$\text{The amount of 2-MBT (mg } L^{-1}) = \frac{Absorbance}{0.0239} \qquad Eq\ .1$$

2. Standard curve of sulfate

Sulfate (turbidimetric) was used to determine the sulfate ion in medium and wastewater suspension. Barium chloride ($BaCl_2$) was used to form barium sulfate that can cause the turbidity. Standard sulfate solution was prepared from Na_2SO_4. 147.9 mg of anhydrous Na_2SO_4 was dissolved in purified water in a 1 L of volumetric flask and diluted to the mark concentration by the water. The standard curve was plotted between absorbance at OD420 and concentration of Na_2SO_4.

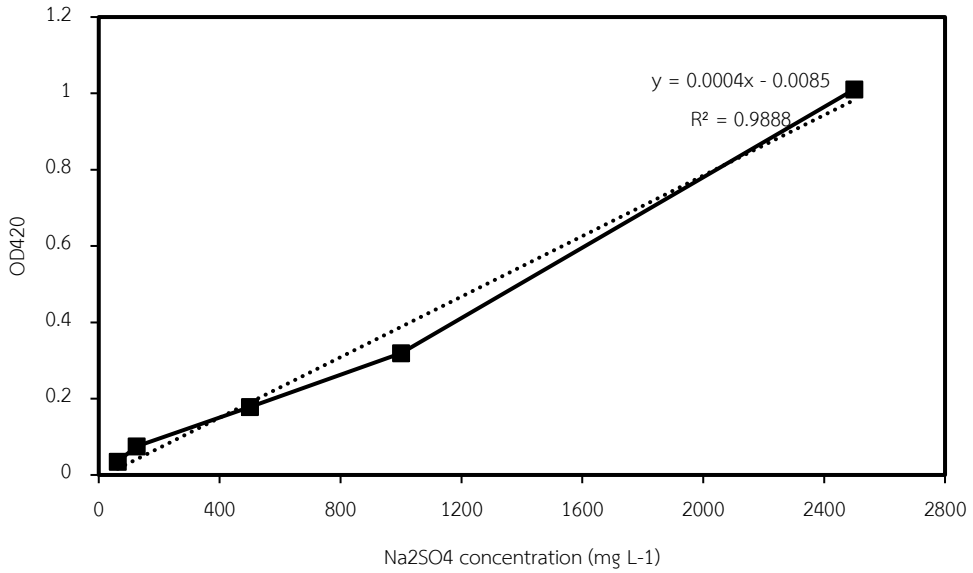

Figure B-2 Na_2SO_4 standard curve for calculation of sulfate concentration

The calculation to determine amount of sulfate in sample is follow

The amount of sulfate (mg) = $\frac{Absorbance+0.0085}{0.0004}$ *Eq 2*

The amount of sulfate (mg L^{-1}) in 1 L = $\frac{amount\ of\ sulfate\ (eq.2)*1000}{volumn\ of\ sample}$ *Eq 3*

3. Standard curve of protein

The research used the Bradford protein assay to measure the concentration of total protein in samples. The principle of this method is that molecules of protein was to bind with Coomassie dye under acidic conditions that presented a color change from brown to blue. A graph of Bradford standard protein assay performed with BSA as a standard. The concentration of BSA was plotted on the x-axis and measured OD at 595 nm values was plotted on the y-axis. The equation was made by linear regression of the data in chart.

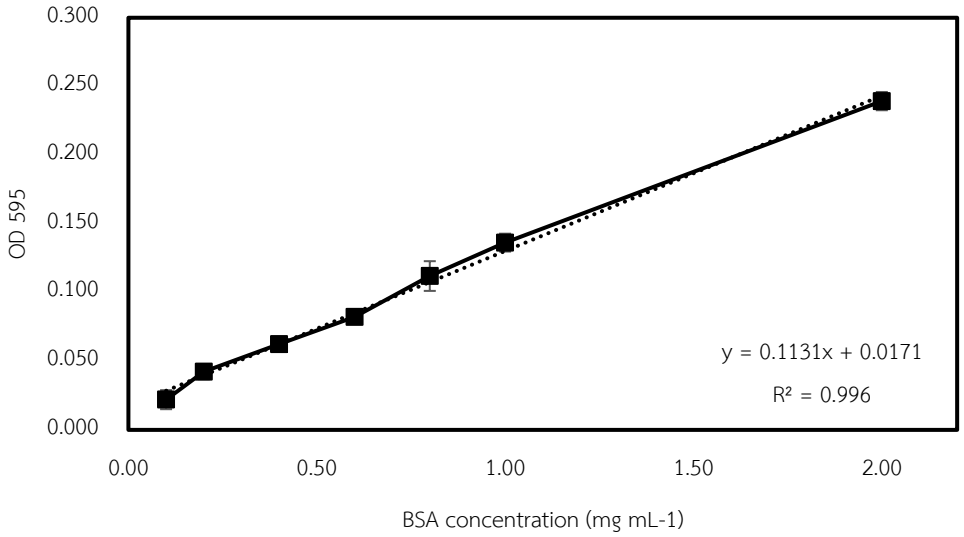

Figure B-3 BSA standard curve for calculation of protein concentration

The calculation to determine amount of sulfate in sample is follow

The amount of protein (ug) = $\frac{absorbance - 0.0171}{0.1131}$ *Eq. 4*

The amount of protein (mg mL^{-1}) = $\frac{amount\ (eq..4) * 1000}{volumn\ of\ sample}$ *Eq. 5*

APPENDIX C

STANDARD CURVE OF QUANTITATIVE PCR

1. **Fasta format of 16s gene sequence of *Pseudomonas aeruginosa* CDRS2 used for a standard curve for qPCR**

>CDRS2

TTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGA
TAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGG
ACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGG
CGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAAT
ACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGT
AATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCA
AGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTAC
GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGT
GGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCT
TAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCA
GACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACG
AGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACG
TGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGAT
CGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATC
AGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCTCCAGAAGT

2. Standard curve of quantitative PCR of *Pseudomonas* genera

Standard curve for the detecting of Pseudomonas spp. used the extractant DNA of pure strain of Pseudomonas aeruginosa. Known amount of copies of the target sequence were measured by quantitative PCR and calibrated in a second assay to copies per ml of sample. The calculation for the number of copies DNA is follow

Number of copies = (amount x (6.022×10^{23})) / (length (bp) x (1×10^{9}) x 650) *Eq 4*

This work generates a standard curve from the pure strain template by plotting the Ct values against the log initial concentration of copies number as presented in *equation 4*

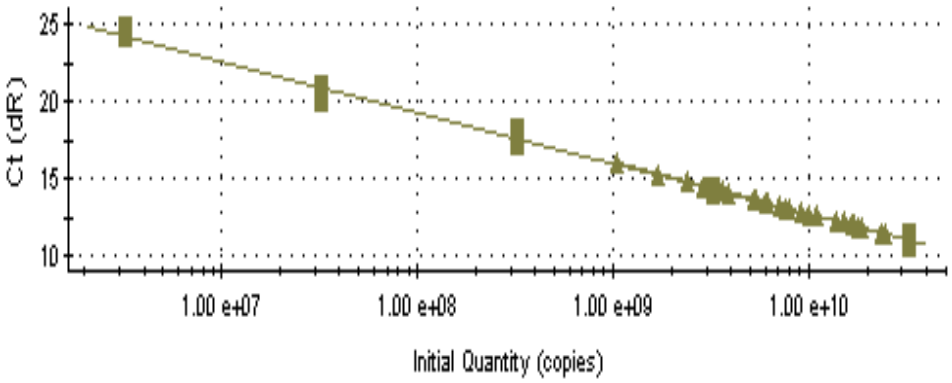

Figure C-1 A standard curve for detecting *Pseudomonas* genera

After obtaining the standard curve, the efficiency of amplification of the standard template is 90- 110% and that the $R^2 > 0.9$. This standard curve presented the equation is follow

$$Y = -3.302 * LOG(X) + 45.72$$

$$R^2 = 0.997$$

The concentration of unknown samples was calculated from the known concentration based on the standard curve generated from pure strain template as shown in Figure C-1.

APPENDIX D

SUPPLYMENTARY DATA OF GC-MS PROFILES

Table D-1 Lists of investigated compounds presented in rubber wastewater samples

Chemical name	RT (min)	m/z	+EI TIC MS		
			Initial wastewater	Treated sterilized wastewater	Treated non-sterilized wastewater
2-Mercaptobenzothiazole	18.394	167	2.21E+07	259371.36	278814.84
Benzothiazole	9.436	135	1.11E+07	759542.5	554112.81
Benzene, 1,3-bis(1,1-dimethylethyl)-	9.844	175	3.90E+06	2903556.75	5517657.5
Methyl stearate	19.563	74	3.63E+06	513553.53	671507.5
Hexadecanoic acid, methyl ester	18.009	74	3.60E+06	3412349	6144126.5
Di-n-octyl phthalate	23.595	149	2.90E+06	2384732.75	2920445.25
Pentadecane	10.178	57	2.40E+06	1999135.88	3059377.25
14-Octadecenal	18.793	82	2.01E+06	341194.72	360537.63
Phenol, 2,4-bis(1,1-dimethylethyl)-	13.293	191	1.85E+06	1794289.88	1980590.13
1-Octadecanol	16.731	55	1.78E+06	1601669.13	2774686.5
Benzothiazole, 2-(methylthio)-	14.523	181	1.66E+06	1053822.63	8856417
Ethylbenzene	3.401	91	1.45E+06	1426706.88	1494671
Undecane, 2-methyl-	6.614	43	1.42E+06	1332774	1855408.38
1-Hexanol, 2-ethyl-	6.144	57	1.29E+06	1057044.13	1292536.13
Acetic acid, 2-phenylethyl ester	3.830	104	1.27E+06	1177587.63	1309157.13

Table D-1 Lists of investigated compounds presented in rubber wastewater samples (Cont.)

Tetradecane	11.831	57	1.20E+06	1098529.38	1590215.13
Decane	5.568	43	1.15E+06	1186748.5	1190424.88
Benzoic acid, methyl ester	7.326	105	1.11E+06	864764.63	868497.06
Tetradecane	9.933	57	1.04E+06	912501.19	1406506.75
Heptadecane	15.658	57	9.57E+05	874378.94	1265580.25
Dodecane	8.958	57	8.74E+05	3535854.75	1075397.13
Decane, 2-methyl-	5.817	43	7.89E+05	871117.88	926871.81
Octadecane	16.799	57	7.60E+05	707971.69	1020500.81
Hexadecane	14.329	57	7.55E+05	744075.94	989846.94
Dodecane, 4,6-dimethyl-	9.643	175	7.00E+05	660341.25	802752.06
Silane, cyclohexyldimethoxymethyl-	8.386	105	6.75E+05	619400.63	967073.75
Decane, 4-methyl-	5.967	43	6.71E+05	700147.56	799510.69
2-Aminobenzothiazole	14.492	150	3.38E+05	369690.63	987184.5
2-Hydroxybenzothiazole	15.371	151	4.90E+05	414772.47	453568.53
2(3H)-Benzothiazolethione, 3-methyl-	16.956	181	341979.41	394132.34	871421.81

VITA

NAME	Saowaluk Krainara
DATE OF BIRTH	19 October 1987
PLACE OF BIRTH	Nakhon Si Thammarat
INSTITUTIONS ATTENDED	In 2006, High school, Stree Thongsong in Nakhon Si Thammarat In 2006-2010, Bachelor degree of Science in Microbiology from Faculty of Science of Prince of Songkla University, Thailand. In 2010-2013, Master's degree of Science in Biotechnology from School of Bioresources and Technology of King Mongkut's University of Technology Thonburi, Thailand In 2014-2020, Doctor of Philosophy in Hazardous Substance and Environmental Management, Inter-Department of Environmental Management, Graduate School, Chulalongkorn University, Thailand
HOME ADDRESS	483 Moo.2 Thum-Yai Thung-Song District Nakhon Si Thammarat, 80110
PUBLICATION	KRAINARA, S., SURARAKSA, B., PROMMEENATE, P., THAYANUKUL, P. & LUEPROMCHAI, E. 2020. Enrichment and characterization of bacterial consortia for degrading 2-mercaptobenzothiazole in rubber industrial wastewater. Journal of Hazardous Materials, 400, 123291.