การคัดแยกและลักษณะสมบัติของแบคทีเรียที่สามารถย่อยสลายไตรโคลคาร์บาน
เพื่อเป็นสารส่งเสริมการเติบโตของพืช

นางเมอร์รี่ ชิปาฮูทาร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ISOLATION AND CHARACTERIZATION OF TRICLOCARBAN DEGRADING BACTERIA AS PLANT GROWTH PROMOTER

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เมอร์รี่ ซิปาฮูทาร์ : การคัดแยกและลักษณะสมบัติของแบคทีเรียที่สามารถย่อยสลายไตรโคลคาร์บานเพื่อเป็นสารส่งเสริมการเติบโตของพืช (ISOLATION AND CHARACTERIZATION OF TRICLOCARBAN DEGRADING BACTERIA AS PLANT GROWTH PROMOTER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อลิสา วังใน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.จิตรตรา เพียภูเขียว, 121 หน้า.

ไตรโคลคาร์บานเป็นสารต้านจุลชีพที่มีการใช้ในผลิตภัณฑ์อุปโภคและทางการแพทย์ การใช้ไตรโคลคาร์บานซึ่งย่อยสลายได้ยากตามธรรมชาติในปริมาณมาก ทำให้สารนี้ตกค้างที่ความเข้มข้นสูงในกากตะกอนของแหล่งบำบัดน้ำเสีย ส่งผลให้มีการปนเปื้อนออกสู่สิ่งแวดล้อม นอกจากนี้การใช้กากตะกอนที่มีการปนเปื้อนไตรโคลคาร์บานเป็นกากชีวภาพในการเกษตรส่งผลให้เกิดการปนเปื้อนตกค้างของสารนี้ในดิน ด้วยเหตุนี้การพัฒนากระบวนการบำบัดไตรโคลคาร์บานทางชีวภาพที่ตกค้างในดินจึงมีความสำคัญต่อระบบนิเวศของพื้นที่การเกษตร ดังนั้น งานวิจัยนี้ได้คัดแยกแบคทีเรียจำนวน 5 ชนิดจากดินในพื้นที่การเกษตรในประเทศไทยที่มีความสามารถในการย่อยสลายไตรโคลคาร์บานและช่วยส่งเสริมการเจริญของพืช ได้แก่ *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, *Pseudomonas* sp. MS45 และ *Pseudomonas fluorescens* MC46 ทั้งนี้ จากแบคทีเรียที่คัดแยกได้ทั้งหมดนี้ ได้นำ 2 สายพันธุ์มาศึกษาต่อเพื่อพัฒนาใช้ในกระบวนการบำบัดสารมลพิษในดิน ได้แก่ MC22 และ MC46 ผลการศึกษาพบว่า MC22 สามารถย่อยสลายไตรโคลคาร์บานภายใต้สภาวะที่ใช้ออกซิเจนและไม่ใช้ออกซิเจน การศึกษาจลนพลศาสตร์และวิถีการย่อยสลายสารพบว่าให้สารมัธยันต์เป็นสารกลุ่มคลอโรแอนิลีนซึ่งถูกย่อยสลายต่อได้อย่างสมบูรณ์ และลดความเป็นพิษได้ดังผลการทดสอบกับพืชถั่วเขียวและถั่วเหลือง สำหรับการศึกษาความสามารถของ MC46 นั้น นอกเหนือจากการศึกษาจลนพลศาสตร์ วิถีการย่อยสลายสารและการลดความเป็นพิษของสารแล้ว ได้พัฒนาแบคทีเรียนี้ในรูปของสูตรหัวเชื้อเพื่อเพิ่มประสิทธิภาพสำหรับการใช้ในการบำบัดไตรโคลคาร์บานที่ตกค้างในดินการเกษตร จากนั้นได้พิสูจน์ผลการใช้ในดินจริงระดับกระถางซึ่งพบว่าการเติมหัวเชื้อแบคทีเรียนี้นอกเหนือจากจะสามารถลดสารที่ตกค้างในดินได้ถึงร้อยละ 74-76 จากความเข้มข้นเริ่มต้น ลดความเป็นพิษของดินที่ส่งผลเสียต่อพืชถั่วเขียวและช่วยส่งเสริมการเจริญของพืชแล้ว ยังเพิ่มแอคติวิตีของเอนไซม์ในดินซึ่งบ่งบอกถึงคุณภาพดินที่ดีขึ้นด้วย ผลการศึกษาดังกล่าวแสดงให้เห็นศักยภาพของ MC22 และ MC46 ในการนำไปใช้จริงในการบำบัดสารไตรโคลคาร์บานและคลอโรแอนิลีนที่ตกค้างในดินการเกษตร รวมทั้งช่วยส่งเสริมการเจริญของพืชด้วย

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MERRY SIPAHUTAR: ISOLATION AND CHARACTERIZATION OF TRICLOCARBAN DEGRADING BACTERIA AS PLANT GROWTH PROMOTER. ADVISOR: ASSOC. PROF. ALISA VANGNAI, Ph.D., CO-ADVISOR: ASST. PROF. JITTRA PIAPUKIEW, Ph.D., 121 pp.

Triclocarban (TCC) is a widely used as antimicrobial agent in household products. High usage volume of TCC results in its accumulation in sludge and lead to contamination in environments. Application of biosolid in agricultural activities leads to contamination of TCC in agricultural areas. So. development of bioremediation technique for TCC is necessary to maintain quality of agricultural ecosystem. For that purpose, this research isolated 5 soil bacteria capable of TCC degradation and with plant-growth promoting (PGP) activities including *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, *Pseudomonas* sp. MS45, and *Pseudomonas fluorescens* MC46. Among them, *Ochrobactrum* sp. MC22 and *P. fluorescens* MC46 were selected for further studies. The results showed that *Ochrobactrum* sp. MC22 could degrade TCC under aerobic and anaerobic conditions. It has ability to degrade TCC in a wide range of concentrations from 0.16-30 mg L^{-1}. The analysis of TCC degradation kinetics and degradation pathway revealed that chloroanilines are degradative intermediates, which could be completely metabolized and detoxified as shown by the toxicity test in pot soil experiment using 2 legume plants. In the case of *P. fluorescens* MC46, not only TCC degradation capability, degradation kinetics, and degradation pathway were analyzed, but it was also developed into a bacterial formula in order to improve its efficiency for TCC degradation and PGP. Moreover, its usage was proven in soil experiment with mung bean plants where the soil was contaminated with TCC. The bioaugmentation of *P. fluorescens* MC46 formula in to the contaminated soil not only reduced TCC by 74-76% of its initial concentration, mitigated TCC toxicity, promoted plant growth, but it also improved soil quality with increasing soil enzyme activities. In conclusion, this study demonstrated the potential use of *Ochrobactrum* sp. MC22 and *P. fluorescens* MC46 for bioremediation of soil contaminated with TCC as well as chloroanilines, while they could promote plant growth.

Field of Study: Biological Sciences Student's Signature .....
Academic Year: 2017 Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

µg	-	microgram
µg h^{-1}	-	microgram per hour
mg	-	milligram
mg l^{-1}	-	milligram per litre
g	-	gram
g l^{-1}	-	gram per litre
kg	-	kilogram
mg kg^{-1}	-	milligram per kilogram
ml	-	millilitre
µl	-	microlitre
mM	-	millimolar
mm	-	millimetre
℃	-	degree Celsius
h	-	hour
d	-	day
k	-	rate constant
$(NH_4)_2SO_4$	-	ammonium sulphate
16S rRNA	-	16 Small ribosomal subunit RNA
ACC deaminase	-	1-Aminocyclopropane-1-carboxylate deaminase
BHC	-	benzenehexachloride
BLAST	-	basic local alignment search tool
BNF	-	biological N_2 fixation
$CaCl_2.2H_2O$	-	calcium chloride dihydrate
CAS	-	chrome-azurol S blue medium
CFU	-	colony forming unit
DCP	-	di-calcium phosphate
DHBA	-	2,3-dihyroxybenzoic acid

DNA	-	deoxyribonucleic acid
dNTPs	-	deoxynucleotide triphosphates
$FeCl_3$	-	ferric chloride
$FeSO_4$	-	ferrous sulphate
H_2S	-	hydrogen sulphide
HCN	-	hydrogen cyanide
HPLC	-	high performance liquid chromatography
IAA	-	indole-3-acetic acid
K_2HPO_4	-	dipotassium hydrogen phosphate
KCl	-	potassium chloride
KH_2PO_4	-	potassium dihydrogen phosphate
LC-MS	-	liquid chromatography mass spectrometry
$MgCl_2.2H_2O$	-	magnesium chloride dihydrate
$MgSO_4$	-	magnesium sulphate
$MgSO_4.7H_2O$	-	magnesium sulphate heptahydrate
$MnSO_4$	-	manganese sulphate
MSM	-	minimal salt medium
N_2	-	nitrogen
Na_2HPO_4	-	disodium hydrogen phosphate
Na_2SO_4	-	sodium sulphate
NaCl	-	sodium chloride
NCBI	-	national center for biotechnology information
NH_4Cl	-	ammonium chloride
NJ	-	neighbor-joining
PCR	-	polymerase chain reaction
PEG	-	polyethylene glycol
PGPB	-	plant growth promoting bacteria
PTE	-	phosphotriesterase
SA	-	salicylic acid
SEM	-	scanning electron microscopy
TCC	-	triclocarban

TCP	-	tri-calcium phosphate
TCS	-	triclosan
Tris-HCl	-	tris-hydrochloride

CHAPTER I

INTRODUCTION

1.1 Background and rationale of this dissertation

Triclocarban [3-(4-chlorophenyl)-1-(3,4-dichlorphenyl) urea] (TCC) chemicals are widely exhausted as extensive range antibacterial agens for several household and pharmaceutical products. The intensive use and recalcitrant property of the compound lead to its accumulation in wastewater, sludge, soil and biosolid. Since industrial sludge and biosolid is generally used as one of the components for fertilizer production, contaminated TCC may be carried over to agricultural soil and caused adverse impact on agricultural environment. Due to its toxicity not only to environmental microbes (Shareef et al., 2009), but also to human at high concentration (Heberer, 2002), the treatment of TCC is needed. Bioremediation is one of the effective treatment techniques that relies on microbial activity (Miller et al., 2010). Recalcitrant contaminants-degrading bacteria do exist in contaminated soil environments (Leigh et al., 2002). Therefore, in current study, bacteria were isolated from contaminated agricultural soils. Because our ultimate goal is to apply these bacteria as a bioaugmented culture to treat toxic contaminants in agricultural field, it is important that these bacteria have positive interaction with plant as well. As a consequence, selection of bacteria having ability to degrade TCC and promote plant growth is focused. Bacteria that can promote plant growth are also able to enhance plant development and increase yield (Lugtenberg & Kamilova, 2009). Strains of *Pseudomonas*, *Bacillus*, and *Serratia* are known as the effective plant growth-promoting bacteria (PGPB). *Pseudomonas fluorescens* strain is found in rhizosphere area. Several *Pseudomonas* strains were revealed to shelter crops from many microorganism bugs.

In present study, bacterial strains that could promote plant growth were conducted to assess their ability to degrade TCC in liquid medium as well as plant growth promoting in TCC contaminated environmental soil. Nevertheless, in various cases PGPB are unsuccessful to show good impacts when used in the land. This is possibly due to not the bacteria could not do colonize rhizosphere and/or plant, which is as an important step needed for exhibiting beneficial effects. The success or failure of PGPB to do the colonization to rhizosphere is the quality of formulations of inoculants containing an effective bacterial strain (Schoebitz et al., 2013). Moreover, this study was not be only investigating the ability of isolates for plant growth promotion, but also improving the cell survival by developing a proper formulation of a likely PGPB strain in a marketable inoculant product.

1.2 Objectives

This research work focuses on these four following objectives:

1) To screen, isolate and characterize TCC degrading-bacteria from contaminated agricultural soils.
2) To examine the toxicity of TCC and its degraded metabolites in microbe, plants, and meristematic cells.
3) To investigate the ability of TCC degrading-bacteria for plant growth promotion
4) To develop an appropriate formulation of plant growth promoting bacterium.
5) To test the effects of different inoculant formulas in the greenhouse experiments and determine the best formula to remove TCC improve plant growth.

1.3 Hypothesis

TCC degrading-bacteria isolated from contaminated agricultural soil have plant growth promoting traits and the proper formulata can improve the cell survival of the plant growth promoting bacteria.

1.4 Expected outcome

1) Potential application of the newly isolated strain as a bioaugmented culture for bioremediation of triclocarban-contaminated site.
2) Successful formulation of inoculant as an important input in sustainable agriculture and environmental solutions.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

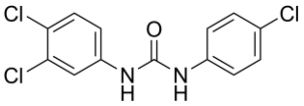
2.1 Triclocarban and its properties

Triclocarban (Table II-1), a polychlorinated binuclear aromatic urea which possesses 36% of chlorine substance (Halden & Paull, 2004b), is commonly used as a broad spectrum antibacterial compound for several household and pharmaceutical products. The intensive use and recalcitrant property of the compound lead to its accumulation in wastewater, sludge, soil and biosolid. Huge amount of triclocarban (up to 98%) in wastewater influent is discarded from the liquid form by activated sludge treatment, and more than an half is changed to the solid phase (i.e. sludge) (Heidler et al., 2006b). Sludge is regularly transferred, thru a various way to cover rule in the US EPA 40CFR503, to approve the application of biosolids for agricultural land. The USEPA estimates that biosolids are produced 8×10^6 dry metric tons every year, and the biosolids are applied to agricultural land (NRC, 2002). Concentration of biosolids-borne TCC was range from 5 to 51 mg kg^{-1} (Chu & Metcalfe, 2007; Halden & Paull, 2005;; Sapkota et al., 2007), with a spucious mean of approximately 20 mg kg^{-1}. A paper which was published in the Targeted National Sewage Sludge Survey (TNSSS) quantified TCC concentrations up to 441 mg kg^{-1} in wastewater treatment sludge and biosolids up to 39±60 mg kg^{-1}. It is very little information about the bioavailability and persistence (i.e. the amount of capability to be taken and present in interaction to organism metabolism) of TCC in the soil or agricultural area after the biosolids application unto it.

Triclocarban (TCC), an antimicrobial agent produced in huge volume, is commonly applied in the ingredient of customer and pharmaceutical care products particularly antimicrobial and antifungal products since twenty years ago (Heidler et

al., 2006b). The N-H group, high chlorine substance and existing of aromatic rings reveals the struggling to TCC biodegradation and the probability of its persevering in the environment for a long time period. Moreover, TCC is hydrophobic and non-volatile in natural environment with low water solubility, and soil adsorption coefficient, thus tending to sorb onto soil and sediment. The half-life of TCC in soil without oxygen predicted more than 3 months (Ying et al., 2007).

Table II-1 Physico-chemical properties of TCC (Cha & Cupples, 2009; Chu & Metcalfe, 2007; Loftsson et al., 2005; Sabourin et al., 2012; Ying et al., 2007).

Properties	Triclocarban
Molecular structure	
IUPAC names	3-(4-Chlorophenyl)-1-(3,4-dichlorophenyl) urea
Other names	Trichlorocarbanilide, TCC, Solubacter
Molecular weight	315.6 g mol−1
CAS registry number	*101-20-2*
Chemical formula	$C_{13}H_9Cl_3N_2O$
Activity	Antimicrobial
Boiling point	254 to 256 °C (489 to 493 °F; 527 to 529 K)
log Koc	4.7
log Kow	4.9
Solubility at 20 °C in water (mg L^{-1})	0.6479; 11
pKa	12.7
Half-life in soil (day)	108 days

Another antibacterial agent contained in a various personal care products is triclosan (TCS). The usage of TCS (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is very familiar to hundreds care goods, including deodorants, soaps, toothpastes, and a variety pf plastic products, comprise up to 0.3% TCS (Schinner & Von Mersi, 1990). Not surprisingly, TCS was discovered more than an half of U.S. streams. The extensive use of TCS in universe built a concern, due to the tinge level of TCC may support the expansion of antimicrobial-resistant microorganims and trigger adverse effects on the environment (Tatarazako et al., 2004). When TCC is displayed to UV, it can potentially be converted into more toxic chemicals like chlorodioxins (Gao et al., 2015). TCS has revealed weak androgenic action in aquatic species and both estrogenic and androgenic retorts in human breast cancer cells (Shareef et al., 2009), implying TCS is an endocrine-disrupting amalgam.

The providence of TCC and TCS in the earth makes a serious attention to environmental scientists and similar regulatory agencies. Incitement for environmental coverage and risk evaluations is delivered by many articles on expansion of microbial defiance after it is used to household biocides, ecotoxicity to aquatic organisms, the possibility for invention of toxic biocide destroys in the environment, and the probability of negative human health effects inferred from study with animal mocks-uo (Chen et al., 2015). Not only their toxic effects, TCC and TCS also expressed their bioaccumulation in aquatic species as well as occur in human milk (Tarnow et al., 2013). Moreover, TCC and TCS were examined to persevere in the land for long time, especially once the condition without oxygen (Ying et al., 2007).

Toxicity of TCS to biofilm algae and bacteria affected an intensification of bacterial transience with a no effect concentration of 0.21 μg/L and TCS was more effective to bacteria than algae (Lozano et al., 2013).

2.2 Biodegradation of triclocarban

Several bacterial species are capable of toxic organic compounds degradation. Evolution made bacteria could survive with many strategies. Bacterial adaptation in toxic and stress environments and in the food resource competition is very important. After the exposing of bacteria to a contaminant area, the bacteria will bear the toxic universe both endogenously and by adaptation. Carbon is often less for bacteria in the soil environment, and environmental stress like malnourishment hints the bacteria to a raised mutation rate. Bacteria reacting to tension condition by creating a new catabolic trail have a prospect to persist. Thus, the capability of bacteria to degrade TCC is important for the niche chances (Johnsen et al., 2005).

The main issue in bacterial degradation is bioavailability. Compounds of bioavailable organic are open for microorganism uptake. Such compounds are generally found in a solvable form for substrate uptake, however its sorption onto soil particles results biodegradation difficulties. Certain bacterial species alter to work together with humic substance-sorbed compounds, though such adaptation capability is deliberated unusual. Biodegradation may occur even if the organic compound bound to humic materials. In contrast, a low temperature environment (less than 10°C) can bring down some organic compounds bioavailability so degradation by bacterial species can be difficult (Ansari et al., 2015). Organic contaminants can be used by bacteria as a carbon source, or degradation may happen by a co-metabolic pathway where the contaminants take after a natural substrate but do not provide energy for bacteria. Nonetheless, high deliberation of contaminants could be toxic to bacteria and prevent biodegradation, so TCC removal by bacteria is probable if concentration of the pollutant is under the toxicity level (Yang et al., 2011). On contrary, a small concentration of pollutant may not supply sufficient energy and carbon for effective biodegradation. Shortly, biodegradation is not efficient if the population of bacteria is

not enough and the bacteria are not able to grow. Therefore, the addition of nutrients (C/N) is effective to aggregate the biodegradation of organic compounds because the feedings effectively stimulate bacterial growth (Jean et al., 2008).

There are two methods to remove contaminants from soil - bioremediation and natural attenuation. These methods are used due to their sustainable and inexpensive. Even though these two methods need less labour than physical methods, the methods are not “no action” or inactive procedures. Alert measurements, validations and monitoring schedules to the process are required. The success of natural attenuation is estimated within a limitation of proper time and no need next activity for purification is necessary. Verdicts regarding natural attenuation must always according to risk assessment, and the action itself should be backed by huge control of the site. Therefore, bioremediation of organic compounds in almost all cases is the best choice, even if the procedure need stimulation (Grant et al., 2007).

It is possible that TCC and phenyl urea herbicides are primarily reduced by bacteria thru hydrolysis of the urea bridge, then producing 4-chloroaniline and 3,4-dichloroaniline (Figure II-1, Reaction 1). For many bacterial strains, 4-chloroaniline is deaminated and hydroxylated by an aniline dioxygenase producing 4-chlorocatechol (Figure II-1, Reaction 2). The removal of 3,4-dichloroaniline is less clear, but may ensue through dehalogenation (Figure II-1, Reaction 4). The ensuing of monochloroaniline may undergo deamination and hydroxylation to 4-chlorocatechol by a dioxygenase enzyme. Intermediate 4-chlorocatechol is then converted in ortho- or meta-cleavage pathways via 1,2- or 2,3-catechol dioxygenase enzymes producing 3-chloro-cis,cis-muconate or 5-chloro-2-hydroxymuconic acid semialdehyde, respectively (Figure II-1, Reactions 3 and 5). These byproducts are thus converted by intermediary metabolic pathways following in carbon and nitrogen digestion.

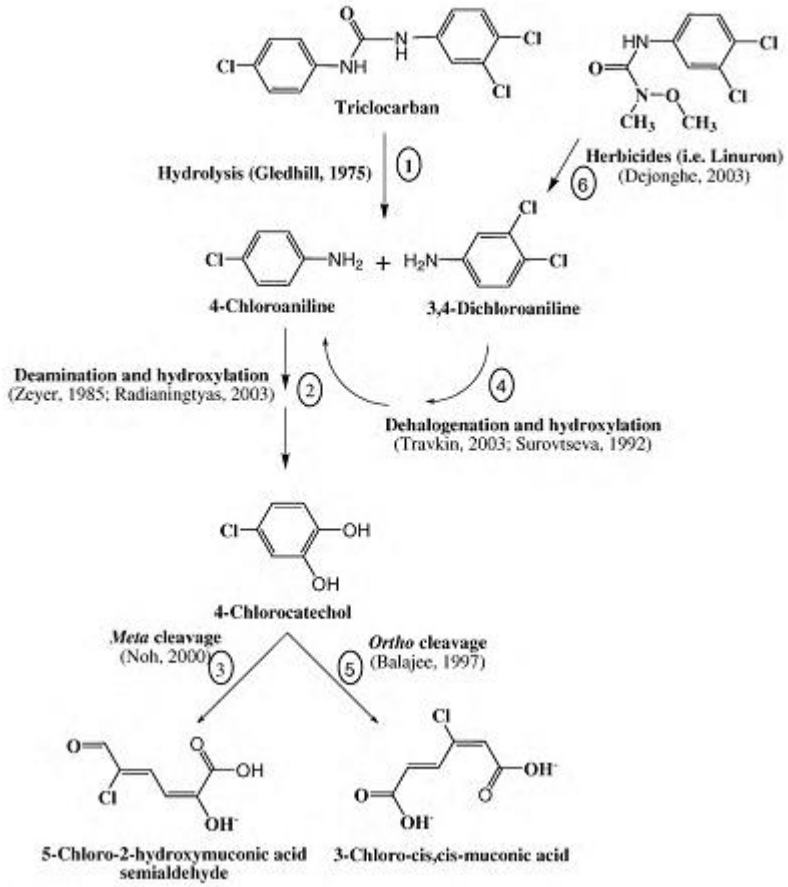


Figure II-1 TCC biodegradation pathway based on previous studies (Miller et al., 2010).

2.3 Biodegradation of triclosan

Biodegradation of TCC in the environment and wastewater has recently become an interesting research topic (Mulla et al., 2016). A previous study reported that approximately 79% of TCC was removed by biological wastewater treatment processes (Shareef et al., 2009), suggesting that (i) biodegradation can be an important removal mechanism in wastewater and (ii) TCS degrading bacteria are present in the activated sludge. These two aspects were supported by two reports. For example, biodegradation of TCS was observed for two wastewater microorganisms, *Sphingomonas* sp. Rd1 (Mulla et al., 2016) and *Nitrosomonas europaea* and by nitrifying activated sludge (Zarate et al., 2012). Still, knowledge about wastewater microorganisms capable of degrading TCS is limited. Recently, a known diphenyl ether degrader, *Sphingomonas* sp. PH- 07, showed an ability to partially degrade TCS and produce three metabolites (hydroxylated triclosan, 4-chlorphenol, and 2,4-dichlorophenol) (Ali et al., 2011). To date, no complete dechlorination of TCS has been observed and TCS degradation kinetics and pathways still remain unclear.

TCS degradation is likely to follow a meta-cleavage pathway. An initial attack of a region selective dioxygenase at the 2,3-position of TCS which has produced in the formation of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, and monohydroxytriclosan (detected) and dihydroxy-triclosan (Lee et al., 2012). Further dioxygenation with simultaneous ether-bond cleavage occurred during diphenyl ether degradation. Then, it is possible that the monohydroxy- and dihydroxy-triclosan were further bound by 2,3-dioxygenase and then subjected to an ether cleavage to produce 2,4- dichlorophenol (shown as reaction a2 or a3 or a4 in Figure II-2). Two other ring-fission metabolites might be resulted; however, none of them were detected in this study. The detection of 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione also suggested another degradation route involving the occurrence of

an ether-bond cleavage of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione (shown as reaction a5 in Figure II-2). Interestingly, strain KCY1 can grow on phenol, but not on catechol, suggesting that the utilization of phenol by strain KCY1 may not follow a catechol pathway, but a hydroquinone pathway. The complete recovery of chloride ions suggests that 2,4-dichlorophenol and 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione were completely dechlorinated (the reaction a6 in Figure II-2).

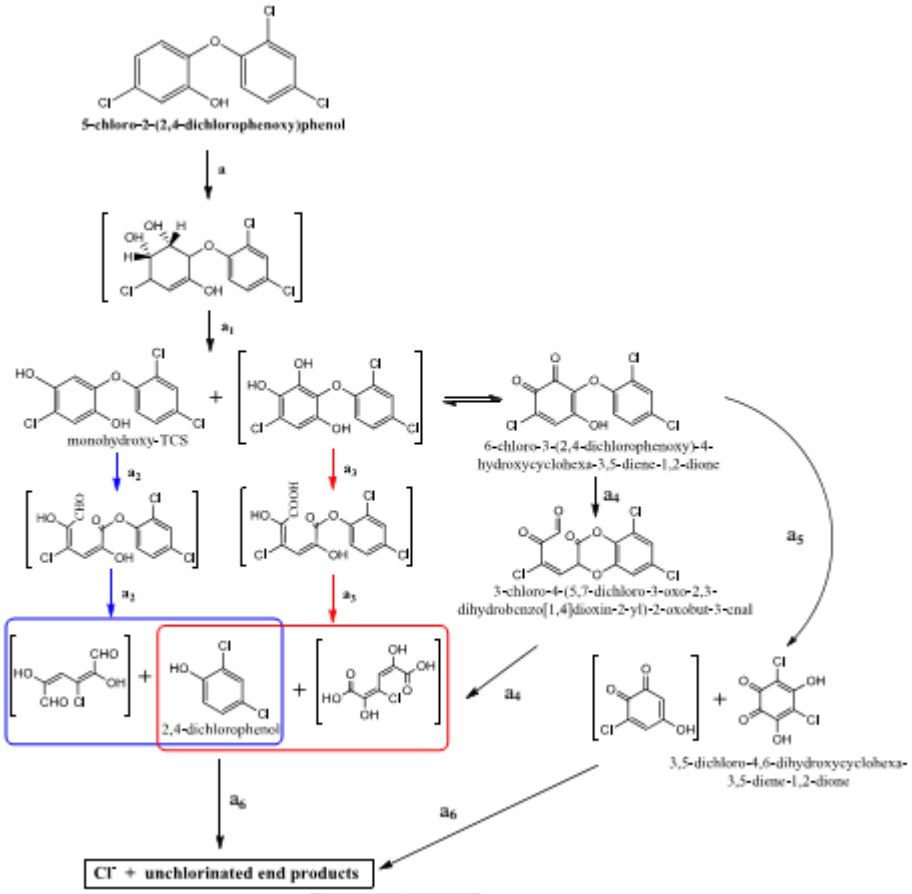


Figure II-2 TCS biodegradation pathway by strain KCY1 on previous study (Lee et al., 2012).

2.4 Plant growth promoting bacteria

Free-living bacteria isolated from agricultural lands and crop plants are often labeled as plant growth-promoting bacteria (PGPB), which colonize the surface of the root or intercellular spaces of the host plant, frequently improving root establishment (Lugtenberg & Kamilova, 2009). In this regard, PGPB have a potential role in developing sustainable agriculture for crop production (Proctor & Woodell, 1975). These mechanisms lead to plant growth promotion of diverse nature such as nonsymbiotic nitrogen fixation, phosphate solubilisation, and production of various phytohormones improving root growth, water absorption, and nutrients (Spaepen et al., 2007). Nevertheless, colonization of plant roots by direct inoculation of free PGPB cells into the soil is not easy because it is susceptible to environmental variations, such as soil conditions, fluctuation of pH and temperature, humidity, protozoa predation, and salt stress (Arora et al., 2008; Wu et al., 2012). This fickleness of PGPB inoculation success on plants is mainly due to quality of formulations of inoculants containing an effective bacterial strain and determines the success or failure of a biological agent (Bashan et al., 2014). Unfortunately, most studies about PGPB strains stop at identification of new isolates having PGPB capacities, without ever reaching the formulation stage.

Plant growth promoting bacteria (PGPB) are an important group of microbial community that exhibit beneficial effects on plant growth and development. Rhizosphere is influenced by the physical, chemical and biological processes of root, which is an ideal place for the proliferation of these microorganisms (Sorensen, 1997). These microbes generally exist more or less near the roots due to the presence 54 of root exudates, which are used as a source of nutrients for microbial growth (Abdel-Aziez et al., 2014). The chemicals which are secreted by plant roots into the soils are generally called as root exudates. Many of these microorganisms depend on the plant root exudates for their survival (Glick, 2012). The exudates may have a wide

range of chemical compounds which in turn modifies the physical and chemical properties of the soil and thus, regulates the structure of soil microbial community in the immediate vicinity of root surface (Deaker et al., 2004).

The PGPB residing in the soil environment can cause dramatic changes in plant growth by the production of growth regulators and improving plant nutrition by supplying and facilitating nutrient uptake from soil (Zaidi et al., 2015). In addition, many of these rhizobacterial strains can also improve plant tolerance against salinity, drought, flooding, heavy metal toxicity, bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds like pesticides and, accordingly, enable plants to survive under non-favourable environmental conditions (Ahmad et al., 2013; Glick, 2010; Raj et al., 2014).

2.5 Formulations

Bacterial formulation is a crucial issue for inoculants containing an effective bacterial strain and can control the success or failure of a biological agent. Microbial inoculant formulations are expected to match the above characteristics and overcome two major problems for living organisms: 1) loss of viability during short storage, and 2) long shelf life and stability over the range of -5 to 30°C (Bashan et al., 2014).

There are different techniques of cell immobilization which are pellets, granules and liquid formulations that have been evaluated and employed to develop carriers for the field application of biocontrol agents (BCAs). Encapsulation of liquid suspension of BCAs by an appropriate gelant (gel forming material), especially natural polymers (alginate, carragenan, agarose, cellulose, agar, hen-egg white, gelatin etc.) as well as synthetic polymers (polyacrylamide, photo cross-linkable resins etc.) is one of the important approaches to develop an efficient delivery system (Aeron et al., 2011). However, the technique using gelant to encapsulate the BCA has failed commercially

as there is difficulty in handling the formulation, low viability of the organism and shorter shelf life (Khan and Gupta, 1998). Powder or fine granular carrier systems for biocontrol agents are more useful than gelant or liquid formulations, and compatible with existing farm machinery(Khan et al., 2011). The powder formulations available are less expensive as they are developed from the low cost agriculture or industrial wastes or by-products and their handling is also easy. Numerous solid materials are employed in the combination of wheat bran-sand mixture, sawdust-sand-molasses mixture, corn cob-sand-molasses mixture, bagassesand-molasses mixture, organic cakes, cowdung-sand mixture, compost or farm manure, inert charcoal, diatomaceous earth and fly ash have been tested to prepare granular or powder formulations of biocontrol agents. The different solid materials such as wheat bran, sand, sawdust, molasses, corn cob, bagasse, organic cakes, cowdung, compost or farm manure, inert charcoal, diatomaceous earth and fly ash have been used to prepare granular or powder based formulations of biocontrol agents (Khan et al., 2011).

Carrier based starter culture formulations of effective composting microorganisms are generally based on food grains. Few literature exists on innovative carrier materials for starter cultures of bacterial strains for composting and there is abundant scope for promotion of many more, superior and cost-effective materials for use as carriers. In the present investigation sawdust from forest saw mills was assessed for suitability as a user-friendly carrier material for starter cultures of efficient bacterial inoculum developed for faster composting of organic wastes (Jha & Saraf, 2015).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Autoclave HV-110	Hirayama, Japan
Autoclave NLS-3020	Sanyo Electric Co., Ltd, Japan
Centrifuge	Labnet, USA
Chemical hood (FH1200X)	BIOBASE, China
High Performance Liquid Chromatography	Shimadzu, Japan (HPLC LC-20AT)
Hotplate stirrer	Lab Tech, Korea
Incubator shaker, innova 4000	New Brunswick scientific, USA
Laminar flow (BBS-V1300-D)	BIOBASE, China
Light microscope	Olympus BX51 (equipped with Olympus DP70 camera)
Micropipette 20, 100, 200, 1000 ul	Gilson, France
Microplate reader (Multiskane GO)	Thermo Scientific, Germany
pH meter	Mettler Toledo, USA
Scanning electron microscope (SEM)	JEOL Model JSM-5410LV, Tokyo, Japan
Spectrophotometer DU 650	Beckmen Coulter, USA
Vortex (Touch mixer model 232)	Fisher scientific, USA

3.1.2 Chemical

Absolute ethanol	Merck, Germany
Acetic acid, glacial	J.T. Baker, USA

Acetone	J.T. Baker, USA
Acetonitrile	Lab-scan, Thailand
Agar	Scharlau Microbiology, Spain
Agarose	BMA, USA
Aniline	Chem Service Inc., USA
Bis-acrylamide	Bio basic Inc., USA
Bovine serum albumin (BSA)	Sigma, USA
$CaCl_2.6H_2O$ (99.0% purity)	Merck, Germany
Crystal violet	BDH, England
$CuSO_4.5H_2O$	Scharlau Microbiology, Spain
EDTA	BDH, England
$FeCl_3.6H_2O$	BDH, England
$Fe(NH_4)_2(SO_4).6H_2O$	BDH, England
Folin-Ciocalteu's reagent	Carlo Erba Reagenti, Italy
Iodine crystal	BDH, England
H_3BO_3	Merck, Germany
$K_2Cr_2O_7$	Riedel, Germany
K_2HPO_4	Riedel, Germany
KH_2PO_4	Carlo Erba Reagenti, Italy
Methanol	Merck, Germany
$MnSO_4.H_2O$	Merck, Germany
NaCl	BDH, England
$NaNO_3$	Carlo Erba Reagenti, Italy
NaOH	Ajax Finechem, Australia
Na_2HPO_4	Fluka, Germany
$Na_2HPO_4.12H_2O$	Ajax Finechem, Australia
$NaNO_3$	Riedel, Italy

Na_2SO_4	Fluka, Germany
NH_4Cl	May&Baker, England
Peptone	Merck, Germany
Poly(ethylene glycol)	Sigma Aldrich, USA
Safanin O	Fluuka, Germany
Sodium acetate	Ajax Finechem, Australia
Succinic acid	Merck, Germany
Triclocarban (TCC)	Sigma Aldrich, USA
Triclosan (TCS)	Trade TCI Mark, Japan
Triphenylformazan (TPF)	Trade TCI Mark, Japan
Tris	USB, USA
Tri-sodium citrate	Ajax Finechem, Australia
Tryptone	Himedia, India
Urea	Merck, Germany
Yeast extracts	Scharlau Chemic Microbiology, Spain
$ZnSO_4.7H_2O$	Merck, Germany
2,3,5-triphenyltetrazolium	TCI, Japan
3,4-dichloroaniline	Merck Schuchardt OHG, Germany
4-chloroaniline	Merck Schuchardt OHG, Germany

3.1.3 Seeds

Mung bean (*Vigna radiata)* and soy bean (*Crotolaria juncea* L.).

3.1.4 Organism

One organism, namely *Escherichia coli* DH5α obtained from the Laboratory of Biochemistry, Department of Biochemistry, Chulalongkorn University, was used for microbial toxicity assessment.

3.2 Methodology

3.2.1 Isolation and identification of TCC-degrading bacteria

3.2.1.1 *Soil samples collection*

Soil samples treated in this research were rhizhosphere soils gathered from agricultural and fruit fields located in central part of Thailand. The sampling sites were used for intensive agricultural practices since long and exposed to pesticides for several years. Soil samples were dig from 15-20 cm of depth and they were air dried at normal temperature (27±2 °C), mixed completely and sieved using 2 mm mesh screen to discard all wastes.

3.2.1.2 *Isolation and selection of TCC-degrading bacteria*

Isolation of bacteria capable of degrading TCC were conducted via enrichment culture method using soil samples that were historically contaminated with pesticide. Enrichment of TCC-degrading bacteria was begun thru the toting of 5 g of soil sample into 100 mL of mineral salts medium (MSM). TCC (30 μM) was swelled as the sole source of carbon and energy into MSM. The flasks were incubated at 30°C for 1 d on an orbital rotary shaker at 150 rpm. A 0.2 mL aliquot was spread onto MSM agar plates containing 30 μM of TCC as the carbon and energy source. After 2 days of incubation at 30 °C, colonies viable on the petri discs were selected to identify their ability to degrade TCC.

3.2.1.3 *Identification of TCC-degrading bacteria*

Identification of pure bacterial strains showing best biodegradation on TCC and cell growth ability were further performed using partial 16S rDNA sequence. A general primer set for bacteria was sourced for polymerase chain reaction (PCR) amplification: forward primer (27f: 5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer (1492r: 5'-GGT TAC CTT GTT ACG ACTT-3'). The PCR condition was started at a pre-

denaturation step at 94 °C for 3 min which was preceded by 30 cycles of 95 °C (30 s), 57 °C (30 s), 72 °C (1.5 min), and a final 10 min-chain expansion cycle at 72 °C. The amplicons were purified and sequenced. Thereafter, the sequences were exposed to the BLAST found in NCBI to get closely related sequences. Neighbour-hood joining method and Mega software (Version 6) were used to construct a phylogenetic tree. A bootstrap value of 1000 was used to find a certainty approximations for the topologies of phylogenetic tree.

3.2.2 Characterization of TCC biodegradation

3.2.2.1 *TCC biodegradation under aerobic condition and anaerobic condition*

a) Under aerobic condition

All experiments were conducted under growth-dependent. The cells were inoculated at a 8% (v:v) level into 100 mL MSM containing 30 µM TCC before being incubated at 30 °C and 150 rpm in a rotary shaker. The stock solution of TCC (100 mM dissolved by acetone) was added to the flasks (250 mL), and the acetone was allowed to evaporate before addition of MSM media. Cultures without inoculant, as abiotic controls, were also performed the same way as above. The degradation experiments were conducted under aerobic condition. All solution flasks were vigorously shaken and then incubated in room temperature. At predetermined time intervals, the culture samples were withdrawn to measure both TCC remaining and OD_{600}. The degradation of TCC was verified by conveying 1 mL volume from each vial and centrifuging the solutions at 10,000 rpm for 10 min to discard the cell pellets. The residues of TCC in the supernatant was analysed by HPLC as per the following conditions: column temperature: 40°C, wavelength: 254 nm, flow rate: 1 mL/min, mobile phase: acetonitrile (ACN) : UP water (70 : 30), and injection volume: 20µL. Quantitative data

was achieved by matching up the peak area of unknown peaks with that of the standard compounds.

b) Under anaerobic condition

Biodegradation tests under anaerobic condition were carried out under various external electron acceptors and various external donors in 150 ml serum bottles under growth dependent experiment. Fresh sterile MSM medium (90 ml) and 8 % (v:v) culture inoculum were added to each serum bottle. Serum bottles were flushed with pure nitrogen gas and sealed with a butyl rubber stopper and aluminum caps (Lu et al., 2007). TCC was added to all culture samples at initial concentration of 30 μM. To study the influence of external electron acceptor on TCC degradation, 20 μM of each ferric, sulfate, and nitrate was inserted to the cultured medium (Boopathy, 2002). To test the influence of external electron donor on TCC degradation, 1g/L of each succinic acid, glucose, and acetate was added to the cultured medium (Li et al., 2011). Anaerobic control experiments containing no electron acceptor or no electron donor and no cell culture were run in parallel. All solution serum bottles were vigorously shaken and incubated at 150 rpm in room temperature. At predetermined time intervals, the culture samples were withdrawn to measure residual TCC. The degradation of TCC was determined by transferring 1 mL volume from each vial to a new Eppendorf and centrifuging the solutions at 10,000 rpm for 10 min to discard the cell pellets. The residues of TCC in the supernatant was analysed by HPLC (LC-20AT, Shimadzu) as per the following conditions: column temperature: 40°C, wavelength: 254 nm, flow rate: 1 mL/min, mobile phase: acetonitrile (ACN) : UP water (70 : 30), and injection volume: 20μL. Quantitative data was achieved by comparing the peak area of unknown peaks with that of the standard compounds.

3.2.2.2 *Effect of additional carbon and nitrogen sources on TCC degradation*

Additional co-substrates (carbon and nitrogen sources) was investigated to study their influence on TCC biodegradation in MSM liquid culture under growth dependent experiment. The experiment was modified based on previous protocol (Wang et al., 2016): 1 g/L of each carbon sources (1. acetate, 2. carboxymethyl cellulose, 3. succinic acid, and 4. glucose) and nitrogen sources (1. ammonium sulfate, 2. ammonium nitrate, 3. sodium nitrate, and 4. urea). 8 mL of *Ochrobactrum* sp. MC22 cell suspension was added to each flask containing 100 mL of MSM medium, 30 µM concentration of TCC, and each co-substrate. Flasks without the addition of additional substrates served as control. At predetermined time intervals, the culture samples were withdrawn to measure both residual TCC and OD_{600}.

3.2.2.3 *Cell growth and TCC biodegradation kinetic*

The kinetic experiments were carried out under growth dependent at TCC concentration range of 1 - 100 µM. The cells grown overnight (8%, v/v) were injected to TCC-containing MSM medium and incubated at 30 ± 2 ºC under shaking conditions (120 rpm). Media without inoculant at the same concentrations of TCC were relayed as abiotic controls. At determined time period, samples were amassed and centrifuged at 10,000 rpm for 10 min to achieve medium without cells, each of which was then depleted for TCC residual concentration analysis using high performance liquid chromatography (HPLC). Then, Michaelis–Menten model was operated to determine the degradation kinetic by plotting the specific biodegradation rate and the concentrations of TCC. The model equations were resolved using GraphPad Prism 5 software. All kinetic experiments were conducted in triplicate.

3.2.3 Determination of TCC biodegradation pathway

The degradation of TCC was examined by taking 1 mL volume from each vial and centrifuging the solutions at 10,000 rpm for 10 min to discard the cell pellets. The residues of TCC in the supernatant was analysed by HPLC (LC-20AT, Shimadzu) as per the following conditions: column temperature: 40°C, wavelength: 254 nm, flow rate: 1 mL/min, mobile phase: acetonitrile (ACN) : UP water (70 : 30), and injection volume: 20µL. Quantitative data was achieved by comparing the peak area of unknown peaks with that of the standard compounds. The biodegradation intermediates formed during the TCC transformation detected by HPLC and analysed by liquid chromatograph/mass spectrometer (LC/MS) equipped with an electrospray ionization (ESI) source type (microtof-Q II 10335 series).

3.2.4 Test of plant growth promoting (PGP) activities

The plant growth promoting traits (phosphate solulibilzation, of indole acetic acid, exo-polysaccharides and organic acids, siderophore, and ammonia, production) of the five selected bacterial strains (MC22, MC35, MC43, MC46, and MS45) were studied in two concentrations of TCC (30 and 50 µM). Treatment without TCC was performed as a control.

3.2.4.1 *Bioassay for phosphate solubilization (P-solubilization)*

P-solubilization was determined by modified method of (Ahemad & Khan, 2012) 100 mL of Pikovskaya broth subjected with 0, 30, and 50 µM concentration of TCC, was injected with 1 mL of 10^8 cells ml^{-1} of each bacterial strain. The flasks were incubated for 7 d with shaking (120g) at 25 ± 2 °C. A 20 ml culture broth from each flask was discarded and centrifuged (10,000g) for 30 min. And P-solubilized is quantified by previous method (He & Honeycutt, 2005). Briefly, reagents for color reactions were formulated as following: Reagent A: ascorbic acid (0.1 M) and trichloroacetic acid (0.5

M). 0.704 g of ascorbic acid (MW 176.1) and 3.268 g of trichloroacetic acid (MW 163.4) were dissolved in 10 mL water and adjusted the volume to 40 mL. Reagent B: ammonium molybdate (0.01 M). 2.472 g of ammonium molybdate (MW 1235.9) was dissolved in 100 mL of water and adjusted the volume to 200 mL. Reagent C (toxic): sodium citrate (0.1 M), sodium arsenite (0.2 M), and acetic acid (5%). 5.882 g of sodium citrate (MW 294.12) and 5.196 g of sodium arsenite (toxic, MW 129.9) were dissolved in 100 mL, added 10 mL of glacial acetic acid, and adjusted the volume to 200 mL. Samples of 0.32-mL volume were added sequentially with 0.40 mL of reagent A, 0.08 mL of reagent B, and 0.20 mL of reagent C and stirred. The occurring of blue color was measured using spectrophotometer at 600 nm. The quantitative data of P-solubilized was determined by the calibration curve of KH_2PO_4 at 0, 20, 40, 60, 80, and 100 µg ml^{-1}. The change in pH following tri-calcium phosphate (TCP) solubilization was verified as well.

3.2.4.2 *Bioassay for indole acetic acid (IAA) production*

Quantitative data of indole-3-acetic acid (IAA) was analysed by the method of (Ahemad & Khan, 2012). Briefly, the bacterial cells were grown in Luria Bertani (LB) broth (tryptone 10 g; yeast extract 5 g; NaCl 10 g per liter dH_2O and pH 7). A-100 ml of LB broth with 100 µg L-tryptophan ml^{-1} supplemented with 0, 30, and 50 µM concentration of TCC was injected with 1 ml culture containing 10^8 cells ml^{-1} bacterial strain and was incubated with shaking (120g) for 24 h at 25 ± 2 ºC. After incubation period, 5 ml of each culture was centrifuged at 10,000 rpm for 10 min. To 2 ml of supernatant, 2 mL of Salkowsky-reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added and incubated at 28 ºC in darkness for 1 h. The IAA concentration in the supernatant was ascertained using a spectrophotometer at 540 nm against a standard curve of IAA at 0, 20, 40, 60, 80, and 100 µg ml^{-1}.

3.2.4.3 *Bioassay for exo-polysaccharides (EPS) production*

For exo-polysaccharides (EPS) production, cells were cultivated in 100 mL basal medium with 5% sucrose supplemented with 0, 30, 50 µM concentration of TCC and incubated with shaking (120g) for 4 d at 25 ± 2 ºC. Culture broth was spun at 5000 rpm for 30 min and EPS was extricated by adding 3 ml chilled acetone to 1 ml supernatant. The precipitated EPS was continually washed three times substitute with dH_2O and acetone alternately and weighed the pellet after overnight drying (Ahemad & Khan, 2012).

3.2.4.4 *Bioassay for organic acid production*

The culture supernatant used for phosphate solubilization test was also used for organic acids detection. The qualitative and quantitative production of organic acids were confirmed using HPLC Shimadzu Japan equipped with C-18 column with UV detector. The mobile phase consisted of 50 mM KH_2PO_4 with a flow rate of 0.7 mL/min. Organic acids were recorded by monitoring absorbance at 210 nm. Organic acids were identified and quantitated by matching the retention times and peak areas with solutions of pure acids (Rajasankar et al., 2013).

3.2.4.5 *Bioassay for ammonia production*

For ammonia assay, bacterial cells were grown in peptone water broth for five days at 27 ± 2 ºC. A 200 µl culture supernatant was mixed with 1 ml of Nessler's reagent and volume of this mixture was adjusted to 8.5 ml by adding ammonia free distilled water. If the colour is changed from brown to yellow, it indicates ammonia production. The ammonia content in the solution was measured using spectrophotometer at 450 nm (Goswami et al., 2014). The concentration of ammonia was calculated using the standard curve of ammonium sulphate in the range of 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mmol ml^{-1}.

3.2.4.6 *Bioassay for siderophore production*

Siderophore production was assessed using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%) according to the method of Ahemad and Khan (2012). Modi medium subjected with 0, 30, 50 µM concentration of TCC was injected with 100 µl of 10^8 cells ml^{-1} of bacterial cells and incubated at 25 ± 2 ºC for 5 d. Cultures were centrifuged at 5000 rpm and the catechol type phenolates [salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DHBA)] in the supernatant were determined using a modification of the ferric chloride-ferricyanide reagent of Hathway (Reese et al., 1998). Shortly, ethyl acetate extracts was prepared by extracting 2 ml of supernatant twice with 2 ml of ethyl acetate at pH 2. Hathway's reagent was prepared by adding 100 µl of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water and to this, was added 100 µl of 0.1 M potassium ferricyanide. For the bioassay, 1 ml of the reagent was added to 1 ml of sample and absorbance was determined at 560 nm for salicylates (SA) with sodium salicylate as standard and at 700 nm for dihydroxy phenols with DHBA as standard. The calibration curve of SA and DHBA was performed at 0, 20, 40, 60, 80, and 100 µg ml^{-1}.

3.2.5 Toxicity study of TCC and its degradation metabolites

To assess the efficiency of biodegradation, toxicity level of degraded intermediates was analysed in contrast with that in the parental compound (TCC) using microbial toxicity, phytotoxicity, and zytogenotoxicity assessment. The degradation metabolites collected at 168-h during the time-course biodegradation test with 30 and 50 µM concentration of TCC was extracted by acetone, dried, dissolved to the initial volume in distilled water, and exposed to toxicity study.

3.2.5.1 *Phytotoxicity*

Phytotoxicity of treated samples was assessed using seed germination bioassay (Raj et al., 2014). Five seeds of *Vigna radiata* and *Crotalaria juncea* were surface-sterilised (Toyama et al., 2011) and placed on filter paper in each petridish soaked with 2-ml of the test solution (triclocarban or its degraded intermediates of each strain) or deionized water (a control). The plates were incubated in dark condition in a growth chamber at a constant temperature of 25° C, humidity 80% and a cycle of 14 h of light and 10 h of darkness (Yang et al., 2011) for 72 h. At the end of germination, the sprouting length of targets was compared with that of control in which distilled water represented non-toxicity effect to the seeds and the maximum sprouting length.

3.2.5.2 *Cytogenotoxicity*

The assay of cytogenotoxicity was tested towards meristematic root tip cells of a healthy *Allium cepa* as demonstrated by (Prasad & Rao, 2013). Shortly, healthy *A. cepa* bulbs of which outer scales and dried roots discarded were preserved in clean water for 72 h in the dark for root growth. Distilled water was changed daily. Then best fresh root tips (2–2.5 cm) were selected for the treatment. The root tips were subjected to two different concentrations of TCC (30 and 50 μM) and its degraded metabolites for 4 h. Treatment of root tips with distilled water were utilised as negative control. Thereafter, root tips were incubated in 95% ethanol and glacial acetic acid for 90 min, steeped in 1 N HCl for 10 min, and washed in distilled. The root tips were stained with 1% aceto-orcein and compressed in 45% acetic acid on microscopic slide. Cell division and chromosomal aberration of the stained root tips were observed using bright field microscopy at 1000× magnification. Mitotic index was considered as the percent ratio of dividing cells and total number of cells scored (1050).

3.2.6 Growth chamber experiments

3.2.6.1 *TCC degradation under growth chamber experiments*

Sterile seeds of *V. radiata* and *G. max* were pre-germinated for 3 days and aseptically transferred into 60 ml test tubes containing 10 ml of one tenth-strength sterile Hoagland's medium injected with 1 ml of bacterial suspension ($2.5 \pm 0.7 \times 10^8$ CFU/ml). Seedlings growing in medium without inoculum or TCC, used as a control. Three replicates were depleted for each treatment. All test tubes were placed in a growth chamber at a constant temperature of 25° C, humidity 80% and a cycle of 14 h of light and 10 h of darkness (Yang et al., 2011). At the determined time points, the water samples were carefully collected and TCC was analysed quantitatively by using HPLC as described above. Then, the suspensions were 7-8 fold serially diluted and plated on LB agar plates. Colony forming units were counted after incubation at 30 ºC for 24h.

3.2.6.2 *Plant growth and root tissues analysis*

Seeds of *V. radiata* were sterilized (Toyama et al., 2011) and pre-germinated for 3 days, then aseptically transferred into 500 ml bottle jar containing 300 ml of half-strength semisolid Hoagland's solution (0.3%) agar. Three experimental sets of at least six-seedlings were grown under the following conditions: (1) normal growth condition; (2) TCC exposure (30 µM); and (3) TCC exposure with bacterial suspension ($2.5 \pm 0.7 \times 10^8$ CFU mL^{-1}). The cultivation was done in a growth chamber at stable humidity (80%) and temperature (25±2 ℃) with a cycle of 14-h of light and 10-h of darkness (Yang et al., 2011). The plant biomass, plant length and root length were determined after 7 days of cultivation. Anatomical analysis of plant roots was conducted by cross-sectioning the primary root at a distance of 1.2–1.5 cm from the apex to prepare 2-mm-thick semi-thin sections. The cross sections were stained with Safranin O and analyzed for tissue differentiation (Vaculík et al., 2012). The sections were attached on

slides and observed under a light microscope and analyzed for total root area, vascular bundle area, and area of xylem element using RootScan v2.0 image analysis software.

3.2.6.3 *Colonization of bacterial strain on the plant root*

The ability of each bacterial strain to survive TCC contamination and colonize *V. radiata* plant roots until the end of the experiment was investigated. About 1-2 mm of taproot roots were sampled from bottle jar 1 week after planting bacteria inoculated plants in 30 µM TCC. The observation of TCC-degrading bacteria colonization on the plant root surface was also performed using a scanning electron microscope (JEOL Model JSM-5410LV, Tokyo, Japan) at 15 kV.

3.2.7 Formulations of TCC-degrading PGP bacterial strain

One strain (MC46) showing best result in plant growth promoting development and TCC biodegradation activities was selected for formulation experiments. Formulation of TCC-degrading bacterium (MC46) was prepared by applying sawdust as the carrier (Khan et al., 2011; Maheshwari et al., 2015). As shown in Table III.1, stock liquid pure culture of MC46 was prepared in minimum salt mineral yeast (MSMY) medium supplemented with benzoic acid 100 ppm in conical flask and incubated in a rotary shaker at 120 rpm for 1 day at 25±2ºC, resulting in a concentration of approximately 10^{10} CFU/ml. Polyethylene glycol (PEG) at 1% and 5%, polyvinyl pyrrolidone (PVP) at 1% and 5%, 50-50% of carboxymethyl cellulose (CMC) and soluble starch at 1% and 2%, and gum arabic at 0.5% and 1% (Almasi et al., 2010; Commare et al., 2002; Dayamani & Brahmaprakash, 2014; Deaker et al., 2004; Denardin & Freire, 2000) were used as additives to the formulations. Other than that, 1% (v/w) molasses (as nutrient) and 100 ppm of benzoic acid (to inhibit all wild strains) were also supplemented to each formulation. All materials with the exception of sawdust were separately autoclaved at 15 psi pressure at 121 ºC for 20 min. Sawdust (150 g) was

filled in autoclavable polypropylene bags and autoclaved thrice at 15 psi pressure at 121 ºC for 20 min on three consecutive days. Additive singly, 1% molasses and 100 ppm benzoic acid were supplemented to saw dust (150 g) after oven drying at 28± 2 ºC for 24 h to achieve the ultimate dried formulation (Prasad et al., 2002). Thereafter, 60 ml of stock culture were transferred to the mixture under aseptic condition and shaken for uniform distribution. All the sawdust-based formulations with a moisture content 40% were packed and sealed separately in polypropylene bags leaving about 25% airspace to give proper aeration to the inoculants (Aeron et al., 2011). The bags were then stored at room temperature (28±2 ºC) in the dark to prevent damage from ultraviolet (UV) radiation and heat for 20 weeks (Maheshwari et al., 2015). Samples were periodically taken to determine the shelf life of the formulations for CFU and the ability of formulated MC46 inoculant in TCC degradation. Before sampling, the bags were shaken carefully but completely to reach uniform distribution of the inoculant in the entire formulation. One gram was drawn from each formulation of strain MC46 and serially diluted with sterile water. From 10^{-7} and 10^{-9} dilutions, 1 ml suspension was spread over solidified MSMY plus 9.47 mg L^{-1} TCC in Petri plates (0.2 ml/plate) and incubated at 30 ºC in an incubator for 24 h. The development of colonies of MC46 was observed and expressed as log CFU/g of formulation.

TCC degradation test was conducted under growth cell dependent condition. TCC and 8% (w/v) sawdust based MC46 formulated in different polymer additives were added to 250 mL Erlenmeyer flasks containing 100 mL MSM medium. Degradation of TCC was examined by transferring 1 mL volume from each vial and centrifuging the solutions at 10,000 rpm for 10 min to discard the cell pellets. The residues of TCC in the supernatant was analysed by HPLC as mentioned above.

Table III-1 Description of sawdust-based formulations developed in this study.

Formulation ingredients
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 1 % PEG (1.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 5 % PEG (7.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 1 % PVP (1.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 5 % PVP (7.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 1 % CMC+Strach (@ 0.75 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 2 % CMC+Strach (@ 1.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 0.5 % Gum Arabic (0.75 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)
Cell culture of *Pseudomonas fluorescens* MC46 (60 ml) containing 10^{10} CFU/ml + sawdust (150 g) + 1 % Gum Arabic (1.5 g) + 1% (v/w) molasses (1.5 ml) + 100 ppm benzoic acid (15 mg)

3.2.8 Pot soil experiments of formulated TCC-degrading PGP bacterial strain

3.2.8.1 *TCC degradation under pot soil experiments*

Pot soil experiments were performed to investigate the effectiveness of bioaugmentation formulated MC46 for remediating TCC contaminated soil and developing plant growth of mung bean (*V. radiata*). Soil samples collected from the top layer of paddy fields located at R*esearch* Center units of Chulalongkorn University, Saraburi Province. These soils had been revealed to continuous applications of biosolids for a considerable period of time. Soil of upper layer (0-20 cm) was collected and air dried, and the chemical properties of the soil were analyzed by Shri A.M.M Murugappa Chettiar Research Centre, India.

Seeds of mung bean were surface sterilized (Toyama et al., 2011) before sowing to the plastic pots, as described earlier (Prasanna et al., 2013). Plastic pots, each filled with 800 g of soil, were prepared for nine different conditions: 1) SS (soil + seeds); 2) SS+F1 (soil + seeds + inoculated F-1); 3) SS+F2 (soil + seeds + inoculated F-2); 4) S+TCC (soil + TCC); 5) SS+TCC (seeds + soil + TCC); 6) SS+TCC+ UF1 (soil + seeds + TCC + uninoculated F-1); 7) SS+TCC+ UF2 (soil + seeds + TCC + uninoculated F-2); 8) SS+TCC+ F1 (soil + seeds + TCC + inoculated F-1); 9) SS+TCC+ F2 (soil + seeds + TCC + inoculated F-2). Whereas F1 is 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive PEG 1%; F2 is 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive CMC + Starch 1%; TCC is a 100 µM of triclocarban (31.56 mg L^{-1}), UF1 is uninoculated MC46 F1; and UF2 is uninoculated MC46 of F2.

Soil application with formulations containing bacterial cells @ 4 x 10^8 cfu g^{-1} of sawdust (taken from 0 week storage) was conducted with two replications. The water content of the soil samples was accustomed to 40-45% of the maximum moisture by the addition of sink water. All pots were placed in a greenhouse with 12 h light photo period. All the pots were watered regularly.

For TCC degradation in soils, samples (5 g) collected every week up to 5 weeks were extracted with 10 ml of methanol on a fine mixer for 3 hours and centrifuged at 10,000 rpm for 5 min to remove the cells and soil. The residues of TCC in the supernatant was analyzed by HPLC under the conditions described above.

3.2.8.2 *Soil enzyme activities*

This study was focused on the characterization of the potential soil enzyme activity in the presence and absence of inoculant MC46 in relation to soil contaminated by TCC. Soil enzyme assays were selected to represent a range of processes involved in nutrient cycling or microbial activity. All selected enzymes analysed were hydrolases elaborated in carbon, nitrogen and phosphorus cycling and were selected for their role in key processes in the decomposition of organic remains. The C-cycle enzymes analysed were cellulase and dehydrogenase; N-cycle enzymes analysed were urease and protease; and P-cycle enzymes analysed were acid and alkaline phosphatase.

CM-Cellulase activity was determined in 2 M acetate buffer (pH 5.5) by reduction of Carboxymethyl cellulose (CMC) to glucose. Briefly, 15 ml of buffer and 15 ml of CMC solution (0.7% w/v) were added to 10 g of soil sample. Controls were prepared without substrate to determine the glucose produced in the absence of added CMC. The concentration of glucose released after incubation at 50 ^{o}C for 24 hours was quantified spectrophotometrically at 690 nm, following the method as described by (Schinner & Von Mersi, 1990).

Activity of dehydrogenase was examined in 0.2 M Tris HCl buffer (pH 7.8) by lessening of 2,3,5-tryphenyltetrazolium chloride (TTC) to Triphenyl formazan (TPF). Briefly, 5 ml of buffer and 5 ml of TTC solution (3 % w/v) were added to 5 g of soil samples. Controls were arranged in the absence of substrate to verify the production of TPF in the absence of TTC. The concentration of TPF released after incubation at

25 ^{o}C for 16 hours was quantified spectrophotometrically at 546 nm as described earlier (Matinizadeh et al., 2008).

Activity of urease was examined using the method described earlier (Kandeler & Gerber, 1988) in 0.1 M borate buffer at pH 10. In particular, 20 ml of buffer and 2.5 ml of 0.72 M urea were transferred to 5 g of soil samples. Controls were arranged in the absence of substrate to verify the ammonium generated in the absence of urea. The concentration of NH_4Cl released after incubation at 37 ^{o}C (urease) for 2 hours was determined spectrophotometrically at 690 nm.

Protease enzymatic activity was examined by using the method described by (Ladd & Butler, 1972). The protease activity was determined by adding 5 ml of 0.05 M Tris buffer (pH 8.1) and 5 ml of casein solution (2% w/v) to 1 g of soil samples. Controls were arranged in the absence of substrate to verify the production of tyrosine the absence of casein. The samples were then incubated at 50 ^{o}C for 2 hours. The released tyrosine was quantified with a spectrophotometer at 700 nm.

Activity of acid phosphatase enzymatic was examined using the methods described by (Eivazi & Tabatabai, 1977). The acid phosphatase activity was determined by adding 4 ml of a modified universal buffer (pH 6.5) and 1 ml of 115 mM p-nitrophenyl phosphate solutions to the soil samples. The samples were incubated at 37 ^{o}C for 1 h. Controls were arranged in the absence of substrate to determine the p-nitrophenol (PNP) produced in the absence of added p-nitrophenyl phosphate. Then the released p-nitrophenol (PNP) was quantified with a spectrophotometer at 400 nm.

The activity of alkaline phosphatase enzymatic was assessed using the methods described by (Eivazi & Tabatabai, 1977). The acid phosphatase activity was determined by adding 4 ml of a modified universal buffer (pH 11) and 1 ml of 115 mM p-nitrophenyl phosphate solutions to the soil samples. The samples were incubated at 37 ^{o}C for 1 h. Controls were prepared without substrate to determine the p-nitrophenol (PNP)

produced in the absence of added p-nitrophenyl phosphate. Then the released p-nitrophenol (PNP) was quantified with a spectrophotometer at 400 nm.

3.2.8.3 *Plant growth and chlorophyll content*

After 35 days of crop growing, plants were gently taken out from soil and the roots were washed three times with distilled water to discard any residues of soil. Plant growth characterizations such as plant height and root length were measured by a centimeter ruler. The fresh biomass yield of mung bean was recorded by weighing the biomass of the stem, leaves and root. Number of leaves was quantified per plant.

Root tissues of *V. radiata* and *G. max* were also quantified on cross root sections at a distance of 1.2-1.5 cm (Vaculík et al., 2012). Approximately 2 mm thick semi-thin sections were prepared and stained with Safranin O. Sections from six different roots were analysed with an Olympus CX31 light microscope, equipped with CANON EOS 650D camera. The tissue areas were determined by Root Scan v2.0 image analysis software.

Chlorophyll a, chlorophyll b, and total carotene content in the leaf tissues of mung bean plants were quantified by the method described previously (Ansari et al., 2015). For the analysis, 0.1 g of leaf was taken from the plants of each condition and was crushed in the mortar. The samples were normalised with 10 mL of acetone followed by centrifugation at 15,000g for 10 min. The absorbance was measured with a spectrophotometer at 470 nm, 645 nm and 662 nm against acetone blank and chlorophyll content was estimated using the following equations (Şükran et al., 1998).

C_a (chlorophyll a) = 11.75 A_{662} – 2.350 A_{645}

C_b (chlorophyll b) = 18.61 A_{645} – 3.960 A_{662}

C_{x+c} (total carotene) = 1000 A_{470} – 2.270 Ca – 81.4 C_b/227.

3.2.9 Statistical analysis

The data were statistically analysed and significant differences among the treatment means were calculated at $P \leq 0.05$ by one-way ANOVA with Dunnett's multiple comparison test using Graphpad Prism, v5.03 (CA, USA).

CHAPTER IV

RESULTS

4.1 Isolation, identification, and cell growth of TCC degrading bacteria

4.1.1 Isolation and identification of TCC degrading bacteria

The soil samples were collected from agricultural areas and fruit fields. In order to isolate potential microorganisms that could degrade TCC, a selective enrichment technique was kept on by supplying TCC as a sole source of carbon. Twenty seven tolerant bacterial strains were isolated from the agricultural areas and fruit fields throughout enrichment culture technique using MSM media procedures and after incubation on agar plates supplemented media. Out of twenty seven bacterial isolates, only four strains were able to tolerate TCC at 30 μM and one strain tolerating TCS at 40 μM. These strains were finally screened for TCC tolerance using cell growth method in MSM liquid medium at OD_{600} (Table IV-1).

Table IV-1 Screening of TCC tolerant bacterial isolates

Bacterial isolates	TCC or TCS concentration (μM)				
	1	5	10	20	30 or 40*
MC22	0.414±0.087	0.398±0.069	0.376±0.091	0.344±0.081	0.323±0.072
MC35	0.221±0.039	0.217±0.076	0.202±0.056	0.198±0.079	0.172±0.067
MC43	0.317±0.071	0.294±0.065	0.256±0.098	0.234±0.086	0.221±0.091
MC46	0.486±0.098	0.412±0.093	0.384±0.089	0.323±0.099	0.316±0.079
MS45**	0.439±0.078	0.407±0.084	0.358±0.075	0.302±0.087	0.259±0.083

*TCS concentration

**TCS tolerant strain

Among the five bacterial isolates, two strains were able to endure TCC up to 30 μM and one strain was able to sustain up to 30 μM (Table IV-1). Therefore 30 μM and 40 μM concentration of TCC and TCS, respectively were selected for further studies.

4.1.2 Identification of TCC degrading bacteria

The highly efficient TCC tolerant bacterial strain plate cultures are presented in Figure IV-1. All of these strains showed higher colony forming unit at 30 μM of TCC or 40 μM of TCS so these concentrations were chosen for next studies. All the bacterial isolates obtained were non-spore forming, motile, and rod-shaped bacteria. As shown in Figure IV-2 all the bacterial strains were found as Gram-negative.

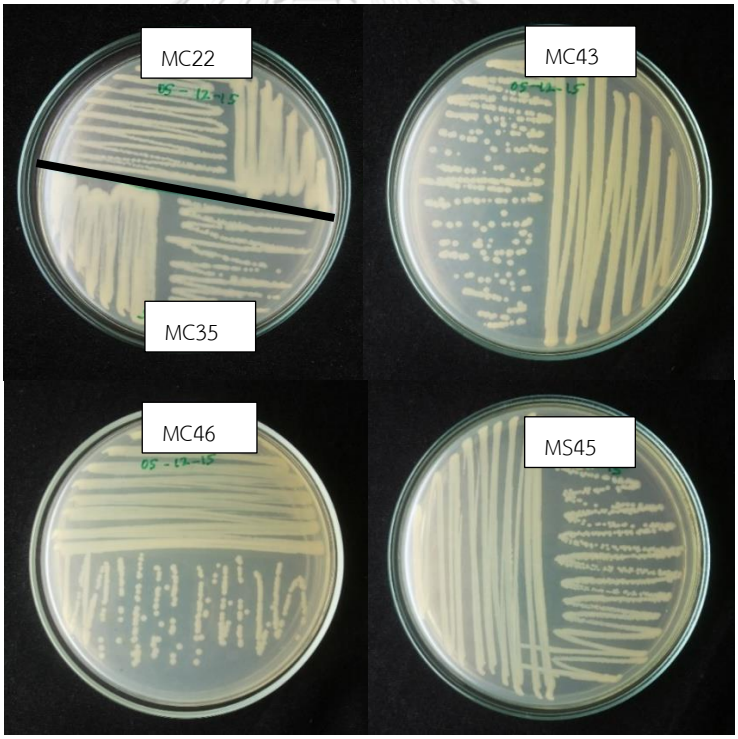


Figure IV-1 TCC tolerant bacterial isolates (MC22, MC35, MC43, and MC46) and TCS tolerant bacterial isolate MS45, obtained by enrichment culture technique.

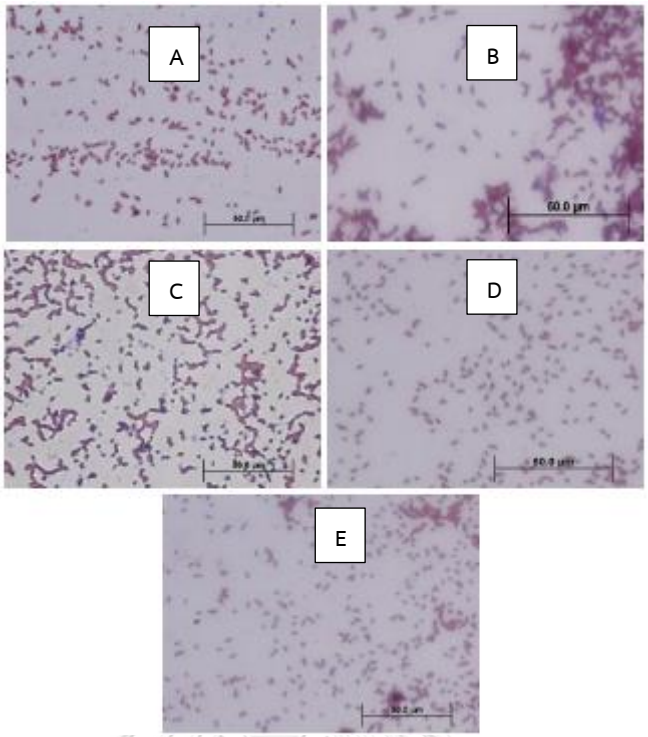


Figure IV-2 Gram staining of TCC tolerant bacterial strains MC22 (A), MC35 (B), MC43 (C), and MC46 (D), and TCS tolerant bacterial strain MS45 (E).

The molecular identification according to 16S rRNA gene sequence analysis was applied to discover the identification of TCC degrading bacterial strains. The small ribosomal sub unit RNA (16S rRNA) sequences of TCC tolerant bacterial isolates (MC22, MC35, MC43, and MC46) were 1415, 1463, 1467, and 1498 base pair nucleotides length, respectively, while 16S rRNA sequence of TCS tolerant strain MS45 was 1286 base pair nucleotides length.

Analysis of the 16S rRNA gene sequences of isolates MC22 and MC35 allowed determine their relationship to *Ochrobactrum* sp. P1 (2013) KF987808 (100 %) as shown in Figure IV-3. MC43 strain was closely related to *Sphingobacterium* sp. N7 KC843944 (99%) (Figure IV-3). MC46 strain was closely related to *Pseudomonas fluorescens* Bp-11 KJ888139 (100%) (Figure IV-3). In the same way, MS45 strain was affiliated with genus *Pseudomonas* sp. The phylogenetic tree was sketched to analyse evolutionary

relationships among sequences of isolated bacteria and nearest neighbours (Figure IV-3). Thus from the BLASTn analysis, MC22, MC35, MC43, MC46, and MS45 were designated as *Ochrobactrum* sp. MC22, *Ochrobactrum* sp.MC35, *Sphingobacterium* sp. MC43, *Pseudomonas fluorescens* MC46, and *Pseudomonas* sp. MS45, respectively.

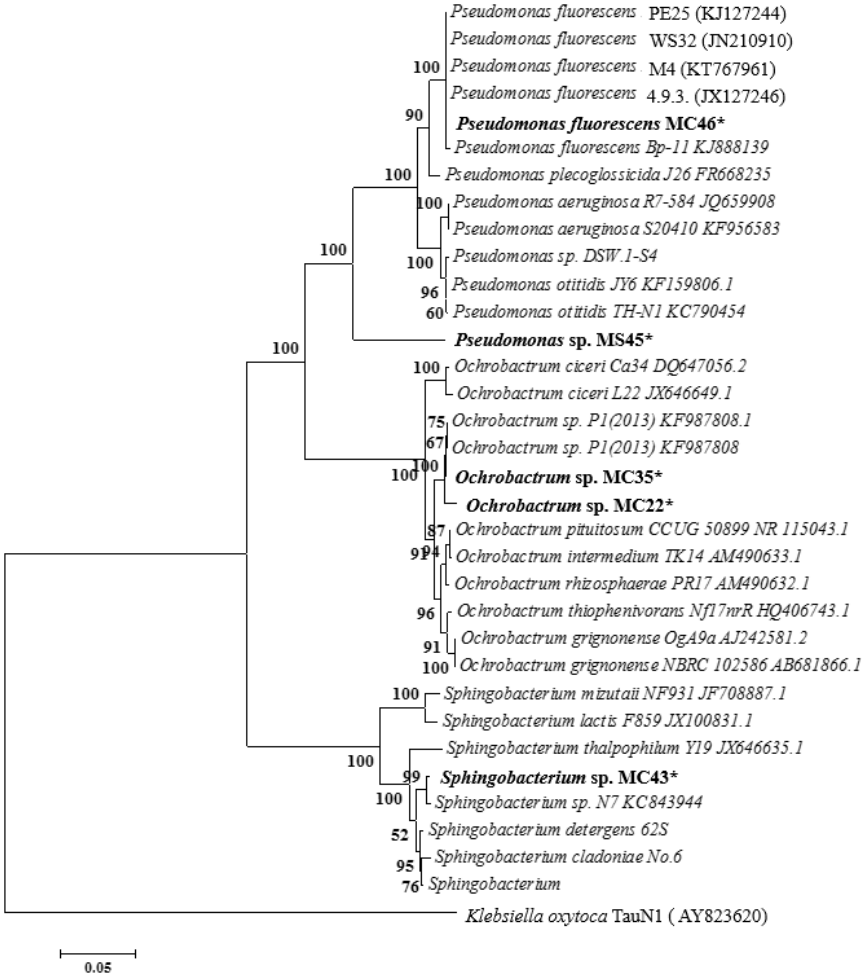


Figure IV-3 The neighbour-joining tree of MC22, MC35, MC43, MC46, and MS45 were constructed using MEGA 6. The scale bar relates to 5 substitutions per 100 nucleotide positions.

4.2 Characterization of TCC biodegradation

4.2.1 Biodegradation under aerobic and anaerobic conditions

4.2.1.1 *Biodegradation under aerobic condition*

The growth of TCC tolerant bacterial strains *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43 and *P. fluorescens* MC46 in MSM with 30 µM of TCC are presented in Figure IV-4. The maximum growth of *Ochrobactrum* sp. MC22 and *Sphingobacterium* sp. MC43 were observed on 48 h of incubation in the MSM medium. The maximum growth of *Ochrobactrum* sp. MC35 and *P. fluorescens* MC46 were obtained on 120 h of incubation. For the growth of all bacterial strains were not found any lag phase in MSM medium thwarted with TCC (Figure IV-4).

HPLC was employed to observe the degradation of TCC. The biodegradation of TCC was assessed using individual bacterial strains. Under aerobic condition, the degradation of TCC was found to be pronounced with *Ochrobactrum* sp. MC22 than by other individual bacterial strains. As presented in Figure IV-4, *Ochrobactrum* sp. MC22 was able to degrade 78% of the initial dose (30 µM) of TCC within 144 h of incubation in the aqueous MSM medium. *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43 and *P. fluorescens* MC46 were just able to degrade TCC by 48%, 51%, and 70%, respectively at the same dose and incubation time. No degradation was observed for TCC in the sterile control with TCC (Figure IV-4).

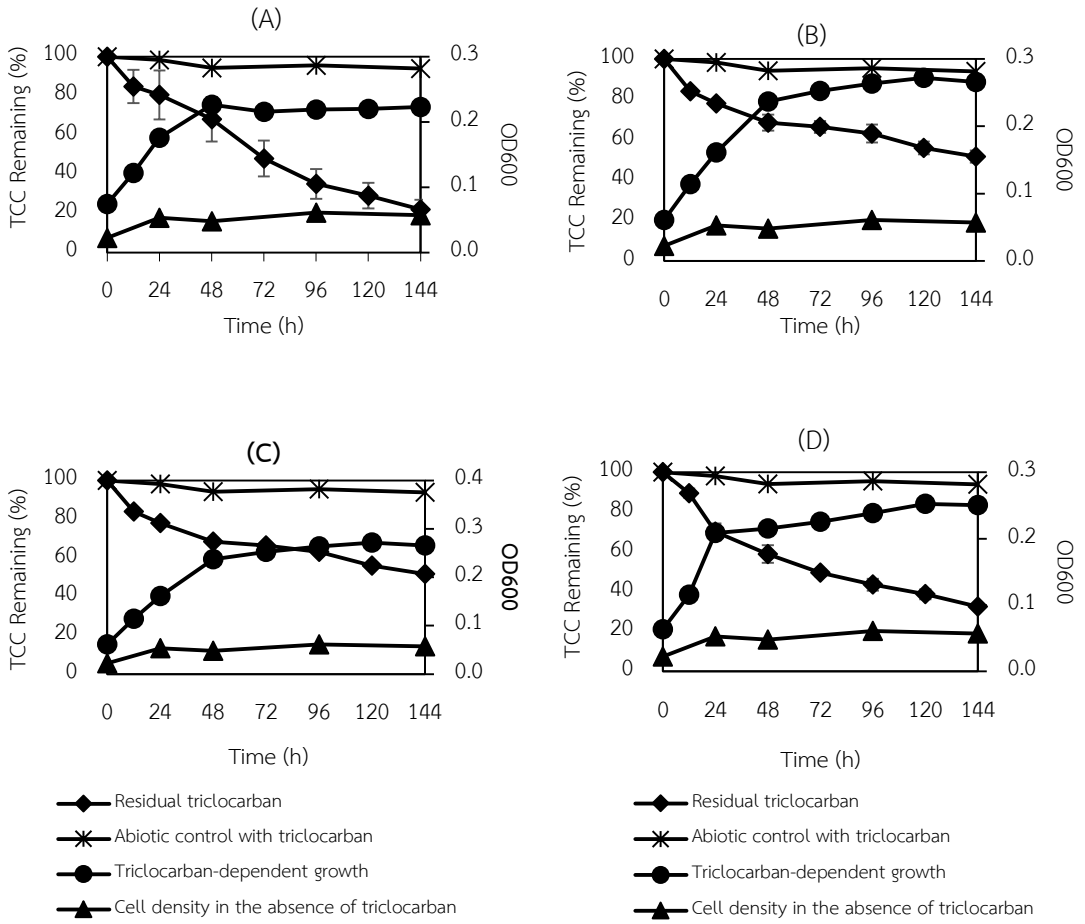


Figure IV-4 TCC-dependent growth of individual bacterial strains and time-course TCC degradation in MSM medium supplemented with 30 μM TCC under aerobic condition by (A) MC22, (B) MC35, (C) MC43, and (D) MC46.

However, *Pseudomonas* sp. MS45 was significantly able to degrade TCS at 40 µM level of concentration. After 144 h of incubation, TCS was found to be 57% degraded by *Pseudomonas* sp. MS45 (

Figure *IV-5*). The growth of TCS tolerant bacterial strain MS45 in MSM supplemented with 40 µM of TCS is presented in

Figure *IV-5*. The growth of *Pseudomonas* sp. MS45 was crushed without TCS in MSM medium, while in MSM supplemented with TCS exhibiting superior growth rate and the maximum growth of *Pseudomonas* sp. MS45 was observed at 72 h of incubation.

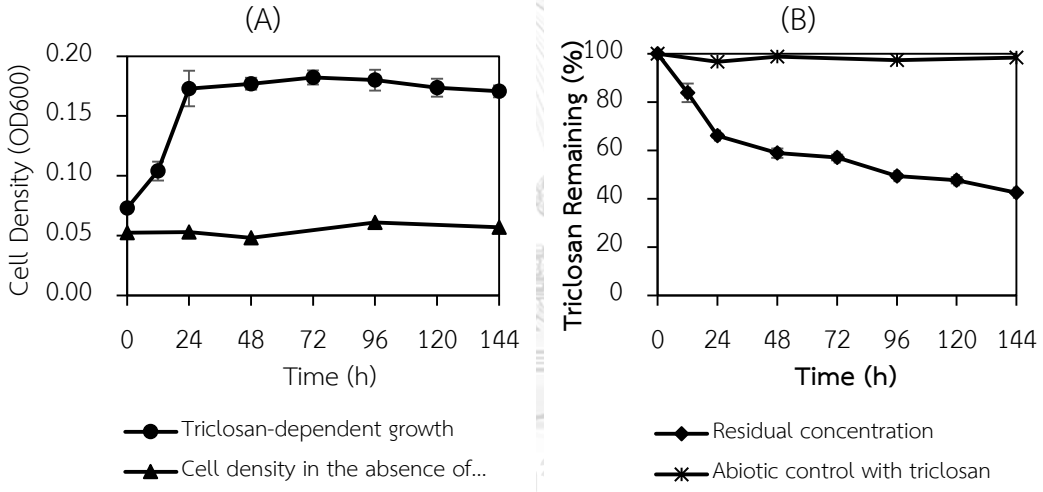


Figure IV-5 TCS-dependent growth of individual bacterial strain (A) and time-course TCS degradation (B) in MSM medium supplemented with 40 µM TCS under aerobic condition by *Pseudomonas* sp. MS45.

4.2.1.2 *Biodegradation under anaerobic condition*

Under anaerobic conditions, no biodegradation of TCC was observed using *Ochrobactrum* sp. MC35, *Sphingobacterium* MC43, or *P. fluorescens* MC46. In contrast, high removal efficiency of TCC by *Ochrobactrum* sp. MC22 under anaerobic conditions was observed in the experiments. Individual of various electron acceptors (ferric,

sulfate, and nitrate) and electron donors (succinic acid, acetate and glucose) were added into the culture medium to investigate the effect of these electron matters on the anaerobic biodegradation of TCC. The evolution of the total concentration of TCC during the incubation is shown in Figure IV-6. The biodegradation efficiency of TCC was greatly increased by adding these electron matters. The anaerobic biodegradation of TCC was greatly enhanced in the presence of ferric as electron acceptor or acetate as electron donor. The maximum TCC removal in the presence of ferric and acetate were 43 % and 50%, respectively of the initial dose (30 μM) of TCC within 14 d of incubation in the aqueous MSM medium. While the maximum specific biodegradation rate of the anaerobic was observed in the control (0.006 ± 0.0002 μmole d^{-1}) was much lower than those in other treatments.

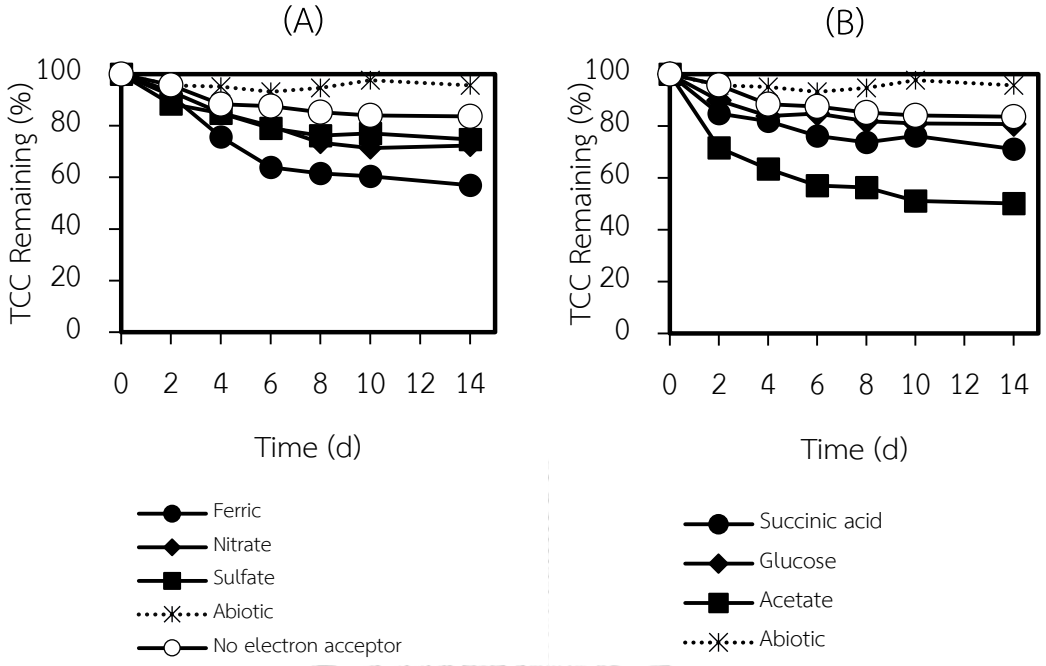


Figure IV-6 TCC-dependent growth of individual bacterial strain and time-course TCC degradation in MSM medium supplemented with 30 μM TCC under anaerobic condition by *Ochrobactrum* sp. MC22 in the presence and absence of (A) various electron acceptors, and (B) various electron donor.

4.2.2 Effect of additional carbon and nitrogen sources on TCC or TCS degradation

The effect of secondary carbon or nitrogen sources: glucose, succinic acid, CMC, sodium acetate, ammonium sulfate, ammonium nitrate, or urea on TCC biodegradation by *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46 were investigated.

The supplementation of additional carbon or nitrogen source did not significantly enhance the biodegradation of TCC biodegradation by all bacterial strains tested. However, adding of glucose clearly inhibited TCC biodegradation by MC22. The capability of MC22 to degrade TCC was decreased 73.15 % than that in MSM medium

alone (Table IV-2). The addition of each glucose as well as succinic acid has decreased the ability of MC35 to degrade TCC up to 78.43 and 43.14 %, respectively. For MC43 and MC46, TCC biodegradation was decreased 90 and 82 %, respectively in the supplementation of glucose. But in addition of urea, MC46 could enhance TCC degradation up to 24% of the MSM alone. Similar to TCC degrading bacterial strains, specific degradation rate of TCS by *Pseudomonas* sp. MS45 in addition of carbon and nitrogen sources mostly showed insignificant different. Only by the addition of urea, *Pseudomonas* sp. MS45 resulted a slight increase in specific degradation rate of TCS (up to 51%). In contrast, the addition of glucose declined the biodegradation efficiency of TCS by MS45 up to 83%.

Table IV-2 Effect of additional carbon or nitrogen source (each at 1 g L^{-1}) on TCC degradation at 30 μM by MC22, MC35, MC43, and MC46; and, - on TCS degradation at 40 μM by MS45.

Treatment	Specific deg. Rate (ng substrate h^{-1} mg cell $prot^{-1}$)				
	MC22	MC35	MC43	MC46	MS45
MSM only	1.49±0.09	0.51±0.04	0.74±0.12	1.88±0.06	7.39±1.48
Glucose	0.4±0.05	0.11±0.03	0.07±0.01	0.34±0.09	1.27±0.02
Succinic acid	1.26±0.14	0.29±0.07	0.18±0.02	1.4±0.07	8.65±0.68
CMC	1.62±0.24	0.37±0.02	0.19±0.02	0.62±0.05	7.28±0.29
Sodium acetate	1.09±0.21	0.62±0.16	0.39±0.01	1.48±0.03	6.63±0.35
Ammonium sulfate	1.43±0.07	0.57±0.08	0.33±0.04	1.86±0.08	6.03±0.61
Ammonium nitrate	1.77±0.03	0.78±0.07	0.43±0.01	2.08±0.04	8.3±0.82
Sodium nitrate	1.75±0.08	0.67±0.06	0.37±0.01	1.92±0.09	8.28±0.43
Urea	1.66±0.15	0.6±0.14	0.37±0.01	2.33±0.13	11.21±2.18

4.2.3 Cell growth and biodegradation kinetics

To fully recognise performance of bacterial characteristic and biodegradation, so bacterial growth kinetic and biodegradation kinetic under aerobic condition were determined at present study.

4.2.3.1 *Cell growth kinetics*

Kinetics of bacterial growth for TCC degradation by *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46 in the batch reactors were determined in terms of specific growth rate. Specific growth rates (μ, h−1) of individual bacterial strains at different TCC concentrations were calculated by Haldane inhibition model using non-linear regression as the following equation:

$$\mu = \frac{\mu_{max} S}{S + (S^2/K_i) + K_s}$$

The saturation constant (K_s), inhibition constant (K_i), and maximum specific growth rate (μ_{max}) were determined from the graph constructed in the correlation amongst specific growth rate and TCC or TCS concentrations (Table IV-3 and **Error! Reference source not found.**). The growth kinetic parameters of K_s, K_i, and μ_{max} provided by Haldane inhibition model of *Ochrobactrum* sp. MC22 were obtained at 5.7 ± 0.918 μM, 55.32 ± 8.062 μM, and 0.0.019 ± 0.0014 h^{-1}, respectively and R^2 = 0.9874. The growth kinetic parameters of K_s, K_i, and μ_{max} by *Ochrobactrum* sp. MC35 were found at 21.11 ± 10.03 μM, 27.87 ± 13.59 μM, and 0.037 ± 0.012 h^{-1}, respectively and R^2 = 0.9359. The growth kinetic parameters of K_s, K_i, and μ_{max} by *Sphingobacterium* sp. MC43 were achieved at 11.46 ± 3.709 μM, 27.79 ± 8.59 μM, and 0.079 ± 0.0153 h^{-1}, respectively and R^2 = 0.9649. While the K_s, K_i, and μ_{max} by *P. fluorescens* MC46 were achieved at 9.265 ± 5.005 μM, 43.22 ± 22.79 μM, and 0.046 ± 0.0133 h^{-1}, respectively and R^2 = 0.8803.

Table IV-3 TCC-dependent specific growth kinetics of MC22, MC35, MC43, and MC46 fitted with Haldane model.

TCC Concentration (uM)	**Specific Growth Rate ($h^{-1}.10^{-3}$)**			
	MC22	MC35	MC43	MC46
5	9.01±0.29	7.64±0.49	4.07±0.51	16.23±1.22
10	10.69±0.26	10.18±0.39	5.28±0.69	21.62±1.74
20	11.59±0.16	12.33±0.48	6.41±0.84	22.27±1.89
30	11.42±0.44	9.75±0.39	5.07±0.66	21.82±0.99
50	10.17±0.42	7.35±0.14	3.74±0.51	25.33±2.45
100	6.24±0.13	4.89±0.08	2.48±0.34	9.84±0.86

Table IV-4 TCS-dependent specific growth kinetics of MS45 fitted with Haldane model.

TCS Concentration (uM)	**Specific Growth Rate ($h^{-1}10^{-3}$)**
5	16.45±1.37
10	19.16±1.99
20	18.86±0.53
40	15.05±2.48
60	11.69±1.36

4.2.3.2 *Biodegradation kinetics*

Biodegradation kinetics of TCC was performed using Michaelis–Menten kinetics model with non-linear regression to fit the experimental data of various concentrations of TCC, as the following equation:

$$\frac{dS}{dt} = -V_{max}\frac{S}{S + K_s}$$

Where K_s is half saturation concentration and V_{max} is maximum specific biodegradation rate. As shown in Table IV-5 and IV-6, plots of initial concentrations against specific degradation rate resulted the values of K_s, V_{max} and V_{max}/K_s. The constant K_s is an indicator of the bacterial affinity for the substrate. Value of K_s (μM), V_{max} (nmole h^{-1} mg cell $protein^{-1}$), and V_{max}/K_s by *Ochrobactrum* sp. MC22 were determined at 6.03 ± 1.59, 5.3 ± 0.9, and 0.88±0.198, respectively with R^2 = 0.977. The value of K_s (μM), V_{max} (nmole h^{-1} mg cell $protein^{-1}$), and V_{max}/K_s by *Ochrobactrum* sp. MC35 were obtained at 4.45±1.42, 1.4±0.09, and 0.32±0.063, respectively. While value of K_s (μM), V_{max} (nmole h^{-1} mg cell $protein^{-1}$), and V_{max}/K_s by *Sphingobacterium* sp. MC43 were achieved at 18.78±4.63, 2.6±0.75, and 0.14±0.162, respectively. And value of K_s (μM), V_{max} (nmole h^{-1} mg cell $protein^{-1}$), and V_{max}/K_s by *P. fluorescens* MC46 were determined at 8.15±1.38, 6.4±0.25, and 0.78±0.178, respectively. While value of K_s (μM), V_{max} (nmole h^{-1} mg cell $protein^{-1}$), and V_{max}/K_s by *Pseudomonas* sp. MS45 were determined at 21.98±6.58, 11.8±1.46, and 0.54±0.221, respectively (Table 4.7). The ratio V_{max}/K_s admitted as the specific affinity is considered as a useful index for the enzymatic reaction.

Table IV-5 TCC biodegradation kinetics of MC22, MC35, MC43, and MC46 fitted with Michaelis-Menten kinetic model.

TCC Concentration (μM)	Specific degradation rate (nmole h^{-1} mg cell $prot^{-1}$)			
	MC22	MC35	MC43	MC46
5	2.31±0.147	0.91±0.074	0.84±0.042	1.97±0.063
10	2.65±0.039	0.89±0.015	0.93±0.054	2.11±0.032
20	4.86±0.104	1.21±0.249	1.26±0.131	2.42±0.204
30	5.14±0.739	1.03±0.117	1.58±0.393	2.79±0.153
50	4.27±0.056	1.23±0.134	2.04±0.243	3.36±0.059
100	4.64±0.064	1.62±0.083	2.34±0.513	3.01±0.221

Table IV-6 TCS biodegradation kinetics of MS45 fitted with Michaelis-Menten kinetic model.

TCS Concentration (μM)	Specific deg. Rate (nmole h^{-1} mg cell $prot^{-1}$)
5	2.41±0.212
10	3.67±0.529
20	5.36±0.595
40	7.87±0.158
60	8.56±2.687

4.2.4 Biodegradation pathway

Biodegradation pathway of the target pollutant is a necessary information to understand the fate of pollutant in environment, consequently the possible degradation pathway of TCC in liquid medium was proposed based on the HPLC and LC–MS analysis results. The LC/MS chromatogram of the cell-free medium sample amassed at 72 h throughout the time course biodegradation experiment with 30 μM of TCC from cultures of strain *Ochrobactrum* sp. MC22 or *P. fluorescens* MC46 for TCC degradation contained several possible intermediates. 3,4-Dichloroaniline (m/z = 161.99), 4-chloroaniline (m/z = 128.02) and aniline (m/z = 93.0) as the major intermediates were confirmed by mass spectra (Figure IV-7).

Figure *IV-7*).

A putative pathway for TCC biodegradation by *Ochrobactrum* sp. MC22 or *P. fluoresecens* MC46 was proposed (Figure IV-8) based on the LC/MS analysis. This pathway presented the hydrolysis of the urea bridge as the first degradation of TCC by *Ochrobactrum* sp. MC22 or *P. fluoresecens* MC46 to produce 4-chloroaniline and 3,4-dichloroaniline. By the process of dehalogenation and hydroxylation, 4-chloroaniline was converted to aniline. And aniline was then degraded by further metabolites.

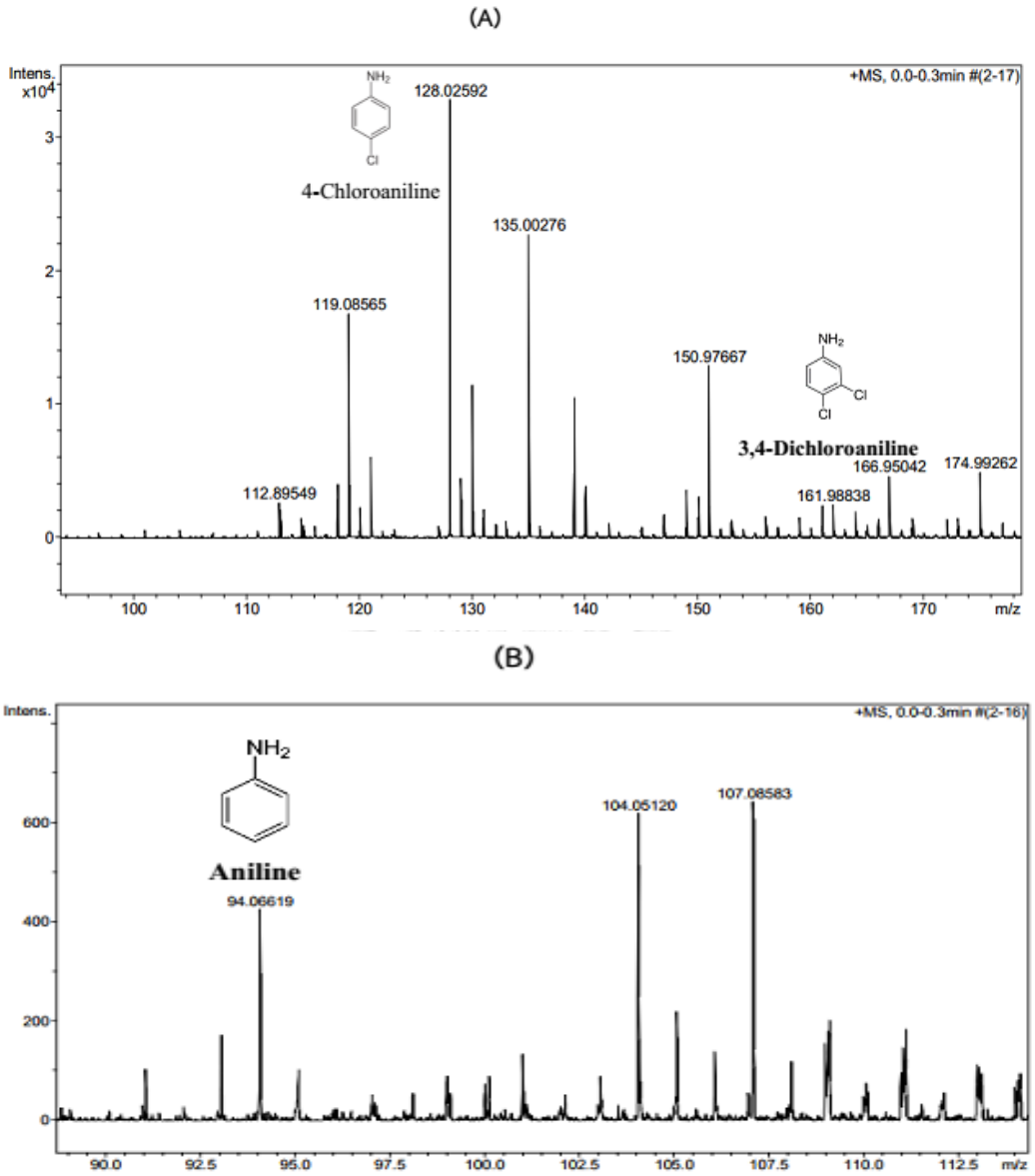


Figure IV-7 Metabolites detected during the degradation of 30 µM concentration of triclocarban were: A) 4-chloroaniline (MW = 128) and 3,4-dichloroaniline (MW = 162) produced by Ochrobactrum sp. MC22 or *P. fluorescens* MC46, and B) aniline (MW = 94) produced by *Ochrobactrum* sp. MC22. These metabolites were validated by Mass Spectrometry analysis.

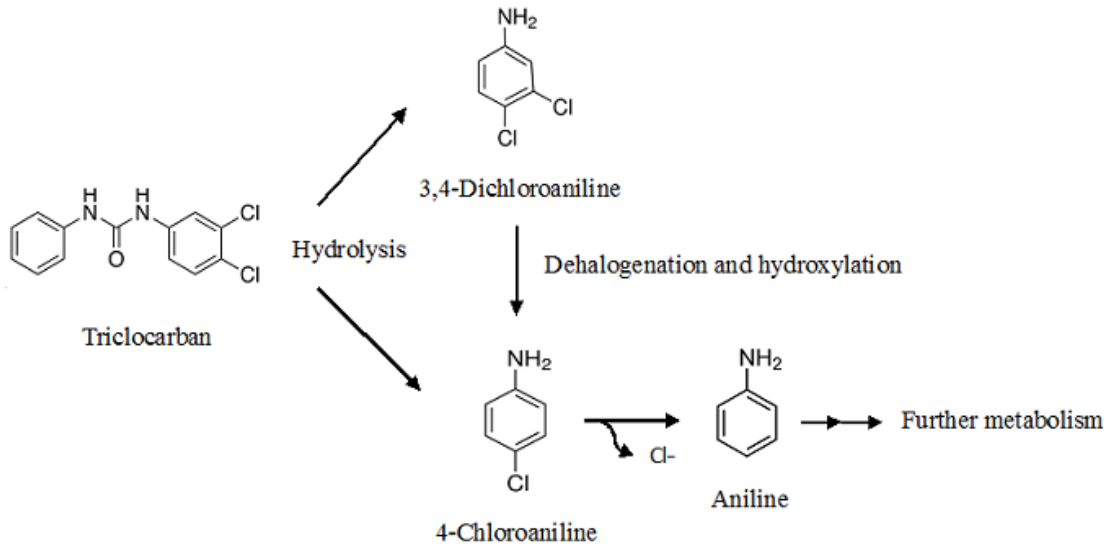


Figure IV-8 Biodegradation pathway for TCC by *Ochrobactrum* sp. MC22 or *P. fluorescens* MC46.

4.2.5 Biodegradation of TCC metabolites

As only *Ochrobactrum* sp. MC22 could revealed all the three metabolites during TCC biodegradation process, therefore, these metabolites biodegradation were just performed with *Ochrobactrum* sp. MC22 inoculation. Cells of MC22 were incubated in MSM medium containing each intermediate. After 6 d of incubation, MC22 was able to completely degraded 3,4-dichloroaniline, 4-chloroaniline, and aniline (Figure IV-9). This is confirming the ability of MC22 to metabolize all the intermediates formed during the biotransformation of TCC under aerobic condition.

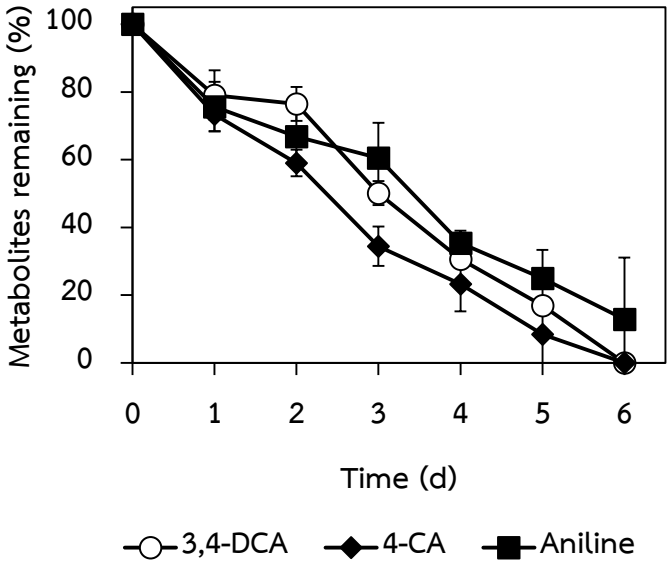


Figure IV-9 Degradation of 3,4-dichloroaniline, 4-chloroaniline, and aniline at 30 μM concentration by *Ochrobactrum* sp. MC22 under aerobic condition.

4.3 Test of plant growth promoting (PGP) traits

The ability of *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46 as TCC-degrading bacteria to restore and enhance soil fertility has been confirmed by their plant growth promoting activities involving P-solubilisation, production of organic acid, exo-polysaccharides (EPS), indole acetic acid (IAA), ammonia, and siderophores. All the TCC-degrading bacterial strains were found to have great potential in P-solubilisation in the absence and presence of stress condition. P-solubilisation treated in the presence of TCC was less but not significantly different than that in the absence of TCC. In present study, these TCC-degrading bacterial strains secreted gluconic, formic acids, and succinic acids as the most prominent organic acids both in the absence and presence of TCC. Additionally, the potential secretion of organic acids was not involved in presence of TCC. (**Error! Reference source not found.**7).

All the individual bacterial strains showed positive results for the EPS assayed mutually with and without the subjection of TCC. One strain, *Ochrobactrum* sp. MC22, showed a decline EPS production at the presence of TCC (25% reduced of the EPS production in the absence of TCC). Whilst the other strains had a higher EPS production ability in the presence of TCC stress condition (**Error! Reference source not found.**).

The influence of two concentrations of TCC on hormone IAA synthesized by the individual bacterial strains varied considerably. *Ochrobactrum* sp. MC22 produced the highest amount of IAA both in the absence and presence of TCC. *Ochrobactrum* sp. MC35 showed IAA production increased gradually with graded decline of TCC higher IAA in the higher concentration of TCC. *Whilst P. fluorescens* MC46 produced the lowest IAA both in the absence and presence of TCC (**Error! Reference source not found.**7).

Two strains, *Ochrobactrum* sp. MC22 and *P. fluorescens* sp. MC46, were able to yield salicylic acid (SA) and 2,3-dihydroxy benzoic acid (DHBA) type siderophores in the absence and presence of TCC. The siderophores were not produced by *Ochrobactrum* sp. MC35 and *Sphingobacterium* sp. MC43 mutually with and without the subjection of TCC (**Error! Reference source not found.**7).

Table IV-7 Plant growth promoting activities of TCC-degrading bacterial strain in the presence of varying concentration of

Strain	Treatments	Phosphate solubilized		EPS ($\mu g\ mL^{-1}$)	IAA ($\mu g\ mL^{-1}$)	Siderophore		Organic acid production		
		Liquid medium ($\mu g\ mL^{-1}$)	Final pH			SA ($\mu g\ mL^{-1}$)	DHBA ($\mu g\ mL^{-1}$)	Gluconic Acid ($\mu g\ mL^{-1}$)	Succinic Acid ($\mu g\ mL^{-1}$)	Formic Acid ($\mu g\ mL^{-1}$)
MC22	Control	137.7 ± 0.25^{a}	2.61^{a}	54.5 ± 4.9^{a}	22 ± 0.89^{a}	29 ± 1.3^{a}	10 ± 3.6^{a}	4.3 ± 0.14^{a}	9.2 ± 0.11^{a}	-
	TCC (30 μM)	130.6 ± 3.34^{b}	2.72^{a}	45.5 ± 2.1^{b}	24 ± 0.88^{b}	27 ± 2.7^{b}	9 ± 0.4^{a}	4.9 ± 0.14^{b}	9.4 ± 0.18^{a}	-
	TCC (50 μM)	121.92 ± 10.46^{c}	3.26^{b}	41 ± 2.8^{c}	25 ± 0.13^{b}	26 ± 2.1^{c}	8 ± 2.4^{b}	4.4 ± 0.25^{a}	9.7 ± 0.06^{b}	-
MC35	Control	131.9 ± 2.27^{a}	2.71^{a}	19.5 ± 2.1^{a}	15.5 ± 0.39^{a}	-	-	4.6 ± 0.25^{a}	9.4 ± 0.19a	-
	TCC (30 μM)	125.1 ± 1.39^{a}	3.35^{b}	28.5 ± 2.1^{b}	18.4 ± 0.33^{b}	-	-	4.7 ± 0.41^{a}	9.8 ± 0.33a	-
	TCC (50 μM)	107.2 ± 5.91^{b}	2.77^{c}	35.5 ± 4.9^{c}	26.9 ± 0.77^{c}	-	-	4.8 ± 0.42^{a}	-	0.9 ± 0.18
MC43	Control	148.5 ± 0.89^{a}	4.56^{a}	44.5 ± 3.5^{a}	15.2 ± 0.26^{a}	-	-	-	-	10.3 ± 0.29a
	TCC (30 μM)	139.2 ± 7.15^{b}	3.85^{b}	43 ± 1.41^{a}	15.1 ± 0.22^{a}	-	-	5.9 ± 0.12	-	4.4 ± 0.18
	TCC (50 μM)	133.2 ± 1.46^{c}	2.77^{c}	24.5 ± 3.5^{b}	14.9 ± 0.34^{a}	-	-	8.4 ± 0.26	-	-
MC46	Control	89.2 ± 4.9^{a}	2.62^{a}	44 ± 2.8^{a}	5.1 ± 0.2^{a}	33.9 ± 1.9^{a}	14.8 ± 0.6^{a}	5 ± 0.17^{a}	-	-
	TCC (30 μM)	95.7 ± 5.8^{b}	2.67^{a}	34 ± 2.8^{b}	5.2 ± 0.3^{a}	34 ± 1.9^{b}	13.9 ± 0.9^{a}	5.3 ± 0.10^{a}	-	-
	TCC (50 μM)	84.1 ± 4.6^{c}	3.04^{b}	23 ± 2.1^{c}	4.7 ± 0.2^{a}	31 ± 1.6^{c}	12.6 ± 0.8^{b}	5.2 ± 0.16^{a}	-	-

4.4 Toxicity study of TCC and its degradation metabolites

To achieve the ultimate goal of pollutant biodegradation work, that is reducing the whole toxicity affected by the parental pollutant or toxic byproducts produced during the biodegradation, henceforth toxicity study is compelled as an ultimate assessment for threat assurance. The current study performed three toxicity assessment techniques to guarantee the no or less toxicity of TCC and the aerobic degraded metabolites by *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46.

4.4.1 Phytotoxicity assessment

Legume seeds of *Vigna radiata* and *Crotalaria juncea* L. were selected for the toxicity test of TCC and the aerobic degraded metabolites of *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46 (at 7 d of degradation period) at each concentration of 30 and 50 µM. After 48 h incubation, the seeds treated with 30 and 50 µM concentration of TCC were found significantly different to the control with distilled water and the degraded metabolites (

Figure IV-10). The maximum sprouting length treated with distilled water (control) were of 2.2 ± 0.15 cm, 2.58 ± 0.08 cm for *V. radiata* and *C. juncea* seeds, respectively. TCC at 30 and 50 µM concentrations inhibited the sprout elongation of *V. radiata* about 46% and 57% and *C. juncea* about 55% and 64%, respectively. This results reinforced that TCC even at low concentration has significant toxicity to plants, thus the aerobic biological treatment with TCC-degrading bacterial strains clearly reduced the toxicity effect to the test plants.

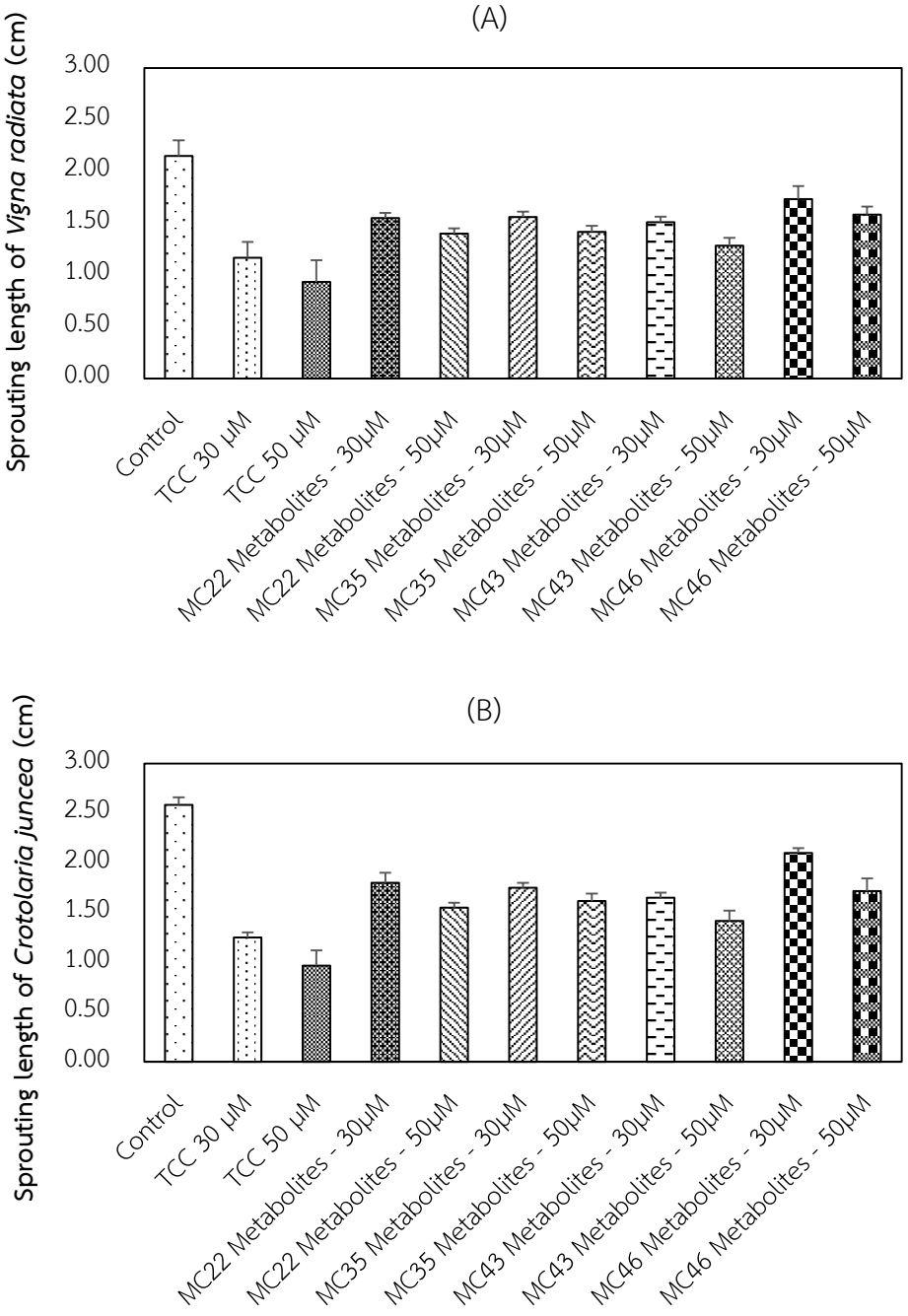


Figure IV-10 Phytotoxicity test of TCC at 30 µM and 50 µM and degraded metabolites with (A) *V. radiata* and (B) *C. juncea* L.

4.4.2 Cytogenotoxicity assessment

The chromosomal aberration percentage and the mitotic indexes of *A. cepa* meristematic cells exposed to TCC and its degraded metabolites were presented in Table 3.1. Mitotic index (MI) % was estimated as number of dividing cells per total number of cells observed × 100. Aberration Index (AI) % was assessed as number of damage cells per total number of cells observed x 100. Low percent of mitotic indexes as 7.9 ± 0.32% and 7.0 ± 0.28% and high aberration indexes as 1.05 ± 0.07% and 1.27 ± 0.05% were detected on *A. cepa* meristematic cells subjected to each 30 and 50 µM concentrations of TCC. The cytotoxicity effect by TCC at the two concentrations tested in which the MI value of the root tip cells was diminished from that in the distilled water (control). The mitotic index decreased as the concentration of TCC increased (**Error! Reference source not found.**8).

The cells treated with degraded metabolites were found close to the cells exposed with distilled water (control). Additionally, representation of chromosomes morphology in the *A. cepa* root tips subjected to distilled water, TCC, and the degraded metabolites were also investigated (Figure IV-11). Several types of chromosome aberrations i.e. polyploid interphase, disoriented metaphase, laggards and spindle at metaphase, anaphase with chromosomal losses, anaphase with chromosomal bridge, extended interphase, prolonged and abnormal kinetics at prophase, chromosomal mis-segregation, chromosome breaks at anaphase, and apoptosis were recorded as a result of the exposure TCC (30 and 50 µM). Spindle abnormality and polar deviation, such as multipolarity which is typical of cancer cell and can lead to abnormal kinetics of chromosome and prolonged prophase, are detected in TCC treated cells.

Insignificant aberrations with chromosomal destruction were mostly detected in cell stages of *A. cepa* treated with degraded metabolites. Meristematic root tips subjected with distilled water (as control) displayed normal cell structures.

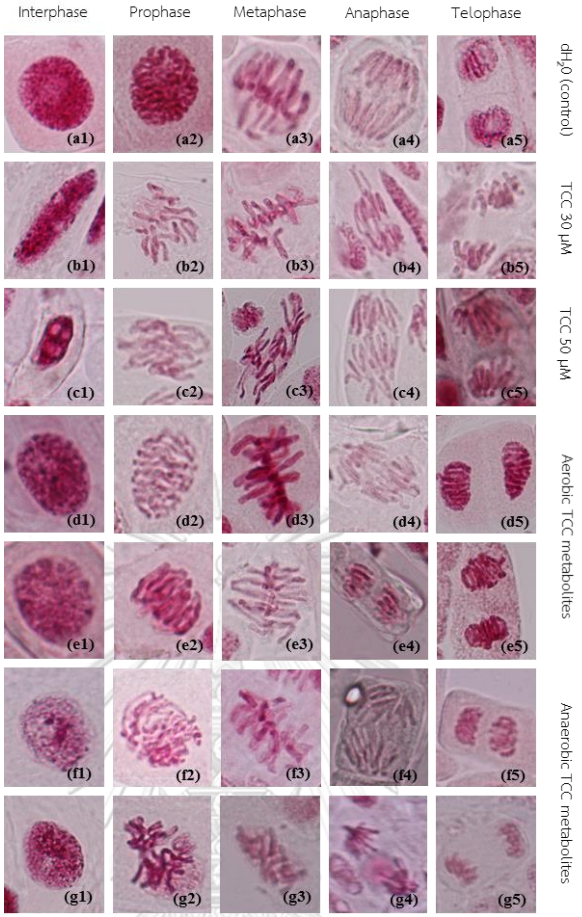


Figure IV-11 Cytogenotoxicity assessment of TCC and its degraded intermediates in meristematic cells of *A. cepa*. *A. cepa* roots were exposed with the following test solutions: (a1–a5) distilled water; (b1–b5) 30 µM TCC; (c1-c5) 50 µM, (d1-e5) aerobic degraded metabolites, and (f1-g5) anaerobic degraded metabolites collected at 3 and 14 days, respectively.

Table IV-8 Mitotic indexes and chromosomal aberration index tested in *A.llium cepa* root tip cells exposed to TCC, the degraded metabolites and distilled water (as control).

Treatment	Concentration of the compound (μM)	No. of dividing cells	Mitotic Index (MI) %	Aberration Index (AI) %	Chromosomal aberrations					
					ML	MC	MA	AB	AL	CB
Distilled water (control)		191[a]	18.2 ± 0.9[a]	0.06 ± 0.03[a]	-	-	-	-	-	-
TCC	30	98[b]	9.4 ± 04[b]	1.05 ± 0.07[b]	+	-	-	+	-	+
	50	87[b]	8.3 ± 0.3[b]	1.27 ± 0.05[b]	-	+	+	-	+	+
Degraded metabolites of MC22	30	133[c]	12.6 ± 0.3[c]	0.39 ± 0.09[c]	+	-	-	-	-	-
	50	121[b]	11.5 ± 0.2[b]	0.53 ± 0.11[d]	-	-	+	-	-	+
Degraded metabolites of MC35	30	127[d]	10.1 ± 0.4[d]	0.58 ± 0.06[c]	-	-	+	-	-	-
	50	115[c]	9.2 ± 0.37[c]	0.71 ± 0.07[c]	+	-	+	-	+	-
Degraded metabolites of MC43	30	122[d]	9.8 ± 0.35[d]	0.56 ± 0.04[c]	-	+	-	-	+	-
	50	107[c]	8.6 ± 0.42[d]	0.68 ± 0.03[c]	-	+	+	-	+	-
Degraded metabolites of MC46	30	146[c]	11.7 ± 0.5[c]	0.22 ± 0.03[c]	-	-	-	+	-	-
	50	125[b]	10 ± 0.47[b]	0.45 ± 0.08[d]	+	+	-	-	-	-

Chromosomal aberration per 1050 cells. ML: metaphase lagging chromosome; MC: metaphase cluster; MA: metaphase aberration;

AB: anaphase bridges; AL: anaphase lagging chromosome, CB: chromosome breaks. Error bar represents the standard deviation of the triplicates.

4.5 Growth chamber experiments

The present study just focused on investigation of whether TCC-degrading bacteria (*Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46) can mitigate TCC harmfulness to *V. radiata* and promote plant growth in TCC contaminated environments. The *V. radiata* seedlings were placed into Hoagland's solution containing individual bacterial strains. The investigation of TCS-degrading bacterium MS45 to mitigate TCS toxicity to *V. radiata* is not performed due to TCS has no effect on the emergence and growth crop species (Prosser et al., 2014).

4.5.1 TCC degradation under growth chamber experiments

Under hydroponic conditions, all the TCC-degrading bacterial strains promoted TCC degradation in the contaminated water. Among all the TCC-degrading bacterial strains, *P. fluorescens* MC46 was the superlative strain to mitigate TCC harmfulness to *V. radiata* and it could grow in the TCC contaminated water much better than others (Figure IV-12).

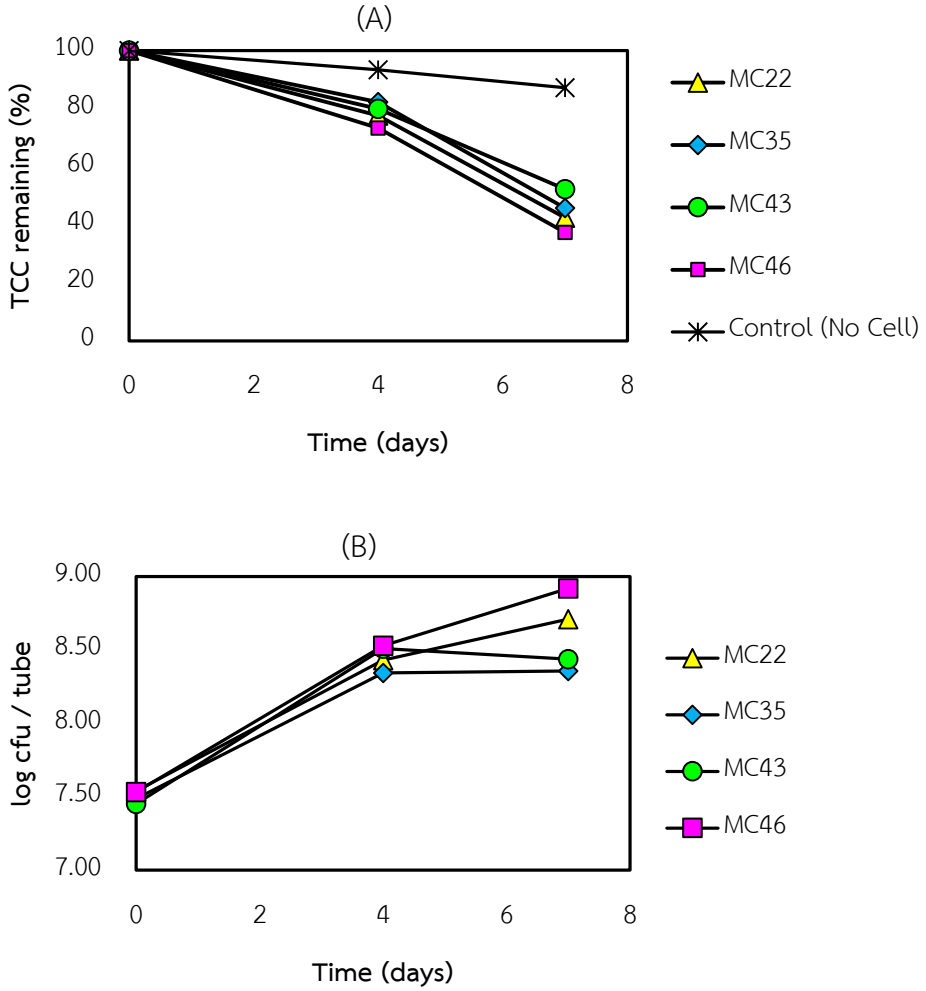


Figure IV-12 TCC degradation (30 μM) and cell growth on *V. radiata* by individual bacterial strain under hydroponic condition (one tenth Hoagland solution) measured after 7-d of incubation.

4.5.2 Plant growth and root tissues analysis

1) Plant biomass, plant length, and root length

As shown in Figure IV-13, *V. radiata* seeds grown in Hoagland's solution amended with TCC together with PGP bacterial strains showed well growth compared to non-inoculated seeds. On the contrary, an extensive shrinkage in the growth of *V. radiata* was detected with the application of TCC alone. A decrease in the plant weight, plant length, and root length of *V. radiata* was noted in the presence of TCC by 53%,

28%, and 44%, respectively. However, the inoculation of individual TCC-degrading bacteria in the subjected with TCC increased the plant weight, plant length, and root length of *V. radiata* by 62-78%, 16-20%, and 29-49%. *P. fluorescens* MC46 had the highest positive impact in promoting *V. radiata* growth over course of the test, indicating that *P. fluorescens* MC46 was an active bacterium. Moreover, the improvement of plant growth is associated with the reducing of TCC absorption by *V. radiata*.

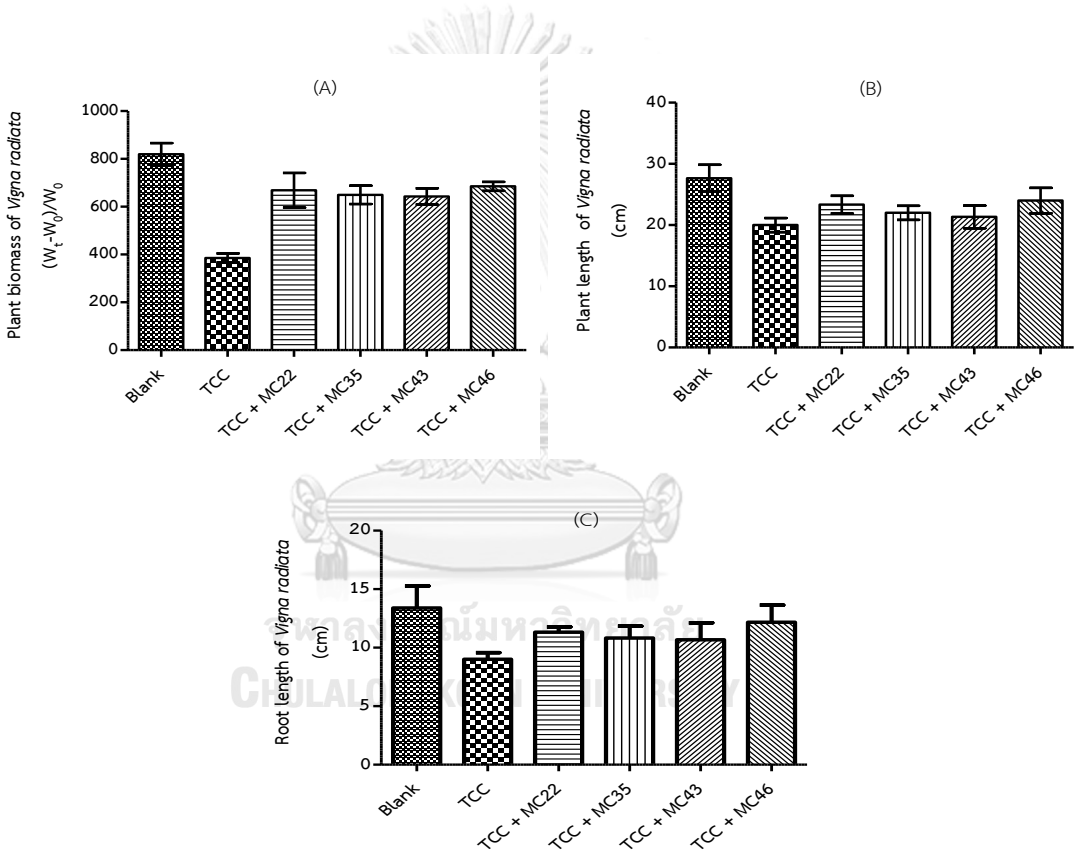


Figure IV-13 Plant characteristics of *V. radiata*: A) plant biomass, B) plant length, and C) root length measured after 7-d of incubation.

2) Cross section area, total stele area, and ylem vessel area

Changes in the sizes of root cross section area, total stele area, and xylem vessel area were also detected in the roots of TCC - *V. radiata* indulged. Anatomical study revealed the decreased root cross section area, total stele area, and xylem vessel area by 19 %, 33%, and 44% in seedlings subjected with TCC alone. However, the symptoms of TCC toxicity were consecutively declined in the roots of *V. radiata* cultivated by bacterial strains. Among of the strains, *P. fluorescens* MC46 was observed as best strain to enhance the root cross section area, total stele area, and xylem vessel area which is consistent with the control (blank) as shown in Figure IV-14.

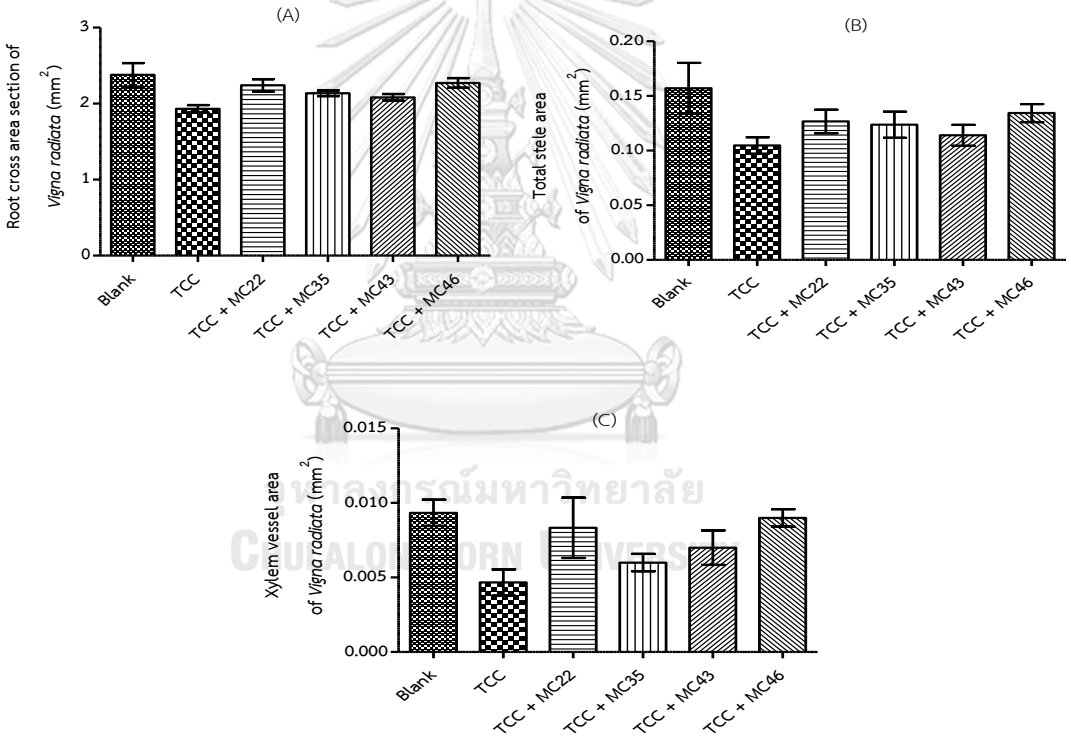


Figure IV-14 Root cross section area (A), total stele area (B), and xylem vessel area (C) of *V. radiata* root. Root was measured after 7-d of incubation and cut approximately 1.5 cm from the apex.

4.5.3 Colonization of bacterial strain on the plant root

Experiments of bacteria colonization on plant root showed that the population of the TCC-degrading bacteria remained relatively constant in the TCC–hoagland's medium during the 7 day experiments. These experiments confirmed TCC detoxification by TCC-degrading bacteria under the symbiotic condition with *V. radiata*. All TCC-degrading bacteria could colonize plant root surfaces with high density on the root surface zone. The root surface was much heavier colonized by *Ochrobactrum* sp. MC22 and *P. fluorescens* MC46 than other two bacterial strains (Figure IV-15).

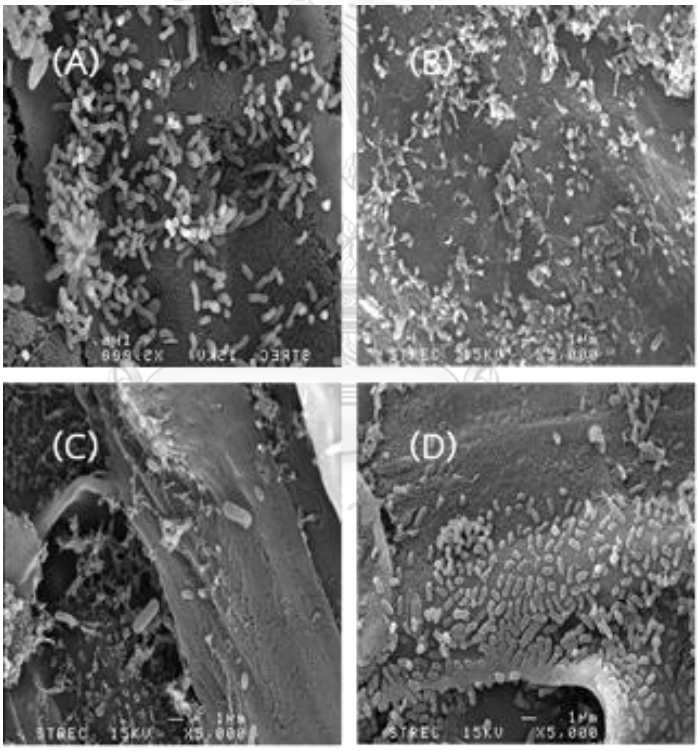


Figure IV-15 Scanning electron micrographs showing the colonization of A) *Ochrobactrum* sp. MC22, B) *Ochrobactrum* sp. MC35, C) *Sphingobacterium* sp. MC43, and D) *P. fluorescens* MC46 on the root surface of the *V. radiata* grown for 7 days in a haft-strength Hoagland's nutrient solution subjected with 30 µM TCC.

4.6 Formulation of TCC-degrading PGP bacterial strain

Considering results from growth chamber experiments above expressed that MC46 was the most affirmative strain to TCC biodegradation and plant growth characteristics. It expressed potential use of *P. fluorescens* MC46 as a bioaugmented culture for TCC contaminated soil treatment, thus further studies were performed to ensure the effectiveness of *P. fluorescens* MC46 when applied to soil. Therefore, *P. fluorescens* MC46 inoculant is formulated, and evaluated for its viability and activity after storage and in pot soil experiments. At present study, sawdust was selected as a solid carrier because of its high water-holding capacity and high surface area for the colonization of large number of bacterial population. To this formula, molasses was also supplemented as a low-cost carbon source.

4.6.1 Shelf life of formulated bacterial stain

The application of different polymer additives (PEG 1%, PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, gum arabic 0.5%, and gum arabic 1%) to the sawdust based formulation caused no inhibition to the growth of *P. fluorescens* MC46. The growth of *P. fluorescens* MC46 was well maintained until at least 8-week storage, and no antagonist was detected. The survival of *P. fluorescens* MC46 estimated after 20 weeks in the sawdust carrier, was 10.3 log cfu g^{-1} material with CMC+starch 1% additive at 6-week storage. At the same time, the survival rate in other additives was only 8.65 to 9.62 log cfu g^{-1} material. The populations of *P. fluorescens* MC46 started decreasing after 10 weeks, and maximum decreased was found after 12-week storage: 10 % decreased in addition of CMC+starch 1%; 15% decreased in PEG 1%, PVP 1%, and PVP 5%, 18% decreased in PEG 5% and CMC+starch 2%; 19% decreased in gum Arabic 0.5%; and 21% decreased in gum Arabic 1%. Nonetheless, after 20-weeks storage, the populations of *P. fluorescens* MC46 was found minimal reduced in PEG 1% (8% only), whereas in PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, gum arabic

0.5%, and gum arabic 1% was 21%, 15%, 22%, 20%, 15%, 18%, and 24%, respectively (**Error! Reference source not found.**9).

Table IV-9 Shelf life of *P. fluorescens* MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with different additives (PEG 1%, PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, Gum arabic 0.5%, and Gum arabic 1%). Colony is in TCC-containing MSMY agar plates.

Formulation	Log No of CFU g^{-1} inoculant										
	Week-0	Week-1	Week-2	Week-3	Week-4	Week-6	Week-8	Week-10	Week-12	Week-16	Week-20
PEG-1%	8.77±0.14	9.03±0.17	9.11±0.47	9.64±0.65	9.32±0.56	9.37±0.47	9.42±0.42	8.61±0.34	7.41±0.39	8.49±0.43	8.34±0.51
PEG-5%	8.61±0.15	9.02±0.24	9.06±0.21	9.26±0.35	9.23±0.31	9.24±0.32	8.76±0.45	8.32±0.23	7.02±0.31	7.32±0.52	7.19±0.48
PVP-1%	8.52±0.12	9.86±0.06	9.43±0.51	9.82±0.44	9.52±0.21	9.49±0.08	9.39±0.65	8.38±0.34	7.27±0.27	8.22±0.41	8.03±0.42
PVP-5%	8.51±0.21	10.12±0.11	10.23±0.07	10.06±0.41	9.63±0.52	9.62±0.43	9.27±0.55	8.28±0.24	7.25±0.43	8.19±0.35	8.02±0.31
CMC+starch-1%	8.49±0.13	9.95±0.09	9.89±0.18	10.22±0.71	10.23±0.31	10.32±0.37	9.66±0.55	8.55±0.43	7.63±0.41	8.21±0.29	7.95±0.21
CMC+starch-2%	8.58±0.12	9.51±0.22	9.34±0.56	9.87±0.45	8.67±0.42	8.65±0.41	8.84±0.45	8.35±0.38	7.01±0.46	8.04±0.23	7.97±0.26
Gum arabic-0.5%	8.66±0.17	9.24±0.06	8.92±0.24	9.12±0.41	8.87±0.31	8.88±0.41	9.01±0.62	7.96±0.29	7.01±0.33	7.43±0.39	7.32±0.54
Gum arabic-1%	8.86±0.13	9.04±0.17	9.68±0.34	10.06±0.55	9.91±0.53	9.61±0.53	9.47±0.61	8.01±0.22	7.03±0.18	7.43±0.39	7.33±0.41

4.6.2 TCC degradation by formulated bacterial strain

In growth dependent reaction, degradation rates of TCC by formulated *P. fluorescens* MC46 at 0-week storage in PEG 1%, PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, gum arabic 0.5%, and gum arabic 1% were found to be 0.25 μM h^{-1}, 0.22 μM h^{-1}, 0.2 μM h^{-1}, 0.32 μM h^{-1}, 0.34 μM h^{-1}, 0.19 μM h^{-1}, 0.22 μM h^{-1}, 0.19 μM h^{-1}, respectively. Biodegradation rates of TCC by formulated *P. fluorescens* MC46 at 0-week storage in PEG 1%, PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, gum arabic 0.5%, and gum arabic 1% were found to be 0.23 μM h^{-1}, 0.22 μM h^{-1}, 0.15 μM h^{-1}, 0.16 μM h^{-1}, 0.23 μM h^{-1}, 0.19 μM h^{-1}, 0.17 μM h^{-1}, 0.17 μM h^{-1}, respectively. And biodegradation rates of TCC by formulated *P. fluorescens* MC46 at 0-week storage in PEG 1%, PEG 5%, PVP 1%, PVP 5%, CMC+starch 1%, CMC+starch 2%, gum arabic 0.5%, and gum arabic 1% were found to be 0.26 μM h^{-1}, 0.2 μM h^{-1}, 0.2 μM h^{-1}, 0.2 μM h^{-1}, 0.27 μM h^{-1}, 0.2 μM h^{-1}, 0.18 μM h^{-1}, 0.18 μM h^{-1}, respectively (data not shown). Whereas, the degradation rate of TCC by unformulated *P. fluorescens* MC46 was calculated only 0.15 μM h^{-1} (Figure IV-16).

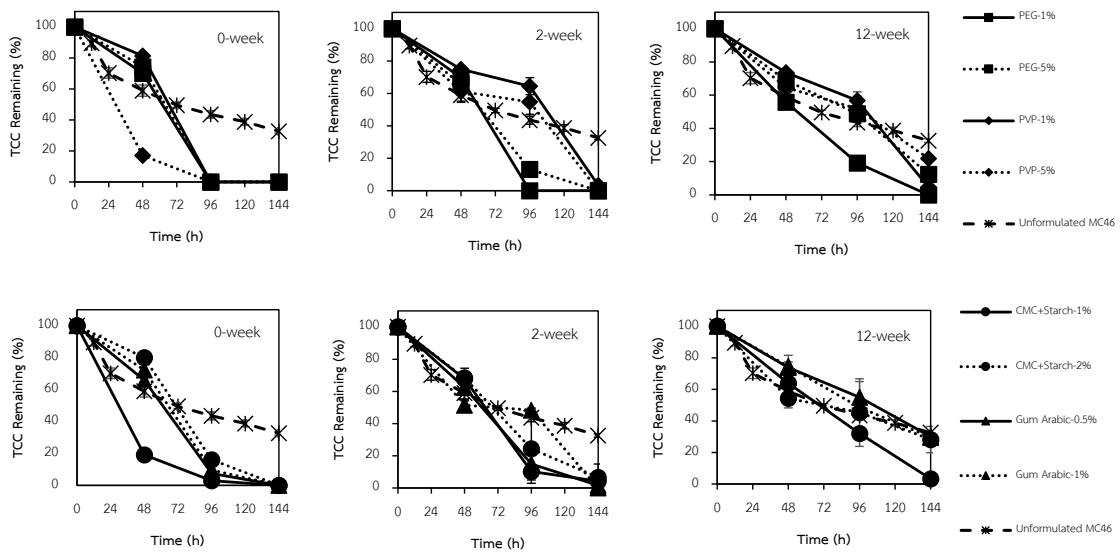


Figure IV-16 TCC degradation with an initial TCC concentration of 30 μM by formulated *P. fluorescens* MC46 at 0-week, 2-week, and 12-week storages conducted in MSM liquid medium.

4.7 Pot soil experiments of formulated TCC-degrading PGP bacterial strain

4.7.1 TCC degradation under pot soil experiments

At present study, we compared the TCC biodegradation activity of native soil microorganisms in a TCC-spiked soil to bioremediation of the same soil bioaugmented with formulated *P. fluorescens* MC46. TCC biodegradation in soil was observed since the first week of the incubation in the inoculation of formulated *P. fluorescens* MC46 both in PEG 1% additive (SS+TCC+F1) and CMC + Starch 1% additive (SS+TCC+F2). Even though TCC biodegradation rate of SS+TCC+F1 (0.09 μM h^{-1}) and SS+TCC+F2 (0.08 μM h^{-1}) in pot soil experiments was 3-fold lesser than TCC biodegradation rate in the liquid MSM medium by *P. fluorescens* MC46 formulated either in PEG 1% additive (F1) or *P. fluorescens* MC46 formulated in CMC+starch 1% additive (F2), however, the biodegradation rate either in SS+TCC+F1 or SS+TCC+F2 was 2-fold higher than the biodegradation rates in the uninoculated soils (S+TCC, SS+TCC, SS+TCC+UF1, and

SS+TCC+UF1). Total TCC could be degraded in SS+TCC+F1 and SS+TCC+F2 was 76% and 74%, respectively. In contrast, TCC only could be degraded 32% to 39 % in soils without inoculation of *P. fluorescens* MC46 at 5 weeks incubation time. Moreover, TCC degradation in addition of formulation materials to the soils did not clearly different with that in normal soil (Figure IV-17).

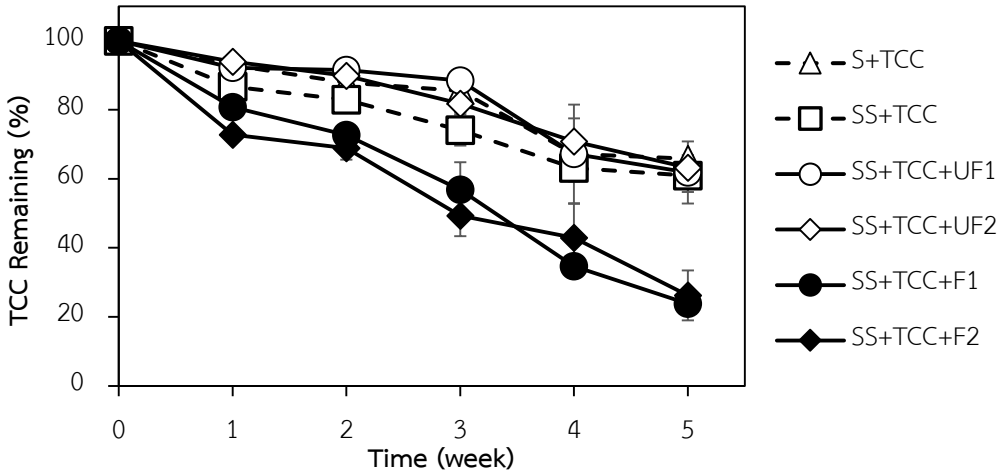


Figure IV-17 TCC degradation by formulated *P. fluorescens* MC46 in pot soil experiments with *V. radiata*. Soil was mixed with 100 µM TCC prior to use. S = soil; SS = soil and seeds; TCC = triclocarban 100 µM; F1 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive PEG 1%; F2 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive CMC+starch 1%; UF1 = uninoculated F1 (without MC46); and UF2 = uninoculated F2 (without MC46).

4.7.2 Soil enzyme activities

None of the enzyme activities tested appeared to be negatively affected by the inoculation of the *P. fluorescens* MC46. On the contrary, most enzyme activities yielded significant decreased due to TCC (**Error! Reference source not found.**). Dehydrogenase, CM-Cellulase, acid phosphatase and alkaline phosphatase activities were always lower in the TCC treated soils, whereas protease and urease activities

were not significantly influenced by TCC (Table IV-10). Normal (uninoculated without TCC) soil produced protease activity 153 µg Tyrosine g^{-1} soil $2h^{-1}$, 154 µg Tyrosine g^{-1} soil $2h^{-1}$, and 179 µg Tyrosine g^{-1} soil $2h^{-1}$ at 0-week, 2-week, and 5-week incubation, respectively. The addition of *P. fluorescens* MC46 enhanced protease activity of soil without TCC by 23% and 40% at 2-week and 5-week experiment, respectively. Moreover, *P. fluorescens* MC46 also enhanced the protease activity of soil with TCC by 28% and 51% at 2-week and 5-week incubation, respectively (**Error! Reference source not found.**).

Soil inoculated with *P. fluorescens* MC46 enhanced urease activity of soil without TCC by 26% and 33% at 2-week and 5-week experiment, respectively (**Error! Reference source not found.**). Normal soil inoculated with *P. fluorescens* MC46 showed 21% higher of urease activity than that in the uninoculated one.

The amount of dehydrogenase activity produced in soil inoculated with *P. fluorescens* MC46 without TCC was increased 2-fold from 0-week to 5-week incubation in soil without TCC. Soil inoculated with *P. fluorescens* MC46 enhanced dehydrogenase activity of soil with TCC by 87% after 5-week incubation (**Error! Reference source not found.**). Additionally, CM-Cellulase activity rose in soil inoculated with *P. fluorescens* MC46 without TCC (∽ 25%) over the normal soil. By contrast, the results showed that TCC decreased CM-Cellulase activity by 51% after 5-week experiment. However, *P. fluorescens* MC46 exhibited CM-Cellulase activity 2-fold higher than that in uninoculated soil (**Error! Reference source not found.**).

The results showed that activity of acid phosphatase was 2-fold superior than the activity of alkaline phosphatasey. After 5 weeks of incubation, acid phosphatase was notably stimulated and increased by 46% in soil inoculated with *P. fluorescens* MC46 without TCC. In contrast, TCC drastically decreased the acid phosphatase by 62% (**Error! Reference source not found.**). Similarly, activities for alkaline phosphatase enzyme were successively greater in soil inoculated with *P. fluorescens*

MC46 without TCC by 42% than that in normal soil. On the contrary, activity of alkaline phosphatase enzyme was 61% inhibited in the addition of TCC after 5-week experiment. However, *P. fluorescens* MC46 enhanced 85% the activity of alkaline phosphatase enzyme, as compared to the uninoculated soil with TCC as shown in **Error! Reference source not found.**10.

Table IV-10 Effects of soil treatments on soil enzyme activities. SS = soil and seeds; F1 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive PEG 1%; F2 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive CMC + Starch 1%; TCC = triclocarban 100 µM, UF1 = uninoculated F1 (without MC46); UF2 = uninoculated F2 (MC46).

Treatment	**Pretease (ug Tyrosine g soil $2h^{-1}$)**	**Urease (ug N g soil $2h^{-1}$)**	**Dehydrogenase (ug TPF soil $16h^{-1}$)**	**CMC-Cellulase (ug Glucose g soil $24h^{-1}$)**	Acid phosphatase (ug pNP g soil h^{-1})	Alkaline phosphatase (ug pNP g soil h^{-1})
SS 0-week	153±6	88±5	165±7	33±1	70±2	24±1
SS-F1 0-week	135±4	88±3	136±8	32±1	69±3	25±1
SS-F2 0-week	142±7	88±2	144±6	33±1	71±2	25±2
SS-TCC 0-week	147±1	91±1	157±7	31±1	71±6	26±1
SS-TCC-UF1 0-week	149±9	96±1	161±5	30±2	68±2	22±2
SS-TCC-UF2 0-week	149±6	95±1	165±12	31±1	73±4	23±1
SS-TCC-F1 0-week	151±6	91±2	130±4	29±1	75±4	26±2
SS-TCC-F2 0-week	153±3	98±1	135±12	28±0	64±1	29±2
SS 2-week	154±5	89±3	188±18	35±1	83±10	33±3
SS-F1 2-week	189±4	112±2	200±14	44±2	98±15	46±2

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Treatment	**Pretease (ug Tyrosine g soil $2h^{-1}$)**	**Urease (ug N g soil $2h^{-1}$)**	**Dehydrogenase (ug TPF soil $16h^{-1}$)**	**CMC-Cellulase (ug Glucose g soil $24h^{-1}$)**	Acid phosphatase (ug pNP g soil h^{-1})	Alkaline phosphatase (ug pNP g soil h^{-1})
SS-F2 2-week	183±1	105±3	203±6	44±2	98±12	47±2
SS-TCC 2-week	127±2	88±5	164±7	25±0	65±6	22±1
SS-TCC-UF1 2-week	149±1	92±3	176±11	26±0	61±5	19±2
SS-TCC-UF2 2-week	143±12	92±8	179±8	25±1	65±7	18±4
SS-TCC-F1 2-week	162±5	108±2	174±10	34±2	85±15	31±2
SS-TCC-F2 2-week	161±3	102±3	176±5	34±1	88±14	34±1

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Treatment	**Pretease (ug Tyrosine g soil $2h^{-1}$)**	**Urease (ug N g soil $2h^{-1}$)**	**Dehydrogenase (ug TPF soil $16h^{-1}$)**	**CMC-Cellulase (ug Glucose g soil $24h^{-1}$)**	Acid phosphatase (ug pNP g soil h^{-1})	Alkaline phosphatase (ug pNP g soil h^{-1})
SS 5-week	179±15	96±1	239±7	45±1	107±6	52±2
SS-F1 5-week	251±5	128±5	289±8	52±2	151±8	62±3
SS-F2 5-week	245±1	121±8	279±3	53±1	156±9	60±4
SS-TCC 5-week	135±8	75±1	103±9	16±1	41±3	20±2
SS-TCC-UF1 5-week	167±7	77±1	104±0	19±1	40±5	17±1
SS-TCC-UF2 5-week	165±3	79±1	107±4	19±1	36±1	17±1
SS-TCC-F1 5-week	204±8	115±5	193±8	31±2	95±7	37±3
SS-TCC-F2 5-week	191±11	109±8	180±2	32±1	100±7	35±4

4.7.3 Plant growth analysis and chlorophyll content

Results demonstrated that the *V. radiata* seeds cultivated in TCC contaminated soil with formulated *P. fluorescens* MC46 showed improved growth compared to non-inoculated plants (Figure IV-18). On the contrary, a significant decrease in the growth of *V. radiata* was detected in the forlomTCC contaminated soil. A decrease in the plant biomass by 27%, plant height by 29%, root length by 38%, root cross section area by 42%, number of leaves by 39%, was observed in the presence of TCC. Similarly, reduction in the chlorophyll-a by 39%, chlorophyll-b by 33%, and total carotene by 38%, was also observed in the presence of TCC. However, the inoculation of formulated *P. fluorescens* MC46 increased the plant biomass by 39%, plant height by 38%, root length by 52%, root cross section area by 65%, number of leaves by 58%, in the presence of TCC. In addition, clearly increase in the chlorophyll-a content by 73%, chlorophyll-b content by 50%, and total carotene of leaves by 63%, was also observed upon application of formulated *P. fluorescens* MC46 in the presence of TCC.

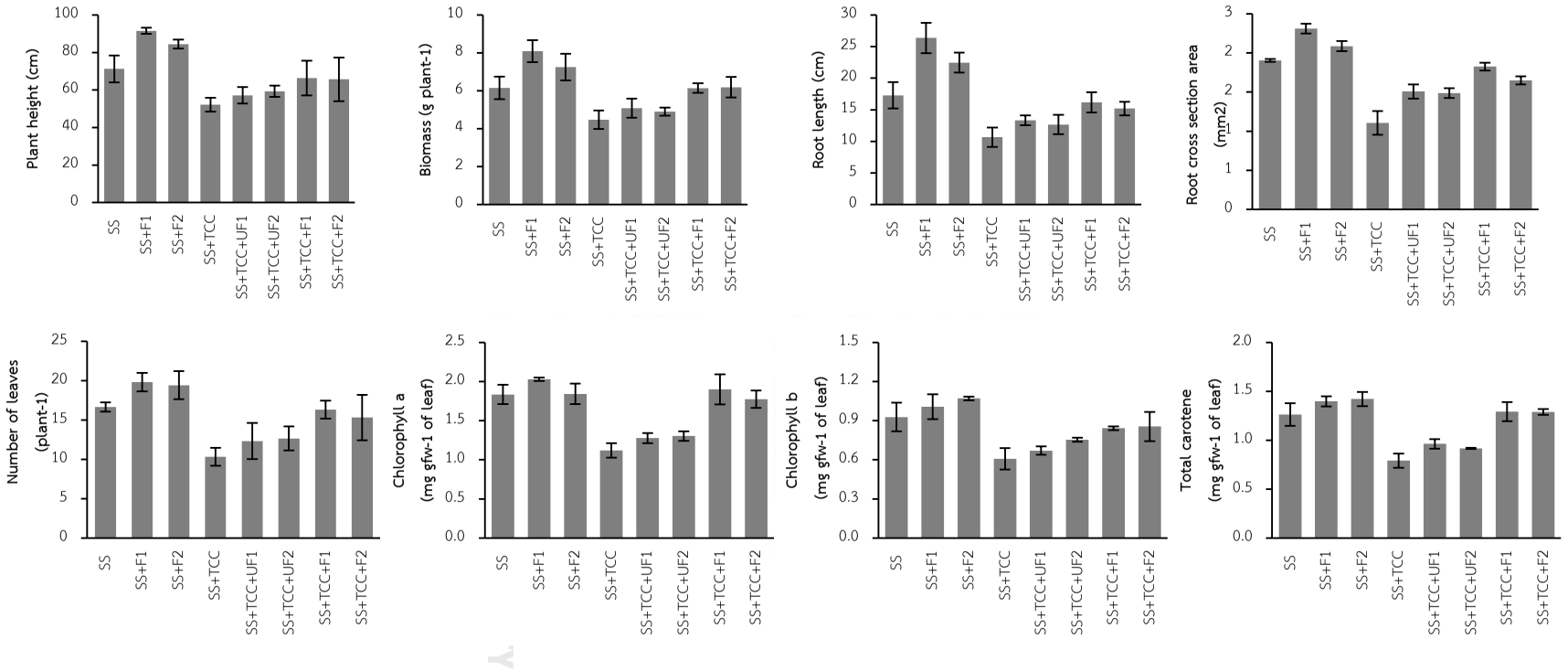

Figure IV-18 Effects of soil treatments on plant and root of *V. radiata* (Mean ± SD) after 5-week experiment. SS = soil and seeds; F1 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive PEG 1%; F2 = 5% of inoculant MC46 formulated in sawdust as carrier, molasses as nutrient and benzoic acid as anti-fungal with additive CMC + Starch 1%; TCC = triclocarban 100 µM, UF1 = uninoculated MC46 F1; UF2 = uninoculated MC46 of F2.

CHAPTER V
DISCUSSION

Many articles have reported the ability of *Ochrobactrum* sp. in degradation of toxic compounds. *Ochrobactrum anthropi* BD-1 isolated from soil was able to degrade dioxin (Chen et al., 2015), *Ochrobactrum* sp. BAP5 isolated from marine sediments capable in benzo[a]pyrene-degradation (Wu et al., 2009). *Ochrobactrum* sp. B2 isolated from soil can degrade both p-nitrophenol (Qiu et al., 2007), as well as methyl parathion (Qiu et al., 2006), and *Ochrobactrum* sp. JAS2 from soil could degrade chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol (Abraham & Silambarasan, 2016). While *Sphingobacterium* sp. has been reported to be an oil and grease-degrading bacterium in wastewater (Nzila et al., 2017). *Pseudomonas* sp. has emerged as a promising candidate for the bioremediation of various toxic pollutants. Some bacteria belonging to *Pseudomonas* sp. have been reported to degrade hexadecane, polyester polyurethane, crude oil, toluene, polycyclic aromatic hydrocarbon, formaldehyde, arsenate, catechol, chloroanilines, carbazole, and pyrene. However, up to now, only two articles reported TCS degradation by *Pseudomonas* sp. (Gangadharan Puthiya Veetil et al., 2012; Meade et al., 2001).

Bacterial ability to degrade TCC was observed in liquid medium and soil. Some studies have been published on TCC biodegradation in the liquid culture at 4 mg L^{-1} or 12.67 μM (Mulla et al., 2016), and in soil experiment at 1 mg kg^{-1} or 3.17 μM (Al-Rajab et al., 2009) then 2 mg kg^{-1} or 6.34 μM (Wu et al., 2009), but those with high TCC biodegradability are limited in number. To our knowledge, this is the first report regarding simultaneous isolation of TCC-degrading bacterial strains from agricultural contaminated soil having the ability to detoxify TCC, promoting plant growth, and degrading TCC in pot soil experiment in an effective manner. In the present study, the concentration of TCC degraded by selected isolates in liquid medium was 30 μM. Such

a concentration is much higher than that in previous studies. Moreover, this study is also the first time to report the kinetic analysis of bacterial growth and TCC biodegradation.

Study in biodegradation of TCC under anaerobic condition is very limited. It was reported that TCC was highly resistant to biodegradation under anaerobic condition with microorganisms soil (Ying et al., 2007). Nonetheless, MC22 was able to degrade TCC under anaerobic condition. The difficulty of degrading TCC under anaerobic condition indicates that this compound tends to persist in the anaerobic condition. Many factors, such as nutrients, redox conditions, and co-metabolic substrates, may affect the anaerobic biodegradation.

Pseudomonas sp. MS45 was able to degrade TCS and utilize it as carbon source for growth. Little biodegradation of TCS was found during 14 days incubation. Specific biodegradation rate of TCS by *Pseudomonas* sp. MS45 in addition of carbon and nitrogen sources mostly showed insignificant different.

Several environmental factors such as pH, oxygen, temperature, salinity, organic carbon, and co-substrates have been suggested as factors affecting the growth and composition of bacterial communities (Robinson et al., 2009). In our study, co-substrate, was selected, and its acceleration on degradation efficiency were tested. It is found that glucose was not able to use for accelerating the TCC degradation efficiency of *Ochrobactrum* MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46. Similar co-substrate, which exhibited similar results, has been used in 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) degradation by *Sinorhizobium meliloti* NM, (Wang et al., 2016) and phenanthrene degradation by *Burkholderia cepacia* PM07 (SUNG et al., 2005). This is suggested that glucose played as carbon catabolite repression in *Pseudomonas, which* not only inhibited the degradation for nonpreferred compounds, but could also generated an important reorganization of the metabolism that requires the activation of several genes (Rojo, 2010). To this point,

all bacterial strains preferred glucose as the first carbon and energy source much more than TCC. The same phenomenon in additional of in-organic nitrogen (no significant effect) and organic carbon (slightly significant) also occur on *p*-nitrophenol (PNP) degradation by *Ochrobactrum* sp. B2 (Qiu et al., 2007).

The transiently accumulated intermediates identified by LC/MS (3,4-dichloroaniline and 4-chloroaniline) were similar with previous observations involving characterizations of a wastewater bacterial consortium (Miller et al., 2010) and *Sphingomonas* sp. strain YL-JM2C (Mulla et al., 2016).

In this study, all bacterial strains almost possessed multiple plant growth promoting activities, such as P-solubilisation, EPS, IAA, siderophore, and ammonia (NH_3) production, both in the absence and presence of TCC stress. It is reported that one of the most readily available for plant growth is solubilisation of minerals such as phosphorus (Kloepper et al., 1988). The production of EPS by PGPB is respected as an valuable activity to protect bacterial cell plants against phagocytosis, desiccation, and phage attack (Tank & Saraf, 2003). IAA production by bacteria promoted the growth of root by directly stimulating plant cell division or cell elongation (Khalid et al., 2004). Although *P. fluorescens* MC46 produced relatively less amount of IAA compared to other bacterial strains in this study and other published PGPB, such as *Acinetobacter* sp. AVLB2 which produced IAA up to 86 μg mL^{-1} in normal condition but the IAA production decreased in the presence of 4-nitroaniline stress (Silambarasan & Vangnai, 2016) as well as *Klebsiella* sp. PS19 that produced IAA up to 42 μg mL^{-1} in normal condition but reduced the IAA in the increasing of insecticides concentrations (Ahemad & Khan, 2011). However, MC46 was able to maintain the IAA production even if in the TCC stress condition at low and high concentration.

Siderophores play as solubilizing agents for iron from organic compounds or minerals under iron starvation restrictions (Indiragandhi et al., 2008). The production of

a noteworthy quantity of siderophores (SA and DHBA) by all bacterial strains directed them as a bio-control against soil borne phytopathogens.

The results of phytotoxicity study and seedling growth of *V. radiata* in Hoagland's solutions suggest that toxicity reduction took place after treatment with the individual bacterial strains, which was possibly due to degradation of TCC during biological treatment. The reduction in phytotoxicity by microbial treatment had previously been reported for pulp paper effluent (Raj et al., 2014; Singhal & Thakur, 2009), and olive mill effluent (Quaratino et al., 2007) and it verifies the assumption that biological treatment with such isolates can lead to obvious reduction in phytotoxicity associated with effluent along with overall advance in effluent characteristics. Similar to phytotoxicity study, cytogenotoxicity assessment using *A. cepa* root tips also indicates the ability of the tested bacterial strains to not only degrade TCC but also to detoxify this pollutant. These results parallel with earlier studies exhibiting the reduction of cytogenotoxicity of azo dye by *Marinobacter* sp. HBRA (Arun Prasad et al., 2013), and of 4-nitroaniline by *Acinetobacter* sp. AVLB2 (Silambarasan & Vangnai, 2016).

In relation to bacterial formulation of *P. fluorescens* MC46, sawdust based formulation with 5% molasses supported large, porous and light substrate for proper colonization and reproduction of the biocontrol bacteria. Sawdust itself is a fancy substrate for the colonization of many bacteria (Morsy et al., 2009). Addition of polymer additive may increase mechanical strength, increase heat resistance, and improve moisture and oxygen of the substrate thus keep the microbial activity accordingly (Tittabutr et al., 2007). The efficiency of sawdust based formulation in addition with PEG 1% for maintenance the population of *P. fluorescens* MC46 is in agreement with that in *Azospirillum* sp. (Dayamani & Brahmaprakash, 2014), and that in *Arthrobacter crystallopoietes* (McAneney et al., 1982). They reported that PEG is modulated by altering water activity of media and it may also be influencing the movement of solutes

in medium as well as across the cell membrane. It was reported that formulations with CMC and starch 1% was found to be the favourable additives for the production of *Bradyrhizobium japonicum*, which is in correspondence with our findings (Fernandes Júnior et al., 2009). Due to the population density of *P. fluorescens* MC46 could be maintained well in PEG1% and CMC+starch 1%, hence, the ability of *P. fluorescens* MC46 in TCC degradation could be maintained as well.

TCC is not easily degraded under soil environmental conditions (Cha & Cupples, 2010; Ying et al., 2007). Similar to earlier results, at the present study without bioaugmentation, natural microorganisms did not demonstrate the capability to effectively remove TCC. The reason for this is unclear but may have to do with the limited bioavailability of the TCC in soils. The bioavailability inadequacy likely affect TCC biodegradation rates to a greater extent (Cha & Cupples, 2010). However, bioaugmentation with formulated *P. fluorescens* MC46 did extensively remove TCC in soils. This reflects that *P. fluorescens* MC46 may have some potential as a bioaugmentation agent.

Minor soil enzymes activity was found in the TCC contaminated soil, which directed the inhibitory impacts of TCC to soil microbes. This is not astounding because since TCC has high antimicrobial activity (Giuliano & Rybak, 2015). Such inhibition of other antibiotics on the enzyme activity has been conveyd by previous studies and these results discovered the stress on the soil microbial community brought by the contamination of antibiotic residues (Pinna et al., 2012; Thiele-Bruhn & Beck, 2005). Soil enzyme activities kept on inhibit until the end of the experiment, is verity to be a thoughtful point since these enzymes acts a vital function in the driving of C, N, and P in the soil. Other than TCC, soil enzyme assays were also affected by bioaugmentation with formulated *P. fluorescens* MC46 to the soils. Activities of enzymes tested were strongly enhanced by *P. fluorescens* MC46, which emphasized the severe impact of

TCC due to biodegradation activity by *P. fluorescens* MC46, therefore, exhibited healthy soil.

Under pot soil conditions, formulated *P. fluorescens* MC46 significantly enhanced the growth and chlorophyll content of *V. radiata* plants over native microorganisms in normal soils. The formulated *P. fluorescens* MC46 also enhanced the growth of *V. radiata* plants even under TCC stress condition, indicating *P. fluorescens* MC46 has potential as a biofertilizer agent. These findings are confirming that *Pseudomonas* is reported as an effective plant growth-promoting bacteria (PGPB) (Glick, 2012). The strains of *Pseudomonas fluorescens* are known to survive in rhizosphere (Souza et al., 2015; Zhou et al., 2016). Several *Pseudomonas* strains were demonstrated to protect plants against many fungal, bacterial and viral diseases (Russo et al., 2001; Sivasakthi et al., 2014). To our knowledge, the present study represents the first report of biofertilizer potential of TCC degrading bacterial strain, with additional traits of enhancing particular soil enzyme activities.

CHAPTER VI

CONCLUSION

According to the findings in this thesis, pure cultures bacteria: *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43, and *P. fluorescens* MC46 which were isolated from agricultural soil, have capability of completely degrading TCC, whereas, *Pseudomonas* sp. MS45 isolated from agricultural soil as well was more capable to degrade TCS. All of these strains are gram negative.

In conclusion:

a) Under aerobic condition, *Ochrobactrum* sp. MC22, *Ochrobactrum* sp. MC35, *Sphingobacterium* sp. MC43 and *Pseudomonas fluorescens* MC46 were able to utilize TCC as a sole carbon source while *Pseudomonas* sp. MS45 was more capable to remove TCS.

b) Under anaerobic condition, only *Ochrobactrum* sp. MC22 was pronounced to degrade TCC in the presence of ferric as electron acceptor and acetate as electron donor.

c) Along with the degradation capability, these bacterial strains possessed plant growth promoting activities , which were steadily maintained under TCC stress condition, can colonize on plant root to promote symbiotic relationship with plant, and simultaneously.

d) Biodegradation pathway by MC22 and MC46 was proposed through metabolite identification.

e) *Pseudomonas* sp. MS45 has enormous potential to act as a green technology for the cleanup of sites contaminated with TCS.

f) Two formulations of MC46 for TCC biodegradation and PGP were well developed, and proven in pot soil experiments using mung bean plants to

exhibit the potential bioaugmentation approach for TCC treatment in contaminated soil.

g) Shelf-life of MC46 and TCC-degrading activity were maintained for 20 weeks by forming it as a sawdust-based formula with low-cost and safe ingredients including molasses, and either PEG or CMC-starch blend as a polymeric additive.

h) This study is the first report to reveal the potential application of the formulated MC46 inoculant for the TCC degradation and plant growth development as well as enzyme activities of indigenous soil microbes.

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APPENDICES

APPENDIX A

Retention Time of TCC

TCC was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of TCC standard was 6.478 ± 0.027 as shown in Figure A-1.

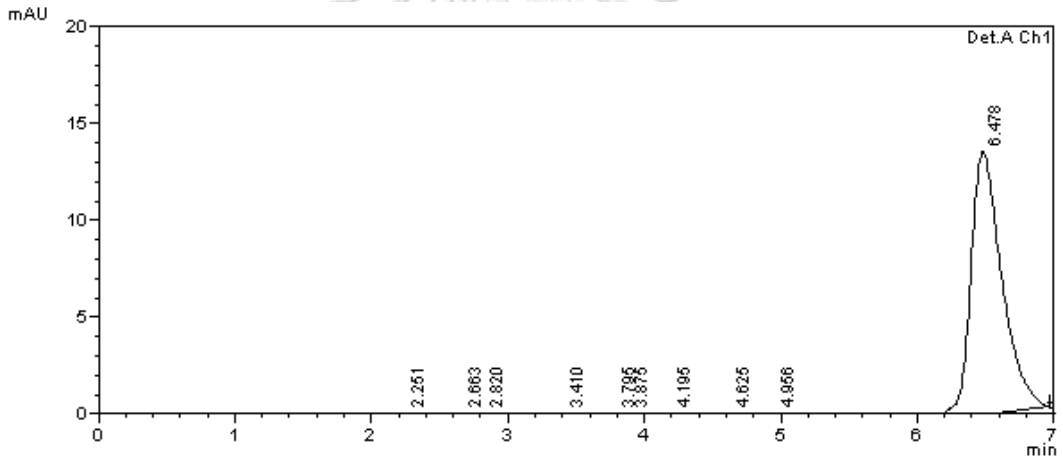

Figure A-1 HPLC chromatogram of TCC standard at concentration 30 µM.

APPENDIX B

TCC standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of TCC product. The TCC standard was freshly prepared in acetone as a stock solution at 100mM. Thus, TCC at various concentrations was obtained by doing a serial dilution from TCC stock solution. The standard curve of TCC was shown in Figure B-1.

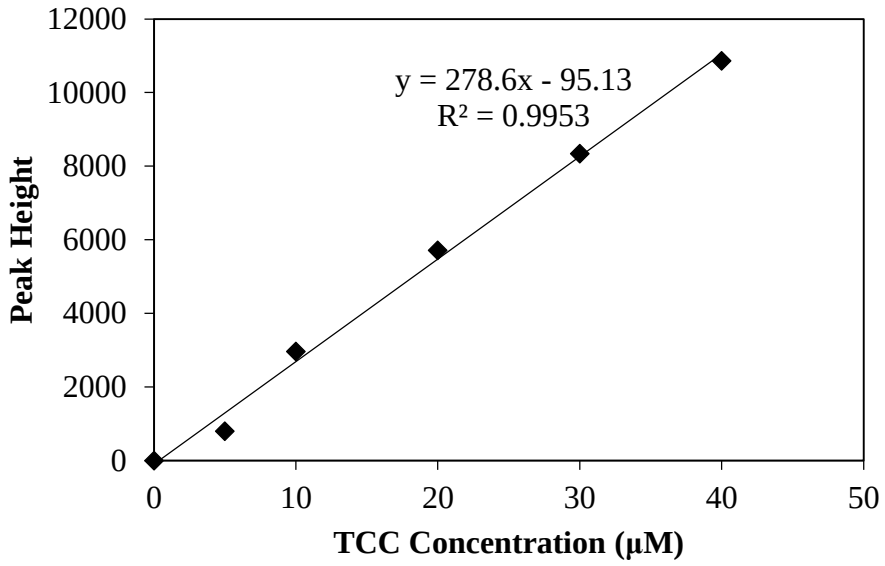

Figure B-1 TCC standard curve for calculation of TCC concentration.

$$\text{The amount of TCC} = \frac{\text{Peak height} + 95.13}{278.6}$$

APPENDIX C

Retention Time of 3,4-Dichloroaniline

3,4-Dichloroaniline was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of 3,4-dichloroaniline standard was 3.858 ± 0.059 as shown in Figure C-1.

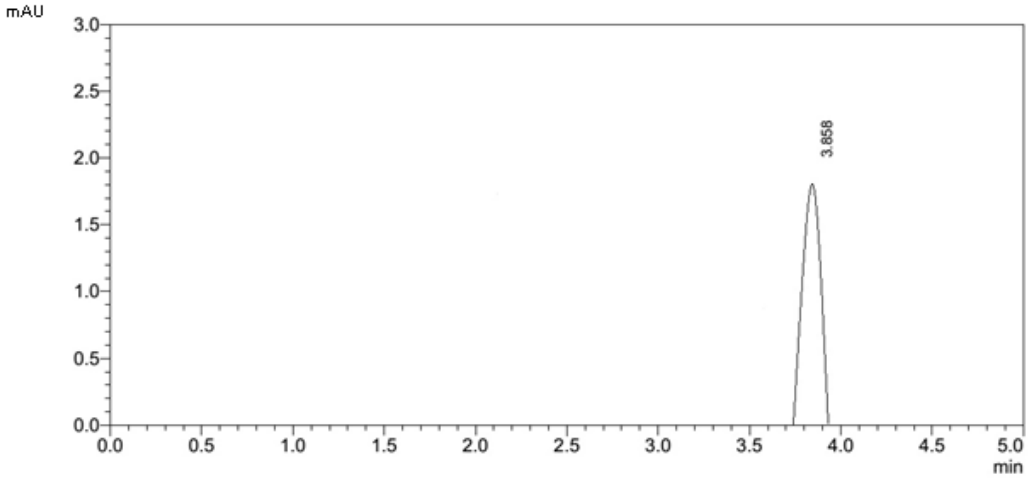

Figure C-1 HPLC chromatogram of 3,4-dichloroaniline standard at concentration 30 µM.

APPENDIX D

3,4-Dichloroaniline standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of 3,4-dichloroaniline product. The 3,4-dichloroaniline standard was freshly prepared in acetone as a stock solution at 100mM. Thus, 3,4-dichloroaniline at various concentrations was obtained by doing a serial dilution from 3,4-dichloroaniline stock solution. The standard curve of 3,4-dichloroaniline was shown in Figure D-1.

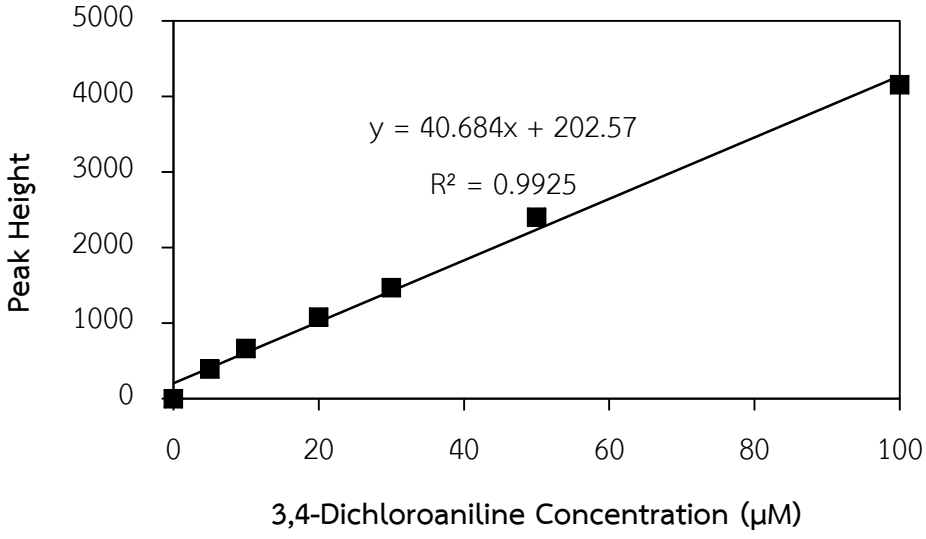

Figure D-1 3,4-Dichloroaniline standard curve for calculation of 3,4-dichloroaniline concentration.

$$\text{The amount of 3,4-dichloroaniline} = \frac{\text{Peak height} - 202.57}{40.684}$$

APPENDIX E

Retention Time of 4-Chloroaniline

4-Chloroaniline was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of 4-chloroaniline standard was 3.612 ± 0.174 as shown in Figure E-1.

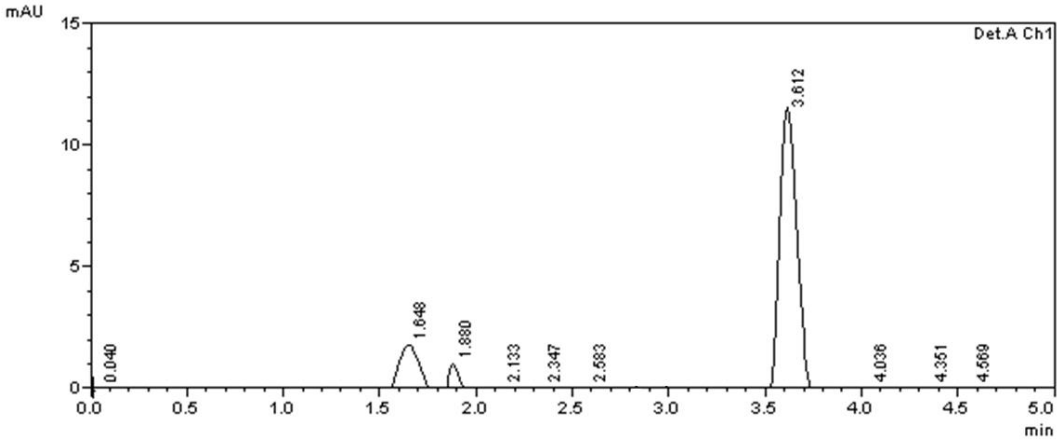

Figure E-1 HPLC chromatogram of 4-chloroaniline standard at concentration 30 µM.

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APPENDIX F

4-Chloroaniline standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of 4-chloroaniline product. The 4-chloroaniline standard was freshly prepared in acetone as a stock solution at 100mM. Thus, 4-chloroaniline at various concentrations was obtained by doing a serial dilution from 4-chloroaniline stock solution. The standard curve of 4-chloroaniline was shown in Figure F-1.

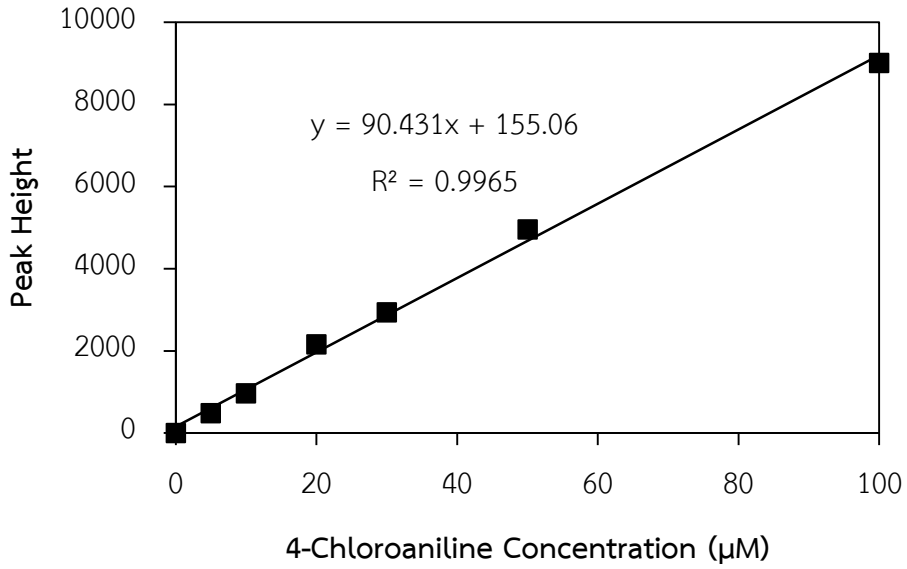

Figure F-1 4-Chloroaniline standard curve for calculation of 4-chloroaniline concentration

$$\text{The amount of 4-chloroaniline} = \frac{\text{Peak height} - 155.06}{90.431}$$

APPENDIX G

Retention Time of Aniline

Aniline was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of aniline standard was 3.218 ± 0.086 as shown in Figure G-1.

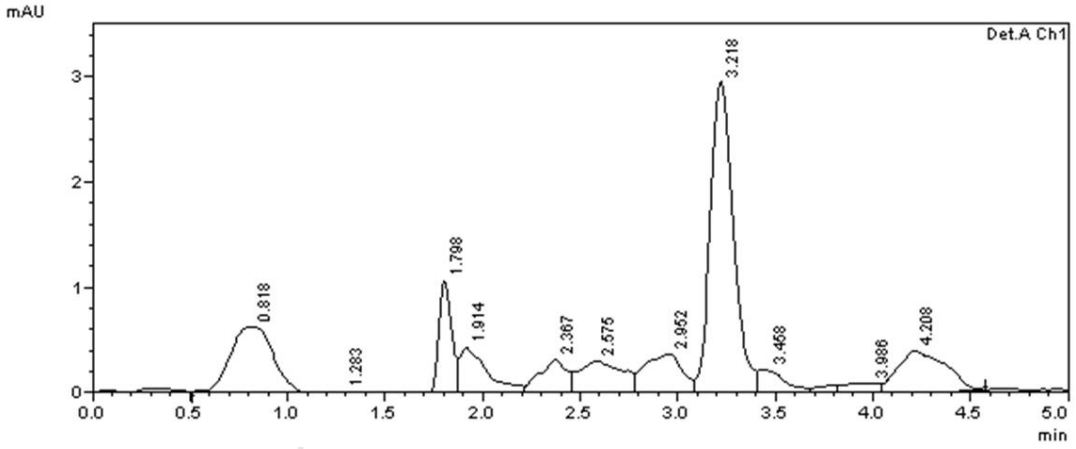

Figure G-1 HPLC chromatogram of Aniline standard at concentration 30 µM

APPENDIX H

Aniline standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of aniline product. The aniline standard was freshly prepared in acetone as a stock solution at 100mM. Thus, 4-chloroaniline at various concentrations was obtained by doing a serial dilution from aniline stock solution. The standard curve of aniline was shown in Figure H-1.

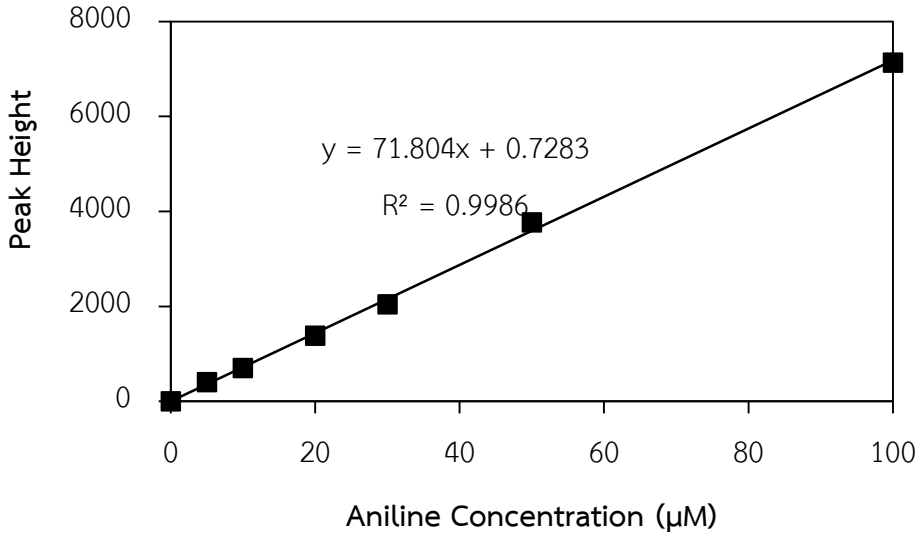

Figure H-1 Aniline standard curve for calculation of aniline concentration.

$$\text{The amount of aniline} = \frac{\text{Peak height} - 0.7283}{71.804}$$

APPENDIX I

Retention Time of TCS

TCS was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of TCS standard was 6.264 ± 0.034 as shown in Figure I-1.

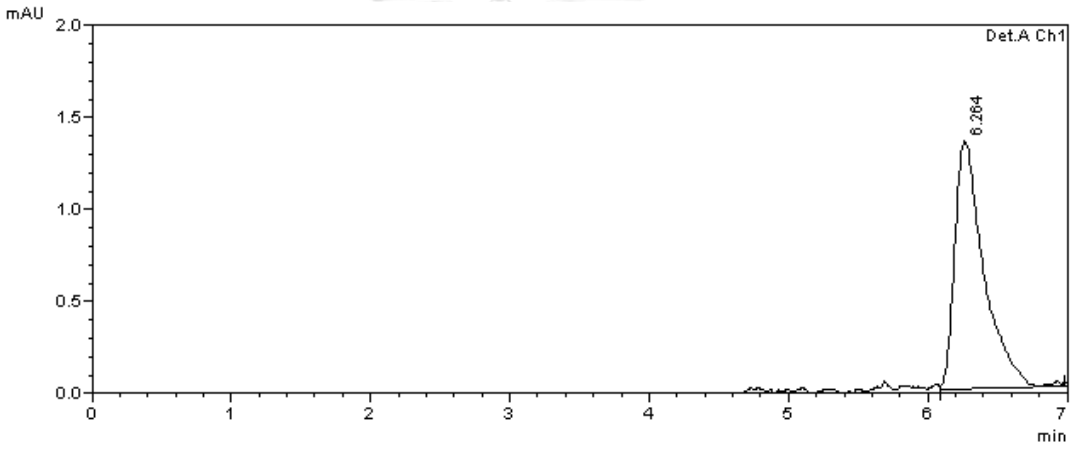

Figure I-1 HPLC chromatogram of TCS standard at concentration 40 µM.

APPENDIX J

TCS standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of TCS product. The TCS standard was freshly prepared in acetone as a stock solution at 100mM. Thus, TCS at various concentrations was obtained by doing a serial dilution from TCS stock solution. The standard curve of TCS showed in Figure J-1.

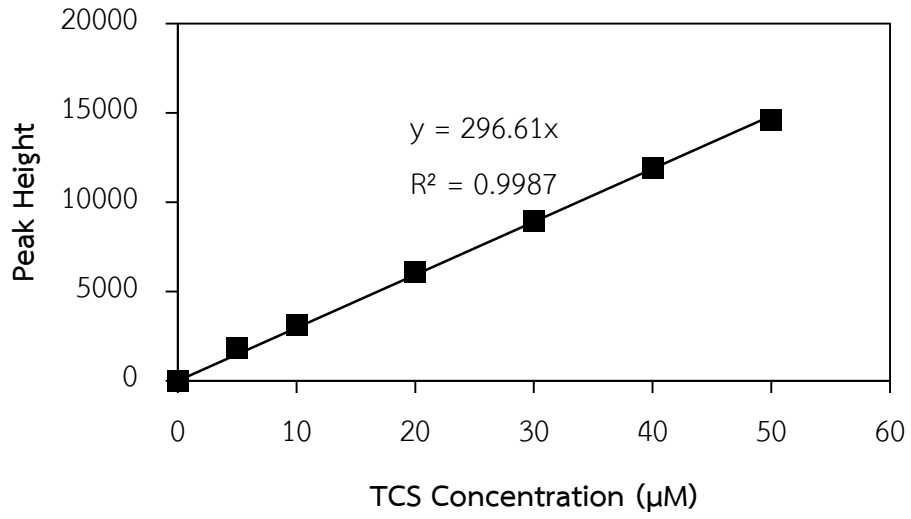

Figure J-1 TCS standard curve for calculation of TCS concentration.

$$\text{The amount of TCS} = \frac{\textbf{Peak height}}{\textbf{296.61}}$$

APPENDIX K

Retention Time of 2,4-dichlorophenol

TCS was analysed using reverse phase HPLC equipped with a UV detector (at 245 nm). The separation was performed at 40 ºC on a C18 column using an acetonitrile : UP water mixture (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min and injection volume was 20 µl. The retention time of 2,4-dichlorophenol standard was 3.982 ± 0.026 as shown in Figure K-1.

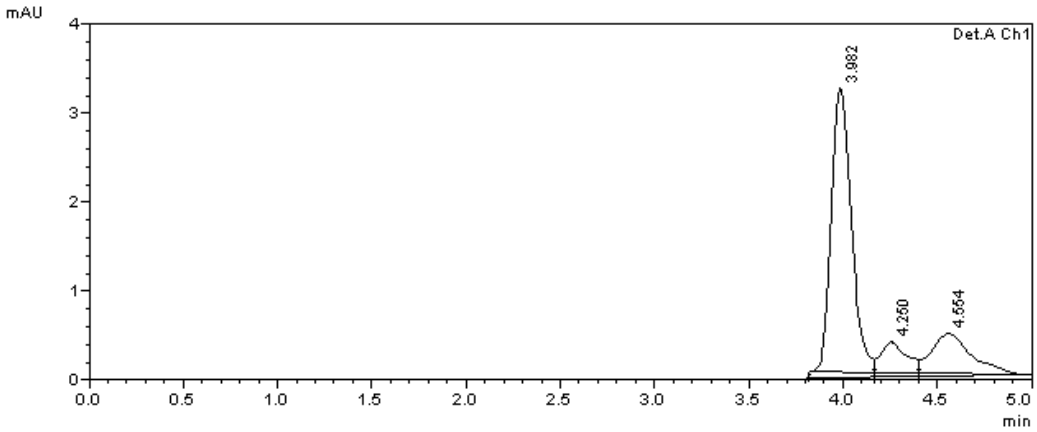

Figure K-1 HPLC chromatogram of 2,4-dichlorophenol standard at concentration 40 µM.

APPENDIX L

2,4-Dichlorophenol standard curve

Standard curve was used to analyse chromatogram of HPLC results in order to calculate the concentration of 2,4-dichlorophenol product. The 2,4-dichlorophenol standard was freshly prepared in acetone as a stock solution at 100mM. Thus, 2,4-dichlorophenol at various concentrations was obtained by doing a serial dilution from 2,4-dichlorophenol stock solution. The standard curve of 2,4-dichlorophenol was shown in Figure L-1.

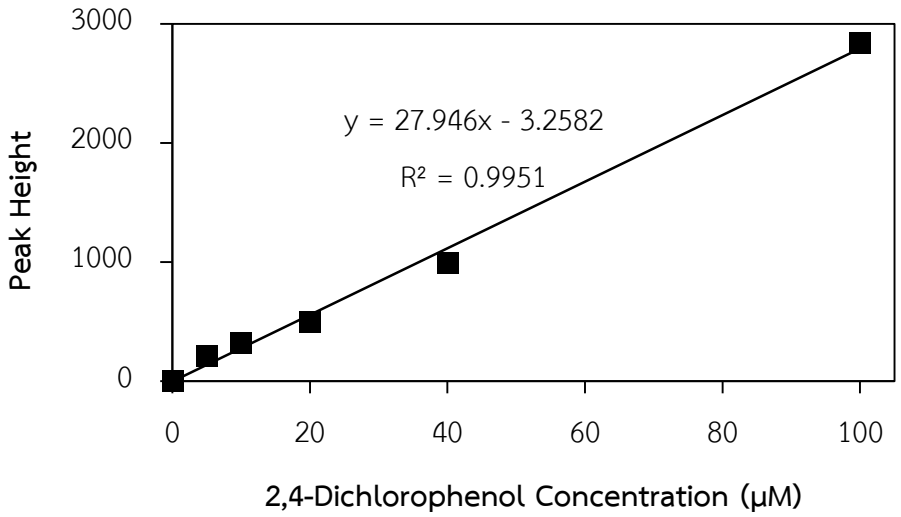

Figure L-1 2,4-Dichlorophenol standard curve for calculation of 2,4-dichlorophenol concentration

$$\text{The amount of 2,4-dichlorophenol} = \frac{\text{Peak height} + 3.2582}{27.946}$$

APPENDIX M

Protein standard curve

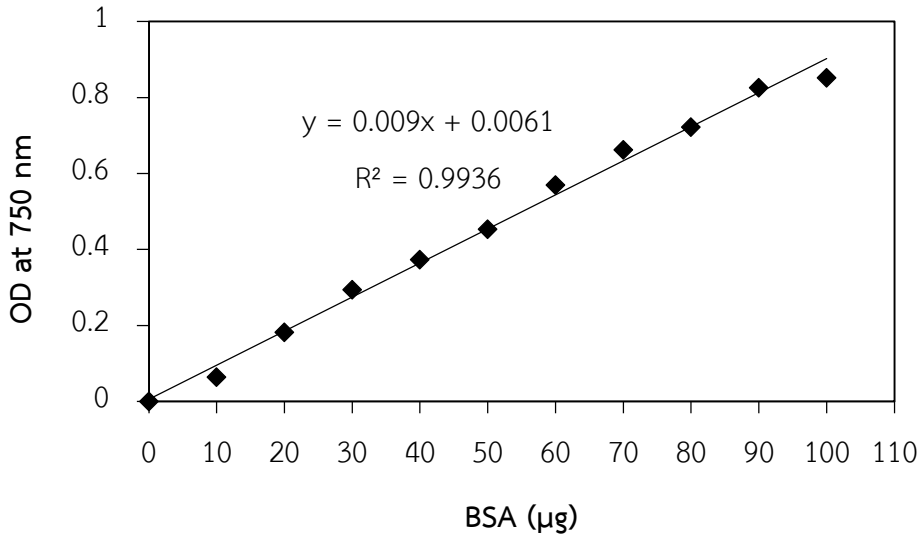

Figure M. Standard curve of BSA from modified Lowry method used to determine cell protein.

Principle : Dulley and Grieve (1975) and Lowry et al. (1951)

Copper + Protein Alkaline pH → Copper-protein complex
(CuSO4.5H2O) Phenol reagent (blue colour)

APPENDIX N

FASTA format of 16S rRNA gene sequence of the isolated bacteria

- FASTA format of *Ochrobactrum* sp. MC22

>MC22_16SrRNA 1415 bp

CGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAACG
CGTGGGAACGTACCTTTTGCTACGGAATAACTCAGGGAAACTTGTGCTAATACCGTATGTGCCCTTCGG
GGGAAAGATTTATCGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT
GAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGG
CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAG
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GGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAG
GAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTTGGGGAGTTTACTCCTTT
CGGTGGCGCAGCTTAACGCATTAAACATTCCGCCTGGGGAAGTACGGTCGCAAGAATTAAAACTCAAAG
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AGCCCTTGACATACCGGTCGCGGACACAGAGATGTGTCTTTCAGTTTGGCTGGACCGGATACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCT
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- FASTA format of *Ochrobactrum* sp. MC35

>MC35_16SrRNA 1463 bp

CGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAACG
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GGGAAAGATTTATCGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT
GAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGG
CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAG
CGCACGTAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACT
GGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAG
GAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTTGGGGAGTTTACTCTTCG
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GCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTC

AAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGCA
CGCGAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAG
TTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCACACCATGGGAGTTGGTTTTACCCGAAGGCGCTGTGCTAACCGCAAGGAGGCAGGCGACCACGGT
AGGGTCAGCGACTGGGGTGAATCTAGGGGGTGCCCCCCAAAAAAAAGGGGGGAAACAAAGGTTTTTAAA
ACAAAAGGGACCCA

- FASTA format of *Sphingobacterium* sp. MC43

>MC43_16SrRNA 1467 bp

CGTCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGGACGGGATCCATTGATAGCTTGCTAT
CAATGGTGAGAGTGGCGCACGGGTGCGTAACGCGTGAGCAACCTGCCTCCATCAGGGGGATAGCCTCTC
GAAAGAGAGATTAACACCGCATAATATCAACAGTTCGCATGTTCAGTTGATTAAATATTTATAGGATGG
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ACTGCTTTTGTCCAGGAATAAACCTAAATACGAGTATTTAGCTGAATGTACTGGAAGAATAAGGATCGG
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GGTGCGTAGGCGGCCTGTTAAGTCAGGGGTGAAATACGGTGGCTCAACCATCGCAGTGCCTTTGATACT
GATGGGCTTGAATCCATTTGAAGTGGGCGGAATAAGACAAGTAGCGGTGAAATGCATAGATATGTCTTA
GAACTCCGATTGCGAAGGCAGCTCACTAAGCTGGTATTGACGCTGATGCACGAAAGCGTGGGGATCGAA
CAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATAACTCGATGTTGGCGATAGACAGCCAGC
GTCTTAGCGAAAGCGTTAAGTTATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCAAAGGAATTGA
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GTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATGTTTAGTTGCCAGC
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ATCATGGCCCTTACGTCCGGGGCTACACACGTGCTACAATGGATGGTACAGCGGGCAGCTAGCTGGCAA
CAGCATGCTAATCTCTAAAAGCCATTCACAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGG
ATTCGCTAGTAATCGCGTATCAGCAATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CAAGCCATGAAAGTTGGGGGTACCTAAAGCATGTGACCGCAAGGAGCGTGTCAGGGTAAAACCGATAAT
TGGGGCTAAGTCGTACAG

- FASTA format of *Pseudomonas fluorescens* MC46

>MC46_16SrRNA 1286 bp

TTTTGGAGGAGCGGGCGTGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCG
GAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAG
ATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGGACGATCCGTAACTGGTC
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TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
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CGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
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GGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCA
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AGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGT
GAAGTCGGAATCGCTAGTAATCGCGAATCAAGAATGTCGGCGGG

- FASTA format of *Pseudomonas* sp. MS45

>MS45_16SrRNA 1498 bp

CCCTCGTAGATTGACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCT
CTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAAC
GGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAG
CCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG
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CGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGA
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GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAGCCTTGAGCTTTTAGT
GGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
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TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCA
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TCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGT
GATTCATGACTGGGGTGAAGTCGTACGGANCCNAAAAANCNCCCCACAG

APPENDIX O

Soil Characteristics Test Report

DST

Shri AMM Murugappa Chettiar Research Centre
MCRC

IITM-AIDB

MURUGAPPA GROUP

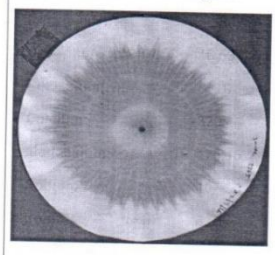
Soil Test Report

Sample ID: 3MCRC 3365. Village: PATUMWAN Collection Time: 02/03/2016

Farmer's Name: EX.PADDY. 375g Taluk: THAILAND Date of analysis: 09/03/16

pH:	5.50		Boron:	0.55	mg / kg
EC:	0.14		Molybdenum:	-	mg / kg
Organic Carbon(OC):	-	kg / acre	Sulfate:	19.47	mg / kg
Organic Carbon(OC):	1.68	%	Humus(HA):	143.38	kg / acre
Nitrogen(N):	104.02	kg / acre	Total Minerals:	209.22	kg / acre
Phosphorous(P):	16.53	kg / acre	Bacteria:	0	10^6 cfu / gm
Potassium(K):	88.67	kg / acre	Azotobacter:	0	10^6 cfu / gm
Calcium:	370.43	mg / kg	Azospirillum:	0	10^6 cfu / gm
Magnesium:	175.16	mg / kg	Rhizobium:	0	10^6 cfu / gm
Sodium:	107.56	mg / kg	Actinomycetes:	0	10^6 cfu / gm
Iron:	8.01	mg / kg	Fungi:	0	10^6 cfu / gm
Manganese:	6.27	mg / kg	Protease:	0	µg Tyr/g/hr
Copper:	1.35	mg / kg	Cellulase:	0	mg Glu/g/day
Zinc:	0.52	mg / kg	Invertase:	0	µmol lu/g/day
			Alk. Phos:	0	µg pnp/g soil/hr

Image of Soil Chromatogram



Nutrient	Low	Medium	High	Nutrient	Low	Medium	High
N (kg/acre)	<113	113-182	>182	Iron (mg/Kg)	<6	6-8	>8
P (kg/acre)	<18	18-36	>36	Mn (mg/Kg)	<1	1.2-2.5	>2.5
K (kg/acre)	<60	60-138	>138	Cu (mg/Kg)	<0.3	0.3-1	>1
OC (%)	<0.75	0.75-1.5	>1.5	Zn (mg/Kg)	<0.5	0.5-1	>1
Mg (mg/kg)	<10	10-15	>15	Sulphur (mg/Kg)	0-10	10-15	>15
HA (Kg/Acre)	<18	18 -31	>31				

* The physico-chemical properties of soil were determined by following an alternative analytical indigenous technology developed by MCRC, IIT (M) with financial assistance from Department of Science & Technology, GOI, New Delhi and Murugappa Group.

Nutrient Level	Ca (mg/kg)	Boron (mg/kg)	Molybdenum (mg/kg)
Sufficient	>300	>0.5	>0.2
Deficient	<300	<0.5	<0.2

Date: 09/3/16 Analysed By: S.P.L. Approved By:

APPENDIX P

Culture medium and TCC solution

- **Mineral salts medium (MSM)**

Mineral salts medium (MSM) was used for screening, isolation, and degradation. MSM medium was contained the following components (Anwar et al., 2009).

Na_2HPO_4	5.8g
KH_2PO_4	3.0g
NaCl	0.5g
$MgSO_4$	0.25g
NH_4Cl	1.0g

The components were dissolved in 1 liter of distilled water and adjusted pH to 6.8-7. The solid media were prepared by adding 20 g L^{-1} agar into the liquid media. The media were sterilized by autoclaving at 121 °C for 20 min.

- **Luria bertani medium (LB)**

Tryptone	10g
Yeast extract	5g
NaCl	10g

LB medium was dissolved in 1 liter, adjusted pH to 7, and sterilized by autoclaving at 121 °C for 20 min.

- **Preparation of TCC solution**

Stock solution of TCC was arranged in acetone at 100 mM concentration then sterilized on 0.45 µm Nylon filter membrane. TCC at each concentration was obtained by doing serial dilution from TCC stock solution.

APPENDIX Q

Chemical Details and Photos

- **Triclocarban**

  Purity: >99%

  CAS Number: 101-20-2

- **Triclosan**

  Purity: >96%

  CAS Number: T1872

- **3,4-Dichloroaniline**

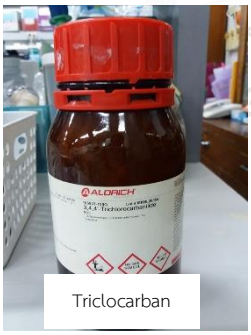
  Purity: >99%

  CAS Number: **106-47-8**

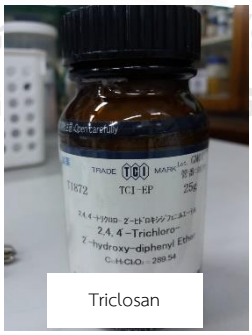
- **4-Chloroaniline**

  Purity: >99%

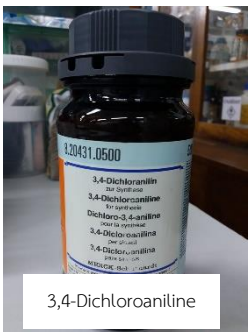
  CAS Number: **106-47-8**



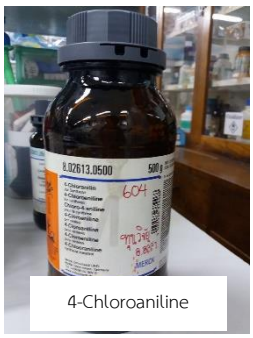
Triclocarban



Triclosan



3,4-Dichloroaniline



4-Chloroaniline

VITA

Mrs. Merry Krisdawati Sipahutar was born on 26th December 1974 in Pematang Siantar, North Sumatera, Indonesia. She received Bachelor's degree from Marine Science and Technology, Faculty of Marine and Fishery, Bogor Agricultural University, Indonesia in 1998. She finished her Master Degree in Management of Natural Resources and Environmental Program, Bogor Agricultural University, Indonesia in 2002. And then continue the PhD Degree in Biological Sciences Program, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

During her study, she produced the following output of the research.

1. M.K. Sipahutar, A.S. Vangnai. (2017). Role of plant growth-promoting Ochrobactrum sp MC22 on triclocarban degradation and toxicity mitigation to legume plants, Journal of Hazardous Materials. 329: 38-48.

2. Sipahutar, M. K., Piapukiew, J., & Vangnai, A. S. (2018). Efficiency of the formulated plant-growth promoting Pseudomonas fluorescens MC46 inoculant on triclocarban treatment in soil and its effect on Vigna radiata growth and soil enzyme activities. Journal of Hazardous Materials 344: 883-892.

3. Sipahutar, M. K., Piapukiew, J., & Vangnai, A. S. (2018). Biodegradability and formulation of triclosan-degrading and plant-growth promoting Pseudomonas sp. MS45.